Approaches to DIVA vaccination for fish using infectious salmon anaemia and koi herpesvirus disease as models

A thesis submitted to the University of Stirling for the degree of Doctor of Philosophy in Aquatic Veterinary Studies

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To my ever supportive parents,

Mum, Cathy and

Dad, Monty

To my sisters, Lauren and Michelle,

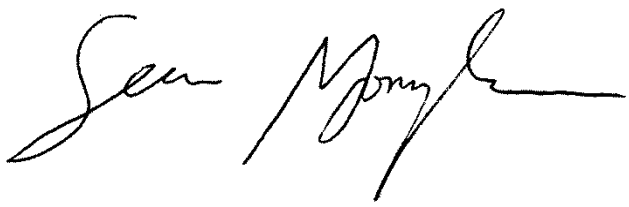
my brother, Daniel

and my nephew, Rhys

Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

Sean J. Monaghan



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List of abbreviations

A_{280}	absorbance at 280 nanometers
A_{495}	absorbance at 495 nanometers
aa	amino acids
AD	Aujeszky's disease
AFCS	Australian foetal calf serum
AI	avian influenza
AIS	antigen induced suppression
AKLH	ascites fluid produced against keyhole limpet hemocyanin
ALVR	agar low viscosity resin
AngHV	anguillid herpesvirus
ANOVA	analysis of variance
AP	alkaline phosphatase
APC	antigen presenting cell
APES	3-aminopropyltriethoxysilane
ARF	aquatic research facility
ASK-2 cells	Atlantic salmon kidney-2 cells
ATCC	American type culture collection
ATPase	adenosine tri-phosphatase
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolylphosphate
BoHV-1	bovine herpesvirus-1
BoHV-5	bovine herpesvirus-5
BSA	bovine serum albumin
BTV	bluetongue virus
BVDV	bovine viral diarrhoea virus
CCB	common carp brain
CCV	channel catfish virus
cDNA	complementary deoxyribonucleic acid
CDR	complementarity determining region
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
CELL	total cell area
CF	condition factor
CND	Canadian dollars
CNGNV	carp nephritis and gill necrosis virus
CO_2	carbon dioxide
CPE	cytopathic effect
CSF	classical swine fever
CSFV	classical swine fever virus
Ct	cycle threshold
CTL	cytotoxic T lymphocytes
Ctx	Chlorella toxin
CyHV	cyprinid herpesvirus
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DIG	digoxigenin
DMEM+	Dulbecco's minimum essential medium plus additive
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dUTP	deoxy-uridine-5-triphosphate
DIVA	differentiating infected from vaccinated animals

DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
dO_2	dissolved oxygen
DPBS	Dulbecco's phosphate buffered saline
dpc	days post challenge
dpe	days post exposure
dpi	days post infection
dpv	days post vaccination
Dsb	disulphide bridge
dTTP	deoxythimidine triphosphate
EAV	equine arteritis virus
EC	European commission
EC	extracellular
EDTA	ethyl-diamino-tetra-acetic acid
e.g.	for example
EGFP	enhanced green fluorescent protein
EITB	enzyme-linked immunoelectrotransfer blot
EIV	equine influenza virus
ELISA	enzyme linked immunosorbent assay
EMEM	Earl's minimum essential medium
Env.	enveloped
ER	endoplasmic reticulum
et.al.	"et alia" and others
ESI-MS	electron ionisation coupled to mass spectrometry
Etx	enterotoxin
EU	European union
FBS	foetal bovine serum
FCA	Freund's complete adjuvant
FcR	Fc receptors
FCS	foetal calf serum
FLI	Friedrich Loeffler Institut
FMD	foot and mouth disease
FITC	fluorescein-5-iso-thiocyanate
× *g*	gravitational force (multiples of gravity)
GAG(s)	glycosaminoglucan(s)
GFP	green fluorescent protein
GLM	general linear model
GMM	genetically modified organisms
h	hour
H_2O	water
H_2SO_4	sulphuric acid
HA	haemagglutinin
DNP	dinitrophenyl
HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
HE	haemagglutinin esterase
HEL	hen egg lysozyme
HEPES	4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid
HEX	hex phosphoramidite
HIER	heat-induced epitope retrieval
HPAI	highly pathogenic avian influenza
hpi	hours post infection
HPR	highly polymorphic region
HRP	horseradish peroxidase
HSWB	high salt wash buffer
IcHV	ictalurid herpesvirus-1
ICTV	International committee on taxonomy of viruses
i.e.	"*id est*" that is
Ig	Immunoglobulin
IF	immunofluorescence

IFAT	indirect fluorescent antibody technique
IFN	interferon
IHC	immunohistochemistry
IHN	infectious hematopoietic necrosis
IHNV	infectious hematopoietic necrosis virus
IL	interleukin
ILTV	infectious laryngotracheitis virus
INM	inner nuclear membrane
ip	intraperitoneal
ISA	infectious salmon anaemia
ISH	*"in situ"* hybridization
kb	kilo base
kbp	kilo base pairs
KCF	koi caudal fin
kDa	kilo dalton
KF-1	koi fin cell line 1
Kg	kilograms
KHV	koi herpes virus
KHVD	koi herpes virus disease
KLH	keyhole limpet hemocyanin
KOH	potassium hydroxide
L	litre
LETV	lung-eye-trachea disease virus
LSD	least significant difference
LSWB	low salt wash buffer
LPAI	low pathogenic avian influenza
LPH	*Limulus polyphemus* hemocyanin
LPS	lipopolysaccharides
M	molar
mA	milliamps
MAb(s)	monoclonal antibodies
MAC	membrane attack complex
MBL	mannose-binding lectin
MEM	minimum essential medium
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
µg	microgram
µL	microlitre
min	minute
mL	millilitre
mM	milli-Molar
M-MuLV	Moloney-murine leukemia virus
MOI	multiplicity of infection
MT	microtubule
MW	molecular weight
NA	non applicable
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
NDV	Newcastle disease virus
NEAA	non-essential amino acids
NIP	4-hydroxy-3-iodo-5-nitrophenyl-acetic-acid
NP	nucleoprotein
NSP	non-structural protein
OD	optical density
OIE	office international des epizooties
OMP	outer membrane protein
ONM	outer nuclear membrane
ORF	open reading frame
p	passage
PBS	phosphate buffered saline

PBST	phosphate buffered saline with tween-20
PCR	polymerase chain reaction
RER	rough endoplasmic reticulum
PFU	plaque forming units
PPRV	peste des petits ruminants virus
PrV	pseudorabies virus
R	reproduction value
RaHV	ranid herpesvirus
Rec.	recombinant
RNA	ribonucleic acid
RNAse	ribonuclease
RT	room temperature
rRT-PCR	real time reverse transcription polymerase chain reaction
RT-PCR	real time polymerase chain reaction
RPV	rinderpest virus
RVFV	Rift valley fever virus
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	second(s)
seg	segments
SHK-1 cells	salmon head kidney cells
SNT	serum neutralisation test
sp.	species
ss	single stranded
SSC	standard saline citrate
SuHV	suid herpesvirus
SV	secretory vesicle
SVC	spring viraemia of carp
SVCV	spring viraemia of carp virus
SVP(s)	subviral particles
TBS	tris buffered saline
TBST	tris buffered saline containing tween-20
$TCID_{50}$	tissue culture infectious dose required for 50% infection of cells inoculated
TCR	T cell receptors
TD	T-cell dependent
TEM	transmission electron microscopy
TGN	trans golgi network
TI	T-cell independent
TK	thymidine kinase
TK PCR	thymidine kinase polymerase chain reaction
TMB	tri-methyl-benzidine
TSA	tryptone soy agar
TSB	tryptone soy broth
TT	tetanus toxoid
TTBS	tris buffered saline with tween-20
UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	variable domain
VHS	viral haemorrhagic septicaemia
VHSV	viral haemorrhagic septicaemia virus
VLP(s)	virus like particle(s)
v/v	volume/volume
WB	western-blot
w/v	weight/volume

Abstract

The expanding aquaculture industry continues to encounter major challenges in the form of highly contagious aquatic viruses. Control and eradication measures targeting the most lethal and economically damaging virus-induced diseases, some of which are notifiable, currently involve 'stamping out' policies and surveillance strategies. These approaches to disease control are performed through mass-culling followed by restriction in the movement of fish and fish products, resulting in considerable impacts on trade. Although effective, these expensive, ethically complex measures threaten the sustainability and reputation of the aquatic food sector, and could possibly be reduced by emulating innovative vaccination strategies that have proved pivotal in maintaining the success of the terrestrial livestock industry. DIVA '*differentiating infected from vaccinated animal'* strategies provide a basis to vaccinate and contain disease outbreaks without compromising 'disease-free' status, as antibodies induced specifically to infection can be distinguished from those induced in vaccinated animals. Various approaches were carried out in this study to assess the feasibility of marker/DIVA vaccination for two of the most important disease threats to the global Atlantic salmon and common carp/koi industries, i.e. infectious salmon anaemia (ISA) and koi herpesvirus disease (KHVD), respectively.

Antibody responses of Atlantic salmon (*Salmo salar* L.), following immunisation with an ISA vaccine, administered with foreign immunogenic marker antigens (tetanus toxoid (TT), fluorescein isothiocyanate (FITC) and keyhole limpet hemocyanin (KLH)) were assessed by antigen-specific enzyme linked immunosorbent assay (ELISA). Although antibodies were induced to some markers, these were unreliable and may have been affected

by temperature and smoltification. Detectable antibodies to ISAV antigen were also largely inconsistent despite low serum dilutions of 1/20 being employed for serological analysis. The poor antibody responses of salmon to the inactivated ISA vaccine suggested that DIVA vaccination is not feasible for ISA. A similar approach for KHV, utilising green fluorescent protein (GFP) as the marker, similarly failed to induce sufficiently detectable antibody responses in vaccinated carp (*Cyprinus carpio* L.). However, as high anti-KHV antibody titres were obtained with an inactivated KHV vaccine (≥1/3200), alternative approaches were carried out to assess the feasibility of DIVA vaccination for carp. Investigations of early KHV pathogenesis *in vivo* and antigen expression kinetics *in vitro* (0-10 days post infection (dpi)) provided valuable data for the diagnostics necessary for DIVA surveillance strategies.

Following viral infection, molecular methods were shown to be the most effective approach for early detection of KHV infected fish prior to sero-conversion, during which time antibodies are not detectable. An experimental immersion challenge with KHV, however, revealed complications in molecular detection during early infection. The KHV DNA was detected in external biopsies of skin and gills, but also internally in gut and peripheral blood leukocytes ≤ 6 hours post infection (hpi), suggesting rapid virus uptake by the host. The gills and gut appeared to be possible portals of entry, supported by detection of DNA in cells by *in situ* hybridisation (ISH). However, many false negative results using organ biopsies occurred during the first 4 dpi. The gills were the most reliable lethal biopsy for KHV detection by various polymerase chain reaction (PCR) assays, with a PCR targeting a glycoprotein-gene (ORF56) and a real-time PCR assay being the most sensitive of the 7 methods investigated. Importantly, non-lethal mucus samples reduced the number of false negative results obtained by all KHV PCR assays during the earliest infection stages with large levels of viral DNA being detected in mucus (up to 80,000 KHV DNA genomic

equivalents 200 μL^{-1}). KHV DNA was consistently detected in the mucus as a consequence of virus being shed from the skin.

Determining the expression kinetics of different viral structural proteins can be useful for DIVA serological tests. Analysis of KHV antigen expression in tissues by immunohistochemistry and indirect fluorescent antibody test was inconclusive, therefore 2 novel semi-quantitative immunofluorescence techniques were developed for determining KHV antigen expression kinetics in susceptible cell lines. During the course of KHV infection *in vitro*, a greater abundance of capsid antigen was produced in infected cells compared to a glycoprotein antigen (ORF56), as determined by detection with antigen-specific monoclonal antibodies (MAbs). The capsid antigen was characterised as a ~100 kDa protein by SDS-PAGE and identified as a product of KHV ORF84 by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF/TOF MS). This antigen was subsequently detected in the serum of >25% of KHV infected/exposed carp (6/17), as well as in carp vaccinated with a live attenuated vaccine (3/4), but not with an inactivated vaccine (0/7), by Western blot making it a potential DIVA target for an inactivated vaccine.

Attempts were made to improve the sensitivity of KHV serological testing by taking advantage of recombinant proteins specific for KHV (CyHV-3), rORF62 and rORF68 and eliminating any interference by cross-reacting antibodies to carp pox (CyHV-1). These proteins successfully reacted with anti-KHV antibodies. The feasibility of DIVA strategies for KHVD was determined using these recombinant antigens to coat ELISA plates. Differential antibody responses were detected from carp sera to an internal virus tegument protein (rORF62) and external region of a transmembrane protein (rORF68). Fish vaccinated with an inactivated vaccine produced significantly lower antibody responses to rORF62 than to rORF68, whereas infected, exposed and live attenuated vaccinated fish recognised both proteins allowing differentiation between vaccinated and infected carp. However, the

sensitivity of the assay was limited, possibly by high levels of natural antibodies detected at the relatively low serum dilutions (1/200) used. As the capsid antigen (ORF84) and tegument protein (ORF62) are derived from internal KHV structural proteins, they induce non-neutralising antibodies, which may be useful for DIVA strategies. Such antibodies are longer lasting than neutralising antibodies and often comprise the majority of fish anti-viral antibodies. This was noted in a fish surviving experimental challenge, which had an antibody titre of 1/10,000, but neutralising titre of 1/45. Such antigens may therefore hold potential for developing effective serological diagnostic tests for KHV and provide the potential for DIVA strategies against KHVD. Natural antibodies will, however, continue to present a challenge to the development of sensitive and reliable KHV serological tests, and hence the application of DIVA strategies.

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Chapter 1

General Introduction

1.1. History of vaccinology and serology

Although the science of 'immunology' has been dominated by research on human and mammalian disease models, and much of what is known in lower vertebrate immunology, i.e. in fish, is based on these findings, the initial concept, rather ironically, all started in starfish! Elie Metchnikoff in 1882 found that after piercing starfish with a 'rose thorn', a coating of cells had developed around the thorn. These cells were later identified as phagocytes (Lydyard *et al*., 2004; Podolski, 2012).

Vaccination aims to mimic the development of natural immunity against disease, which is usually attempted by means of inoculating the host with non-pathogenic, but still immunogenic components of the pathogen or an inactivated or attenuated whole pathogen (Meeusen *et al*., 2007). Vaccination originated from 'cow' when Sir. Edward Jenner in 1796 conferred protection against smallpox in humans by inoculating children with the closely related, but avirulent, cowpox virus ('*vaccinus*' meaning 'from cows') (Hilleman, 2000; Cann, 2005; Moennig, 2005; Meeusen *et al*., 2007). However, the practice of 'variolation' (inoculation of material from small pox lesions) had been practiced in China for many years prior to this (Leung, 2011).

While the discovery of 'innate immunity' is assigned to Metchnikoff, the field of 'acquired immunity' was arguably founded by Louis Pasteur, Robert Koch, Emil von Behring and Paul Ehrlich. Their findings made vast grounds in the fields of bacteriology, medicine and empirical discoveries on immunology, namely antibody response. Ehrlich developed specific methods for the quantification of antibodies making von Behring's theory of passive immunity possible (Hilleman, 2000), and this could possibly be flagged as one of the initial advances into serological research.

Many advances in vaccinology have been made over the last sixty years, through a number of biotechnological approaches yielding whole, subunit, live attenuated, DNA, killed

or recombinant viral or bacterial vaccines (Henderson, 2005; Meeusen *et al*., 2007). These advances in vaccine development can be divided into eras (Hilleman, 2000) as illustrated in Fig. 1.1 A. The adaptive immune response of fish and the production of antibodies, e.g. in salmonids and cyprinids, to pathogenic bacteria, were first recognised in the 1940s (Smith, 1940 cited in Evelyn, 1997). The first report on the development of a fish vaccine was published as early as 1942, as an apparent oral immunisation against furunculosis (Duff, 1942 cited in Evelyn, 1997). It was not until the 1970s, however, that there was any further interest in fish vaccinology. Prior to vaccine development, attention for aquatic disease control was paid predominantly to the application of chemotherapeutants (Evelyn, 1997). Nonetheless, the initial successful vaccination of farmed fish against *Vibrio ordalii* and *Vibrio anguillarum* paved the way for a number of vaccines developed for the aquaculture industry (Evelyn, 1997). Since the 1970s, vaccination has been a major contributor to the success of salmonid culture, and fish have been protected against bacterial diseases, such as vibriosis and furunculosis, while reducing costs and environmental damage associated with the application of antibiotics (Grave *et al*., 1990; Lillehaug *et al*., 2003 Sommerset *et al*., 2005a).

The control of modern day terrestrial animal diseases, however, requires surveillance strategies for which serology has become an increasingly useful tool for the detection of specific antibodies in mammals and birds following immunisation with veterinary vaccines (Pasick, 2004; Suarez, 2005; Meeusen *et al*., 2007). The science of serology was initiated through the discovery of blood groups (i.e. A, B, AB and O) when Karl Landsteiner determined that ‘clumping’ of normal human red blood cells occurred when exposed to foreign healthy blood cells (Weatherall, 2011). The antibody-antigen complexes causing the agglutination instigated the development of immunoassays such as enzyme-linked immunosorbent assay (ELISA) by Eva Engvall and Peter Perlmann (Lequin, 2005).

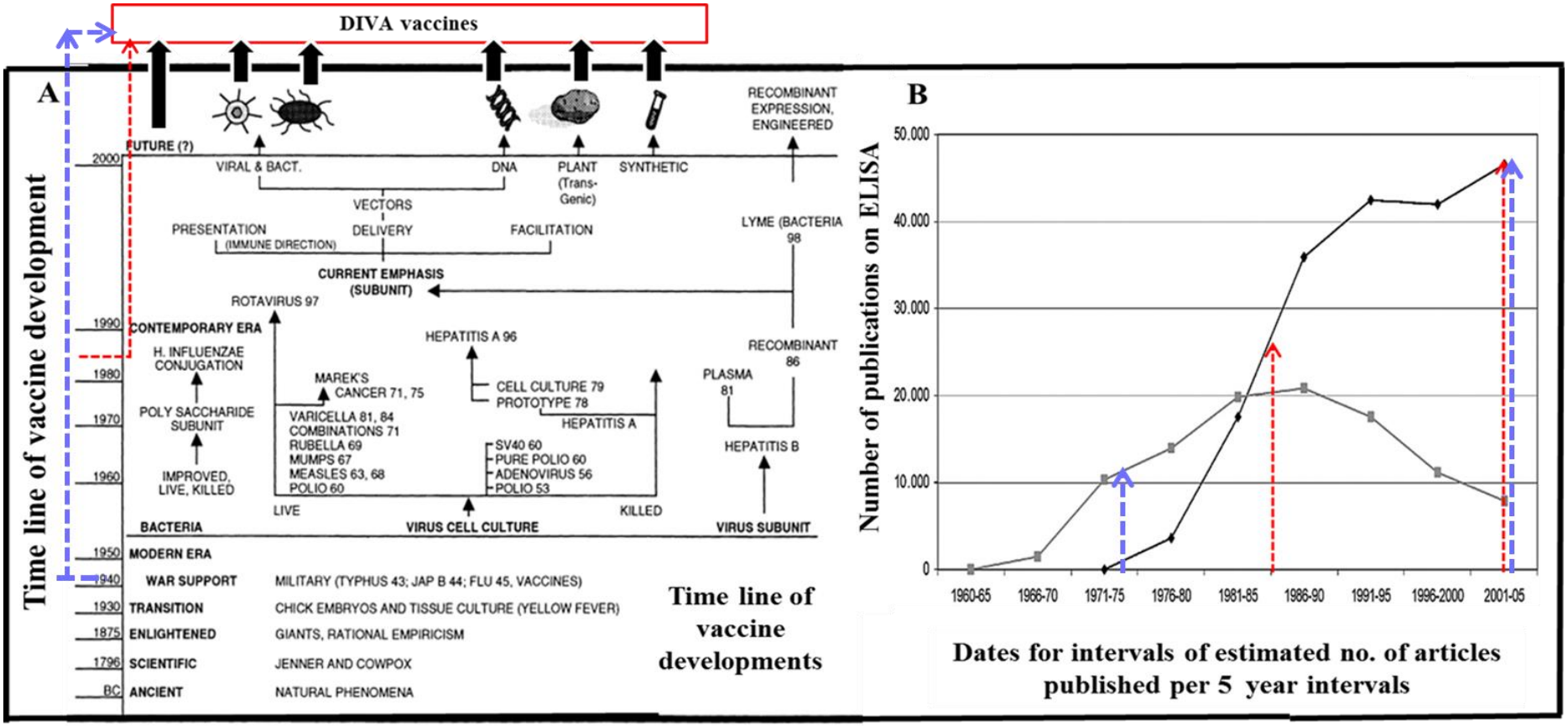


Figure 1.1 The history of vaccine development from BC – 2000 and the application of ELISA in science from 1960-2005. (A) Advances in vaccinology throughout history showing major breakthroughs of vaccines developed against human pathogens, *Modified after Hilleman (2000)*; (B) The estimated number of articles published by PubMed search within 5 year intervals using search terms: enzyme-immunoassay and enzyme-linked immunosorbent assay (ELISA) and immunosorbent assay (black line) and radio-immunoassay (RIA) (grey line), *Modified after Lequin (2005).* Blue dashed line indicates the time line of research and development undertaken for fish. Red dashed line indicates the time line of research and development undertaken on marker/DIVA vaccination.

The importance and application of serology has paralleled that of vaccine development, which is evident by the increasing use of immunoassays, e.g. ELISAs, in research (Fig. 1.1 B). When vaccination and serology are used in combination, disease management can be undertaken most effectively with revolutionary and advanced biotechnological tools. Serological analysis in warm blooded animals is now regarded as a primary epizootiological and diagnostic tool, however, serological procedures for monitoring of fish for infectious viral diseases is not used routinely in aquaculture (La Patra, 1996; Office International des epizooties (OIE), 2012). This is despite establishing 40 years ago that serology could be used to identify exposure of teleost fish to viral infection, e.g. in rainbow trout (*Onchorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) (Plumb, 1973; Jorgensen, 1974 cited in La Patra, 1996; Amend and Smith, 1974). Considering the advances in biotechnological tools available and knowledge of the fish immune response, for which it is known that detectable long-lasting anti-viral antibody responses are induced to certain diseases (La Patra, 1996; Lorenzen and La Patra, 1999; Adkison *et al*., 2005; Fregeneda-Grandes and Olesen, 2007; St-Hilaire *et al*., 2009), serological diagnostics is likely to make a valuable contribution to controlling fish disease in the future (Adams and Thompson, 2006; 2008).

1.2 Control and vaccination against notifiable mammalian and avian diseases

As noted by Moennig (2005), although aquatic animal production is different from animal production on land, the experiences in agriculture are worthwhile reviewing when deciding on and designing disease eradication or control measures in aquaculture.

Eradication programmes are often used as a means of controlling and preventing spread of notifiable diseases (Clavijo *et al*., 2004; Pasick, 2004; Moennig, 2005; Meeusen *et al*., 2007; Vannie *et al*., 2007; Uttenthal *et al*., 2010). Considering the rapidity with which disease can spread amongst farms, especially in regions of intensive culture, eradication measures conducted through what are known as 'stamping-out' policies alone can be extremely damaging (Pasick, 2004). Furthermore, exclusion and slaughter may not be sufficient to eradicate notifiable pathogens promptly (Clavijo *et al*., 2004).

The economical and animal welfare costs associated with eradication programmes has been highlighted from notifiable disease outbreaks in Europe including foot and mouth disease (FMD), highly pathogenic avian influenza (HPAI) and classical swine fever (CSF) (also known as hog cholera) (Clavijo *et al*., 2004; Pasick, 2004). Outbreaks of FMD in the UK in 2001 resulted in losses of up to €4.5 billion with 4.2 million animals slaughtered (Pasick, 2004). Monumental impacts were inflicted as a result of avian influenza (AI) outbreaks in USA, between 1983 and 1984, when 17 million birds were slaughtered (Halvorson, 2002), which was followed by outbreaks that devastated the Italian poultry industry in 1999 - 2000, when 14 million birds were slaughtered resulting in losses of €500 million (Pasick, 2004). However, the culling of birds infected or exposed to avian influenza virus (AIV) is still considered to be a less costly control strategy when the disease outbreak is wide-spread (Avellaneda *et al*., 2010). In 1980 the European Union (EU) adopted a non-vaccination and stamping-out policy against CSF, which relied on pre-emptive slaughter of neighbouring herds in regions with high density pig populations (Pasick, 2004). More than half (56%) of the US$ 2.3 billion worth of losses due to CSF outbreaks in the Netherlands in 1997 - 1998 were attributed to depopulation of infected herds and welfare slaughter (Meuwissen *et al*., 1999). Young piglets were also slaughtered and movement restrictions were put in place (Pasick, 2004). In Canada, the total net economic impact to FMD was

between $CND13.7 – 45.9 billion, based on models, depending on the number of affected premises (Clavijo *et al*., 2004). Emergency vaccination programmes may have been utilised for the control of these outbreaks. The reproduction '*R*' value is a measure of the transmission of a virus defined as "the average number of secondary cases caused by one typical infectious individual" (Van Oirschot, 1999; Bouma, 2005). An infection will fade out if $R<1$, but an infection will spread when $R>1$ (Bouma, 2005). As a result of huge losses due to notifiable viral disease outbreaks, emergency vaccination procedures were considered a useful option to shorten the duration of such outbreaks until the '*R*'value was <1 (Pasick, 2004).

The predominant aim of vaccination for animal agriculture is to provide the most economically beneficial production of stock and provide protection to the consumer against potential zoonotic diseases. Immunisation for livestock and poultry has been estimated to have a greater impact on these economies compared to all other therapeutic and prophylactic treatments combined (Babiuk, 1999). Diseases causing mass mortalities and those that may not cause fatality, but do result in reduced growth rates and welfare concerns, make veterinary vaccination a valuable asset for which innovative vaccine development is important (Meeusen *et al*., 2007; Vannie *et al*., 2007).

For centuries rinderpest (RP) was the most feared of all agricultural diseases, which threatened the livestock and general wellbeing of pastoral communities across much of Asia and Africa (Buczkowski *et al*., 2012). The power of well managed vaccination against animal diseases was demonstrated by the administration of a highly immunogenic, efficacious and safe attenuated live vaccine against rinderpest virus (RPV), for which RPV control was otherwise conducted by rigorous stamping out policies involving quarantine and mass slaughter (Diallo *et al*., 2007; Parida *et al*., 2007; Buczkowski *et al*., 2012; Albina *et al*., 2013).

Conventional efficacious vaccination is intended to prevent disease, reduce clinical symptoms, reduce pathogen shedding, and increase resistance to infection (i.e. the dose required to cause infection), whilst providing a method to manage or eradicate a disease from a region (Vannie *et al*., 1991; Bouma, 2005; Henderson, 2005; Uttenthal *et al*., 2010). The effectiveness of vaccination has also been demonstrated for AD, FMD, AI and CSF, with reduction in virus shedding and transmission to susceptible in-contact animals that subsequently benefit from herd immunity (Donaldson and Kitching, 1989; Halvorson, 2002; Dewulf *et al*., 2003 cited in Pasick, 2004; Bouma, 2005). However, most vaccines do not completely protect the animal from infection or prevent shedding of the pathogen subsequent to infection. In this context, the vaccine effectiveness within a population, i.e. a measure of its ability to reduce virus transmission, is an important characteristic to be considered (Pasick, 2004; Bouma, 2005). Therefore, where notifiable diseases are concerned, it is vital to detect active infection in vaccinated animals to avoid spreading the disease (Clavijo *et al*., 2004; Uttenthal *et al*., 2010). However, as sero-surveillance is used to monitor exposure of animals to the virus, vaccinated animals cannot be distinguished from infected animals as antibodies induced by vaccination cannot be differentiated from those induced by infection. Although vaccines are available to these diseases in the EU, 'decisions to vaccinate – policies' are made by the appropriate authority based on the severity and impact of the disease (Uttenthal *et al*., 2010). The situation differs from the contingency planning for other animal diseases, such as bluetongue, where compulsory vaccination is advocated once a disease outbreak has initiated (Uttenthal *et al*., 2010). Identifying those vaccinated animals by serology may, however, also be problematic. Indeed, vaccination programmes against RPV that eventually led the way to the first successful global eradication of a viral animal disease (Diallo *et al*., 2007; Albina *et al*., 2013), did not enable a disease-free status for many years. This was because of the inability to differentiate between infected ruminants from those that were

vaccinated, which was required for a RPV-disease free status by the World organisation for animal health; OIE. Despite a highly sensitive and specific competitive ELISA being developed to distinguish antibodies to RPV infection from the closely related morbillivirus, peste des petits ruminants virus (PPRV) (Anderson and McKay, 1994), it took many more years to demonstrate sero-naivety after terminating vaccination programmes (Buczkowski *et al*., 2012). Suarez (2005) accurately stated in a review of DIVA strategies for AI that "vaccination by itself cannot be used as an eradication programme. An eradication programme must include strict quarantines, movement controls on animals and equipment, increased biosecurity, increased active and passive surveillance and education of affected groups."

1.3 Control and vaccination of notifiable viral diseases in aquaculture

The aquaculture industry has grown rapidly since 1970 with a mean annual growth rate of 8.8% to satisfy increasing demands. As a result, viruses of fish have become an area of interest to the public due to the increasing number of reports on epizootics and economic losses associated with them (Ilouze *et al*., 2011). Strict surveillance is carried out for a number of notifiable viral diseases caused by members of the rhabdoviridae, orthomyxoviridae and alloherpesviridae that, although effective, have proved both economically and ethically destructive to the aquaculture industry.

Most vaccines available to the aquaculture industry are killed/inactivated whole virus vaccines that are safer than live vaccines, but provide weaker efficacy than live vaccines, which stimulate both cellular and humoral branches of the immune system (Sommerset *et al*., 2005a; Dhar and Allnutt, 2011; Gomez-Casado *et al*., 2011). Furthermore, cost-effective inactivated viral vaccines are difficult to develop for commercial scale production as high doses of intra-peritoneal (ip) administered adjuvanted cell culture-derived antigen are usually

required to provide protection (Sommerset *et al*., 2005a). Nonetheless, the cost-effect margin can be greatly influenced by control measures necessary for particular diseases affecting particular fish species.

Viral haemorrhagic septicaemia (VHS) is one of the most economically important viral diseases of salmonid aquaculture. The large number of susceptible host species of viral haemorrhagic septicaemia virus (VHSV) has made control of this rhabdovirus very difficult (OIE, 2012). In Denmark the rainbow trout industry suffered massive losses with 400 rainbow trout farms infected, and costs in Europe mounted to £40 million per year (Olesen, 1998). Sanitation programmes were subsequently implemented for stamping out VHS in Denmark in 1965, which resulted in a drop in infected farms from 400 to 26, but expenses were all covered by the farmer until financial support was later provided by the EU (Olesen, 1998). After the eradication programme, 454 farms were subsequently free of VHSV (Olesen, 1998). However, these health surveillance schemes coupled to stamping out procedures have been very expensive (Olesen and Korsholm, 1997; Olesen; 1998) requiring all fish on the farm to be destroyed when an outbreak was detected, and the farm was left empty through a fallow period (Olesen, 1998; OIE, 2012). These sanitation programmes proved very effective throughout Europe, however outbreaks have been reported again recently in the UK and Norway (Stone *et al*., 2008; Dale *et al*., 2009) where the latter was attributed to transmission from free-living fish with an isolate previously considered to be avirulent (Dale *et al*., 2009). Despite extensive research into development of a VHSV vaccine, which has included killed, live attenuated and recombinant (through both prokaryotic and eukaryotic expression systems) (Lorenzen and La Patra, 2005) no commercialised VHSV vaccine is available (Lorenzen *et al*., 1998; Lorenzen and La Patra, 2005; Sommerset *et al*., 2005a; Gomez-Casado *et al*., 2011). The most promising vaccine development for salmonid rhabdoviruses has been the development of DNA vaccines. This

led to the successful commercialisation of a DNA vaccine for the closely related infectious hematopoietic necrosis virus (IHNV) in Canada (Anderson *et al*., 1996a; b; Gomez-Casado *et al*., 2011). Nonetheless, for VHSV, vaccination would not be permitted in zones that are VHS-free according to EU regulations (OIE, 2012). Vaccination is not currently approved in VHS or IHN - free farms in the EU. However, killed or non-replicating vaccines may be approved for purposes of stocking infected farms with vaccinated fish (Olesen, 1998), i.e. to limit viral spread, although survivors can also become long-term carriers, meaning that vaccinated animals could continue to spread disease. Surveillance is difficult by virus isolation, which may not be possible in clinically healthy fish (Skall *et al*., 2005) thus detection of virus antigen using cross-serotype reactive monoclonal antibodies (MAbs) of VHSV isolates (Lorenzen *et al*., 1988) or nucleic acid by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Garver *et al*., 2011; Jonstrup *et al*., 2012) are undertaken to confirm VHS-free status. Similar surveillance methods are also used for the other important salmonid rhabdovirus, IHNV (Jorgensen *et al*., 1991).

Twenty years after the introduction of sanitation programmes for the control of VHSV, an even more destructive viral disease began to threaten the success and sustainability of salmonid aquaculture, this time infecting Atlantic salmon. Infectious salmon anaemia (ISA) is one of the most economically important diseases of farmed Atlantic salmon, with a history of outbreaks in both the Northern hemisphere (Lyngstad *et al*., 2008; Kibenge *et al*., 2009a; Murray *et al*., 2010) and southern hemisphere (Godoy *et al*., 2008; Mardones *et al*., 2009; 2011). Due to the serious economic consequences, multiple transmission routes and difficulty in containment and control, ISA previously constituted the only "list 1" classified notifiable fish disease under the former OIE disease classification system (OIE, 2009). The first recorded outbreak of ISA was in Norway in 1984, which resulted in 80% mortality. Subsequently the prevalence of ISA disease outbreaks peaked in 1990 when 80 cases were

reported (Thorud and Djupvik, 1988; Lyngstad *et al*., 2008). Between 1984 and 2005 a total of 437 outbreaks were reported in Norway. However, if it were not for the implementation of regulatory controls in the 1980s and 1990s by Norwegian authorities (Lyngstad *et al*., 2008) these figures may have continued to rise. During this period the use of non-disinfected sea water in hatcheries and movements of fish between sea sites was banned (Lyngstad *et al*., 2008). Furthermore, health certificates were required for operating fish farms and regulations were implemented for disinfection of waste water from processing plants and slaughter houses (Thorud and Håstein, 2003). Since these stringent sanitary measures were employed the number of ISA cases in Norway has reduced, but between 1 and 20 cases are still reported annually (Lyngstad *et al*., 2008). The annual cost of ISA to Norwegian aquaculture is estimated at US$ 11 million.

Infectious salmon anaemia virus (ISAV) has since been detected in all major Atlantic salmon producing regions including New Brunswick, Canada in 1996, Scotland in 1998, Nova Scotia in 1999, Faroe Islands in 2000, Maine, USA in 2001 and Chile in Coho salmon in 2001 (Bricknell *et al*., 1998; Mullins *et al*., 1998; Rodger *et al*., 1998; Bouchard *et al*., 2001; Ritchie *et al*., 2001; Lyngøy, 2003). The first outbreak of ISA outside of Norway occurred in New Brunswick in 1997 resulting in losses of $14 million with 21 farms infected within 3 bays (Hastings *et al*., 1999). Outbreaks of ISA in Atlantic salmon have been reported since and different countries have employed different procedures to deal with the disease (Miller and Cipriano, 2003). In Maine, 2001, 19 cases of ISA were confirmed and precautionary measures to prevent transmission alone were very costly (Miller, 2003). The state's salmonid industry had already voluntarily depopulated about 900,000 salmon worth nearly $11 million. Trade was severely affected as the EU and Chile prohibited importation of trout and salmon eggs from USA, which alone accounted to $2 million (Miller, 2003).

The first outbreaks of ISA in the EU occurred in Loch Nevis, Scotland in 1998 (Rodger *et al*., 1998). The virus spread to 11 farms with another 25 farms suspected and the last confirmed outbreak, during this period, was in the Shetland Isles in 1999, although ISAV was still present on farm sites within surveillance zones in 2000 (Stagg *et al*., 2001). However, eradication programmes have been successfully implemented for the control of ISA, which were achieved in Scotland to control the outbreaks in 1998 – 1999 that had cost the industry £32 million (Bricknell *et al*., 1998; Rodger *et al*., 1998; Hastings *et al*., 1999; Stagg, 2003). The success was attributed to regulated extensive culling, restricted personnel and vehicle movements, and restricted fish and fish product movements. The implementation of control and surveillance zones with regular inspections, during and following the disinfection and fallow period, coupled with improved codes of practice for fish husbandry ensured no disease recurrences (RSE, 2002). However, no compensation was provided for farmers that suffered the effects of ISA outbreaks. Infectious salmon anaemia transmission is very efficient within the transport networks of live fish, harvesting operations, well-boat movements, wild fish and water currents (Nylund and Jakobsen, 1995; Munro *et al*., 2003; Gustafson *et al*., 2007; Munro and Gregory, 2009). Thus when ISA returned to Scotland in 2008 - 2009 at 6 sites in the Shetlands, it was effectively controlled and limited to a localised spread by 'management areas' implemented by Marine Scotland where strict movement restrictions were enforced and depopulation of confirmed sites achieved within 7 weeks (Murray *et al*., 2010). However, because of preventative legislation in Europe, which employed a stamping-out policy, vaccines were not used despite being available in Canada (Sommerset *et al*., 2005a).

The most disastrous effects of ISA were experienced in Chile between 2007 - 2009, where the epidemic had devastating social and economic consequences on the most important animal production system in the country (Mardones *et al*., 2011). From 2008, 159 salmon

farms in Chile were registered as positive for ISA by Chile's National Fisheries and Aquaculture Service, SERNAPESCA (Kibenge *et al*., 2009a), costing the industry $2 billion directly as well as a 30% increase in production costs. Many communities were affected and 15,000 people were made unemployed (Mardones *et al*., 2011). The harvest of Atlantic salmon in Chile in 2009 was 61% lower than the previous year and recovery is not expected until 2015 (Asche *et al*., 2010). Recent epidemiological analysis of the outbreaks suggests that control strategies in such highly populated farmed areas should implement control zones of at least 10 km in any future events rather than the 5 km recommended by the OIE (Mardones *et al*., 2011). This would possibly be more effective, but ultimately also incur more animal slaughter and trade restrictions.

Problems are encountered with the current diagnostic procedures that are recommended for detecting ISAV in suspected sites. The criteria according the OIE 'Manual of Diagnostic Tests for Aquatic Animals' (OIE, 2012) includes the use of virus isolation in cell culture, but many isolates and strains of ISAV have limited cytopathogenicity thus may not replicate within the cell line and/or lack cytopathic effect (CPE) (Dannevig *et al*., 1995; Rolland *et al*., 2003; 2005; Kibenge *et al*., 2006). It may take a number of weeks or months before ISA develops in neighbouring pens and surveillance at present, other than cell culture isolation, is based on virus detection by indirect fluorescent antibody technique (IFAT) and RT-PCR for confirmation, whilst antibody detection is accepted to indicate a suspect case (OIE, 2012). Vaccination has been carried out in North America and Faroe Islands in the last 5 years, but does not provide complete protection in Atlantic salmon (OIE, 2012). The current vaccines available are mostly whole inactivated vaccines (Dhar and Allnutt, 2011) that do not provide clearance of the virus in immunised fish which may subsequently become carriers (Kibenge *et al*., 2004; OIE, 2012). Therefore considering that antibody detection is included as diagnostic criteria for a suspected case, it would be necessary to indicate that

antibody responses to ISAV during surveillance programmes were induced by vaccination and that those fish are not carriers.

During the outbreaks of ISA in Scotland in 1998, another important aquaculture sector, the common carp and koi industry, had suffered major losses as a result of a highly virulent, temperature-dependent disease, koi herpesvirus disease (KHVD). The outbreaks of KHVD have represented a contrasting problem to the epidemiological situation of the notifiable rhabdovirus and orthomyxovirus diseases. A lack of regulation of koi movements in Europe under EC (European Commission) or national health legislation may have represented a major root-cause to the spread of KHVD because fish from the ornamental industry were screened less stringently than food fish at border crosses (Haenen *et al*., 2004; Pearson, 2004). The enormous impacts incurred by KHVD have led to the virus being described as "the worst and most rapidly spreading virus in the past 30 years" (Hedrick pers. comm. cited in Pearson, 2004), which has affected not only carp fisheries (Peeler *et al.*, 2009; Taylor *et al*.; 2010; 2011), the ornamental koi and food carp industries (Perelberg *et al*., 2003; Antychowicz *et al*., 2005; Gomez *et al*., 2011; Azila *et al*., 2012), but also wild carp populations throughout the world (Takashima *et al*., 2005; Grimmett *et al*., 2006; Uchii *et al*., 2009; 2011; Garver *et al*., 2010; Minamoto *et al*., 2011; 2012). Koi herpesvirus disease was recently likened to the global human influenza pandemic of 1918 (Ilouze *et al*., 2011).

The first outbreaks of KHVD occurred in Israel in 1998 (Ariav *et al*., 1999) and in the following three years regular outbreaks occurred throughout the Spring and Autumn at temperatures between 22 - 26ºC (Haenen *et al*., 2004). The disease had spread to 90% of all carp farms in Israel by the end of 2000, costing the carp industry $3 million per year (Perelberg *et al*., 2003). Disease outbreaks also occurred in the USA where carp exhibited similar disease signs, which were initially reported at a koi show in New York in 1998 (Hedrick *et al*., 2000). These exhibitions are thought to have contributed to the spread of

KHVD as fish were transported and held together in tanks or ponds without quarantine (Haenen *et al*., 2004). Isolation of the aetiological agent, koi herpesvirus (KHV), at this time in koi fin (KF-1) cells facilitated the development of sensitive diagnostic methods, including the real-time PCR (Gilad *et al*., 2004), that has since proved pivotal for characterisation and surveillance of KHVD. However, in the absence of sensitive diagnostic tests, incidents of KHVD were becoming very common. Two more outbreaks were reported in Los Angeles and California in 1999 (Gray *et al*., 2002). In Europe, the first outbreaks were reported with mass mortalities in ponds and koi dealerships in 1997 and 1998 (Bretzinger *et al*., 1999; Hoffmann *et al*., 2000 cited in Haenen *et al*., 2004). KHV was isolated in 2001 in a different cyprinid cell line, common carp brain cells (CCB), in Germany following further mass mortalities (Neukirch and Kunz, 2001). This cell line has since been preferred for propagation of the virus for both virological studies and diagnostics. Outbreaks of KHVD in the UK had also occurred following imports from Israel (Way *et al*., 2001 cited in Haenen *et al*., 2004), which suggested possible sources of transmission.

The disease has since been reported extensively worldwide in at least 28 countries (OIE, 2012), throughout Europe (Haenen *et al*., 2004; Bergmann *et al*., 2006; Novotny *et al*., 2010; Doszpoly *et al*., 2011; Toplak *et al*., 2011), Asia (Ariav *et al*., 1999; Perelberg *et al*., 2003; Sano *et al*., 2004; Tu *et al*., 2004; Sunarto *et al*., 2005; Bondad-Reantaso *et al*., 2007; Pokorova *et al*., 2007; Murwantoko, 2009; Pikulkaew *et al*., 2009; Cheng *et al*., 2011; Dong *et al*., 2011; 2013; Gomez *et al*., 2011; Lio-Po, 2011), Africa (Haenen *et al*., 2004), and North America (Hedrick *et al*., 2000; Haenen *et al*., 2004; Garver *et al*., 2010), but not South America or Australasia/Oceania (Pokorova *et al*., 2005). Outbreaks are still continuously being reported with the most recent reports of KHV prevalence in South Korea (Gomez *et al*., 2011), Slovenia (Toplak *et al*., 2011), Hungary (Doszpoly *et al*., 2011), China (Dong *et al*.,

2011; 2013), Romania, Spain and Sweden (OIE, 2012). The virus is likely to be present in more countries where it has not yet been detected (OIE, 2012).

The outbreaks occurring in Asia devastated the carp industries in many countries where carp represents such an important market. After high mortalities (80 – 95%) on carp farms in Indonesia in 2003 (Rukyani, 2002 cited in Haenen *et al*., 2004) that resulted in costs of ~ $15 million, the Indonesian government declared Java and Bali as isolated. Movements of carp from this region to other islands were subsequently prohibited unless quarantine checks were carried out (Sunarto and Rukyani cited in Haenen *et al*., 2004). In Japan 1200 tonnes (t) of carp died during the 2003 outbreaks of KHVD in Lake Kasuminguara, Ibaraki prefecture and in 2004 KHV had spread and was eventually detected in 42/47 prefectures in Japan with more than 100,000 mortalities reported (Iida and Sano, 2005; Ishioka *et al*., 2005; Matsui *et al*., 2008; Yuasa and Sano, 2009). The disease had seriously threatened the $75 million ornamental carp industry and consequently all nishikigoi shows were cancelled in November 2003 (Haenen *et al*., 2004). Strict national disease control measures were subsequently put in place by the Indonesian and Japanese governments in an attempt to contain the outbreaks (Haenen *et al*., 2004).

The spread of KHV is predominantly considered to be through trade in koi carp, particularly before the disease was understood and sufficient diagnostic tools were available to detect the virus. This prevented KHVD being listed as a notifiable disease because absence of disease could not be ascertained and diseased fish could not be confidently identified, which limited the usefulness of legislation (OIE, 2012). However, since being listed, a vast number of studies have been carried out to develop more sensitive, specific, convenient and cost-effective diagnostic tools for KHV surveillance. The most sensitive detection has been achieved through molecular based approaches. These are being continuously developed and improved to detect KHV DNA (Gilad *et al*., 2002; 2003; 2004; Gray *et al*., 2002;

Gunimaladevi *et al*., 2004; Soliman and El-Matbouli, 2005; Bergmann *et al*., 2006; 2009a; 2010a; b; Yoshino *et al*., 2006) and RNA designed to detect replicating virus in fish with possible persistent infections (Yuasa *et al*., 2012a). However, there are still no validated tests that are accepted for declaration of freedom from KHV (OIE, 2012).

Methods to control KHVD generally rely on avoidance of exposure, good hygiene and biosecurity practices (OIE, 2012). Quarantine protocols with sentinel fish at permissive temperatures and depopulation followed by disinfection (Kasai *et al*., 2005) may contribute to limiting KHVD outbreaks, especially as KHV has poor survivability in water (Perelberg *et al*., 2003; Shimizu *et al*., 2006; OIE, 2012). However, controlling the rapid transmission and global spread of KHV is complicated by a number of potential vectors (Bergmann *et al*., 2009a; b; 2010c; Kempter *et al*., 2009; Ilouze *et al*., 2011; Kielpinski *et al*., 2010; Fabian *et al*., 2013), which are often cultured in close proximity with susceptible carp or in the same pond (Kempter *et al*., 2009).

Water temperature is known to have a major influence on the replication kinetics of KHV and subsequent KHVD (St-Hilaire *et al*., 2005; 2009; Yuasa *et al*., 2008; Siwicki *et al*., 2012). Manipulation of water temperature (i.e. raise > 30ºC) has been proposed as a method for controlling KHVD outbreaks (Omori and Adams, 2011). However, carp may subsequently become persistent carriers of the virus. Survivors of KHV are a problem as they may be resistant to KHVD but can still transmit the virus to naïve carp (St-Hilaire *et al*., 2005). Such approaches were previously applied during the Israeli outbreaks in order to produce 'naturally immunised' carp, however, only ~60% were protected (Ronen *et al*., 2003; Michel *et al*., 2010a) and these fish subsequently carry wild-type virus.

Differences in KHV-susceptibility between breeds and strains has been demonstrated (Shapira *et al*., 2005; Hedrick *et al*., 2006; Dixon *et al*., 2009; Rakus *et al*., 2012; Piačková *et*

al., 2013) and breeding programmes for genetically resistant strains has been suggested as a possible strategy to control KHV spread (Rakus *et al*., 2009), however a breeding system could not be implemented for ornamental koi (Ilouze *et al*., 2011).

Vaccination is seen as a very important tool to control KHVD as eradication and disinfection has not been effective (Ronen *et al*., 2003; Costes *et al*., 2008; Perelberg *et al*., 2008; Michel *et al*., 2010a; Ilouze *et al*., 2011). Vaccination against viral disease has been successfully applied for carp, e.g. the first viral vaccine in aquaculture was developed against another notifiable disease, spring viraemia of carp (SVC), caused by a rhabdovirus, spring viraemia of carp virus (SVCV) in 1982 using inactivated vaccines (Sommerset *et al*., 2005a). However, in the case of KHVD, the vaccination strategy is also a crucial consideration. A live attenuated vaccine was developed in Israel to enable emergency vaccination procedures during the mass outbreaks between 1998 - 2000, which provided good levels of protection against KHV challenge. This vaccine (KoVax, KV3) has now been used widely across Israel and has indeed been commercialised. However, there may have been implications as a result of this, including the possible spread and transmission of wild-type virus from exported vaccinated carp. Peeler *et al*. (2009) developed a risk assessment on the impact of importation of carp to the UK in terms of KHV prevalence. They found that the importation of carp vaccinated with KV3 were likely carriers of wild-type virus, which was transmitted to naïve carp in the UK, as carp mortalities were observed in quarantine facilities. Many of these fish were marketed as, or perceived to be, 'safe', which exacerbated the risk of disease introduction as sites intending to 'protect' uninfected stocks actually risked introducing wild type virus to naïve fish (Peeler *et al*., 2009). Although a commercial vaccine has also recently been made available in the USA (Cavoy®, Novartis), such vaccines will continue to present risks to naïve, unvaccinated carp stocks.

Other vaccines have also been developed including inactivated (Yasumoto *et al*., 2006), live attenuated (Ronen *et al*., 2003; Perelberg *et al*., 2008) and recombinant multi deletion vaccines (Costes *et al*., 2008; 2012). However, identifying fish as vaccinated and uninfected provides a safer ground for introducing vaccinated fish to unvaccinated naïve populations.

1.4 Differentiating infected from vaccinated animals: 'DIVA' vaccination

1.4.1 Definition of Marker and DIVA vaccines

The term '**d***ifferentiating* **i***nfected from* **va***ccinated individuals*', i.e. '*DIVA'*, was first proposed by Jan T. Van Oirschot to replace the previously defined concept of 'marker vaccine' (Van Oirschot *et al*., 1986; 1996; Van Oirschot, 1999). A marker vaccine was defined by Van Oirschot *et al*. as "a vaccine (inactivated or live) based on deletion mutants or on isolated microbial proteins that allow the distinction between vaccinated and infected individuals based on the respective antibody responses. Hence, a marker vaccine is used in conjunction with a test that detects antibodies against a (glyco) protein, that is lacking in the vaccine strain" (Van Oirschot *et al*., 1996). Although the 'marker vaccine' was initially based on deletion mutants of the wild-type microbe in conjunction with a differentiating diagnostic test (Pasick, 2004) the term DIVA has now been extended to include subunit and whole killed vaccines (Pasick, 2004; Uttenthal *et al*., 2010), and any other vaccines developed that lack immunogenic proteins of the wild-type strain, e.g. DNA and recombinant vaccines. The accompanying serological diagnostic test has become just as important an area of research as the vaccine strain used for immunisation, as highly sensitive detection of marker-specific

antibodies must be feasible in order to effectively distinguish responses to the vaccine from responses to wild-type virus (Clavijo *et al*., 2004; Uttenthal *et al*., 2010). In the majority of cases the primary concern is to determine whether or not an animal has been infected, regardless of the vaccine status, which led Uttenthal *et al*. (2010) to propose the defined acronym for DIVA to be "**D**ifferentiating **i**nfect*ion in* **v**accinated **a**nimals"

1.4.2 DIVA strategies in practice

Disease control and eradication approaches using marker or DIVA vaccination have never been applied for the aquaculture industry. The challenges and successes experienced in agriculture can provide a guide to successful applications of DIVA strategies against notifiable diseases if this approach were to be adopted in aquaculture.

As well as reducing the impacts of disease, vaccination can also be used to manage or eradicate a disease from a region. It has previously been preferred to terminate vaccination, once a disease-free status had been achieved, as it is expensive for farmers to continue vaccinating (Bouma, 2005). Control of notifiable diseases by mass-culling is generally not acceptable to society (Pasick, 2004; Bouma, 2005), although this approach has been used extensively for both the livestock industry and aquaculture, e.g. for the control of ISA outbreaks in Scotland (Hastings *et al*., 1999). However, once a disease-free status has been obtained in consumer countries, trade may be adversely affected for vaccinating countries, as was observed with Aujeszky's disease (AD) in the Netherlands in the 1980s. At this time, disease free-countries, such as the UK and Denmark, employed a sero-surveillance-identify-cull strategy for AD without vaccination in order to retain disease-free status. Therefore export of swine products, e.g. to Japan and USA, was possible, but no import was allowed from countries where the disease was endemic, or if animals were vaccinated, as this would hamper sero-surveillance strategies (Bouma, 2005). Ultimately Pseudorabies virus (PrV), the

causative agent of AD, became an expensive virus for Dutch farmers, not because of animal losses from AD, but losses from trade as their vaccinated stock could not be exported (Stegeman *et al*., 1997 cited in Bouma, 2005). This is a similar problem for countries where FMD is endemic, as significant constraints are imposed on international trade in live animals and animal products, resulting in high economic impacts associated with loss of export markets and consumer fears (Paarlberg *et al*., 2002; Clavijo *et al*., 2004).

The application of marker vaccines, in combination with additional management measures, such as reduced contacts between herds, can contribute to reducing the *R* value <1 thus improving the possibility of disease eradication, e.g. as seen for PrV (Pasick, 2004; Bouma, 2005), while providing a means to identify uninfected vaccinated animals. The first successful application of a DIVA strategy was achieved for the control and subsequent eradication of PrV through use of a glycoprotein E (gE) negative vaccine and gE specific serological diagnostic test (Van Oirschot *et al*., 1990; 1996; Stegeman, 1995; Van Oirschot, 1999; Vannie *et al*., 2007). However, complications with using DIVA vaccination strategies for controlling notifiable disease also exist for the livestock industry (Bosman *et al*., 2012). Obtaining AD-free status breaks the trading restrictions with countries of the same status, but in the event of new outbreaks, as seen for CSF and FMD, disease can spread quickly to naïve pig populations. The control of AD infected farms would involve emergency vaccination and isolation of the virus by movement restrictions. According to the contingency plans for DIVA vaccination of PrV, a 10 km zone around infected farms (i.e. ring vaccination) would be established and all pigs within this zone would be protectively vaccinated while movement restrictions are imposed. This avoids culling of infected, but apparently healthy animals thus preventing controversial mass culling used for control of FMD and CSF (Bosman *et al*., 2012). However, other animal welfare issues that must be considered during emergency

DIVA vaccination strategies, are the overcrowding and aging of animals that cannot be traded out-with the surveillance zone during the course of the outbreak (Bosman *et al*., 2012).

However, the benefits of DIVA strategies far outweigh any potential negative impacts. DIVA vaccination has been accepted for control of AI by some countries in the EU. This has provided an opportunity for consistent monitoring of stocks, and assurance to trading partners on the infection-free status of vaccinated poultry during low pathogenic avian influenza (LPAI) (Capua *et al*., 2003; 2004; Avellaneda *et al*., 2010). Furthermore, prophylactic use of vaccines against exotic viral infections in production animals is now undertaken exclusively in regions where disease is endemic. For example, DIVA strategies have been used for many years in South America in order to satisfy their OIE status of 'FMD-free with vaccination' in support of exports, e.g. beef. Due to the extensive farming practiced in these countries, clinical surveillance is not easy and sero-surveillance using DIVA offers many advantages (Uttenthal *et al*., 2010). DIVA strategies have also enabled the eradication of FMD from vaccinated pig populations in the far-east and in countries after emergency vaccination, e.g. against FMD in Macedonia and Albania in 1996 (Uttenthal *et al*., 2010). Recently, a 'genetic DIVA' approach has also been applied to populations of wild boars against classical swine fever virus (CSFV) as transmission of the virus to domestic pigs caused outbreaks of CSF (Blome *et al*., 2011). Problems were encountered with diagnosis of dead pigs within the surveillance zones because of false positive PCR results to vaccine strain virus, thus a differential real-time RT-PCR (rRT-PCR) assay was developed in order to differentiate nucleic acid of wild-type virus from the vaccine strain (Blome *et al*., 2011).

Ultimately, implementing a combination of vaccination and eradication programmes through DIVA strategies could enable a 'vaccinate-to-live' policy for notifiable diseases. By emergency 'ring vaccination' with marker vaccines and DIVA sero-surveillance, it may be

possible to reduce transmission, clinical disease, and the presence of infectious virus within animal stocks, and perhaps fish farms, without jeopardising animal trade.

1.4.3 Marker and DIVA vaccine developments

The successful application of DIVA vaccination in eradication programmes for AD in pigs and AI in birds instigated the development of marker vaccines and DIVA systems for a number of other important notifiable diseases that inflict economical and ethical strain on the meat and poultry industries (Pasick, 2004).

The majority of developments carried out on DIVA-compatible vaccines and diagnostic tests initially focused on four of the most economically important trans-boundary diseases in Europe: AD, AI, FMD and CSF (Van Oirschot *et al*., 1996; Babiuk, 1999; Van Oirschot, 1999; Clavijo *et al*., 2004; Pasick, 2004; Bouma, 2005; Suarez, 2005; 2012; Beer *et al*., 2007; Vannie *et al*., 2007; Uttenthal *et al*., 2010). There have since been a vast number of studies and approaches to marker/DIVA vaccine development for many other diseases. Although the purpose of applying DIVA vaccine strategies for many of these diseases is similar, the technological approaches that have been applied are extremely diverse and differ depending on the biology of the RNA or DNA virus, as well as the characteristics of the disease that they inflict and antibody response they provoke (Table 1.1 & 1.2). For example, the groundwork undertaken on PrV using molecular biological approaches enabled mapping of, and functional roles to be assigned to, the various structural proteins of the virus prior to development of the marker vaccine and companion diagnostic test (Van Oirschot *et al*., 1986; 1996; Van Oirschot, 1999). Subsequently, the introduction of DIVA vaccination was largely attributed to collated knowledge of the herpesvirus glycoproteins of PrV (Mettenleiter, 2002). As conventionally attenuated PrV vaccines harbour a deletion within their genomes encoding for an immunogenic glycoprotein (Van Oirschot *et al*., 1990) marker vaccine approaches

initially focused towards several of these characterised envelope glycoproteins. Glycoprotein I or E (gI/gE) (Van Oirschot *et al*., 1996), was one of these targets (Van Oirschot *et al*., 1988; 1990; 1991). This envelope protein had proved an effective marker antigen as antibodies to this glycoprotein persist for >2 years in infected/exposed animals and it is expressed by at least the majority of field strains (Van Oirschot *et al*., 1990).

Different DIVA strategies have been developed since the origin of marker vaccines, some of which require the use of appropriate vaccines and specific companion serologic discriminatory tests (Avellaneda *et al*, 2010). However, many marker vaccines have been developed through more conventional routes without using DNA recombinant technology to engineer the marker vaccine. Conventional inactivated vaccines have been applied successfully for DIVA approaches with companion diagnostic tests targeting proteins involved in virus replication (Mackay *et al*., 1998; Chung *et al*., 2002; Suarez, 2005; 2012; Lambrecht *et al*., 2007; Barros *et al*., 2009; Hemmatzadeh *et al*., 2013). Recent advances in immunology, microbiology, molecular biology, proteomics, genetics, genomics and microbial pathogenesis have led to a wide variety of biotechnological approaches based on DNA mediated vaccine development. Vaccines engineered with gene deletions and additions, live vectored vaccines, chimeric vaccines, peptide and subunit vaccines have all been utilised to induce differential antibody responses (Babiuk, 1999; Henderson, 2005; Meeusen *et al*., 2007). Importantly, many marker vaccines retain the essential properties to: (1) reduce clinical signs after infection; (2) reduce wild-type virus replication after infection; (3) reduce transmission of the virus in the laboratory and in the field (Pensaert *et al*., 1990; Vannie *et al*., 1991; Swayne *et al*., 2000; Uttenthal *et al*., 2010). Many of the approaches to develop marker/DIVA vaccines have utilised virus structural and non-structural proteins, depending on family-specific aspects of the virion particle, and their role in virus pathogenesis and host-pathogen interactions. However, regardless of the approach taken, the essential properties

required to fulfil the DIVA principle is the ability to specifically detect antibodies of infected animals to the marker antigen with a sensitive 'marker assay' (Beer *et al*., 2007). Exploiting various biotechnological tools and expression systems have therefore also contributed to minimised production costs and time lag for development and analysis of diagnostic serological tests (Clavijo *et al*., 2004; Perkins *et al*., 2007a; b; Hema *et al*., 2007; Gómez-Sebastián *et al*., 2008). Furthermore, different expression systems have be used for maximised purity of immunogenic proteins both for development of the vaccine and companion diagnostic test (Van Drunen Little-van den Hurk *et al*., 1997; Wang *et al*., 2002; Clavijo *et al*., 2004; Sørensen *et al*., 2005; Huang *et al*., 2006; Choi *et al*., 2013).

A prerequisite for DIVA vaccination is that all field strains express the marker antigen and that infected animals always elicit antibodies to that protein after infection (Van Oirschot *et al*., 1996; Van Drunen Little-van den Hurk *et al*., 2006). A number of requirements for the DIVA diagnostic test were proposed by Van Oirschot *et al*. (1996):

1. Antibodies must be detectable within three weeks after infection
2. Antibodies must persist for a long period after infection
3. Vaccinated and subsequently infected animals elicit antibodies if wild-type virus replicates within the host
4. Repeatedly vaccinated animals must score negative to the marker
5. A high sensitivity, specificity and reproducibility must be obtained

There is often a lag time before detectable antibody responses are produced to the marker, not only following vaccination, but also following infection, which can vary depending on the disease and antigen used for serological screening (Van Oirschot *et al*., 1996; Van Rijn *et al*., 1996; Bouma *et al*., 1999; De Smit *et al*., 2001; Beer *et al*., 2007). Temperature may also represent an issue with the approach in fish as antibody responses are temperature dependent

for poikliotherms (Bly and Clem, 1992). Therefore, direct virus detection, of either antigen or nucleic acid, is usually necessary, especially if screening individual animals, to confirm their infection status. Furthermore, serology is only currently utilised for detecting suspect cases, but confirmation requires direct pathogen detection methods (OIE, 2012).

Few bacterial DIVA vaccine approaches have been conducted, e.g. a subunit and negative marker vaccine for *Actinobacillus pleuropneumoniae*, the causative agent of Porcine pleuropneumonia, by deletion of the Apx2A gene which expresses Apx2 toxins (Goethe *et al*., 2001; Tonpitak *et al*., 2002; Mass *et al*., 2006). However, copious studies have been conducted for marker vaccines and DIVA approaches for RNA and DNA viral diseases of mammals and birds (Table 1.1 & 1.2). These DIVA approaches have been achieved by taking advantage of the properties of serum immunoglobulin specificity and affinity, particularly immunoglobulin G (IgG) in mammals and IgY in birds.

DIVA approaches have varied considerably depending on the virus type. The approach to developing a DIVA vaccine requires either (1) construction of vaccines that exhibit different immunogenic properties to the wild-type strain or (2) exploit immunogenic variations that exist between vaccine and wild-type strain.

Since no DIVA approach has been applied for aquatic viruses, developments undertaken for mammalian and avian viruses and their success in the field may provide useful models for aquatic DIVA developments.

Table 1.1 Animal RNA virus DIVA vaccine approaches

Virus	Virus family, genome, proteome	DIVA approach		References
		Vaccine	Diagnostic assay	
Avian Influenza A virus (AIV) *Avians*	Env. capsid Neg. ss RNA 13.6 kbp; 8 seg Orthomyxoviridae	(**1**) Inactivated vaccine, (**2**) Rec. H vaccine, (**3**) Rec. H5N8 vaccine, (**4**) Rec. antigenically matched H5N9 inactivated vaccine, (**5**) H5N1 influenza marker vaccine with foreign gene insert, (**6**) Rec live-vectored (FPV, NDV, ILTV, VSV) HA expressing vaccine, (**7**) Inactivated reverse genetics engineered HA NA vaccine, (**8**) DNA vaccine, (**9**) Virus-like particles, (**10**) Reassortment heterologous N (H9N8) vaccine	(**1**) Heterogenous N ELISA to wild-type, homologous H protein, (**2**) *E.coli* or baculovirus expressed NS1-specific ELISA, (**3**) NA inhibition assay, (**4**) MHV 5B19 synthetic peptide ELISA, (**5**) Rec. M2e ELISA, (**6**) NP ELISA, (**7**) M ELISA, (**8**) Recombinant N2 ELISA	Halvorson, 2002; Capua *et al*., 2003; 2004; Liu *et al*., 2003; Tumpey *et al*., 2005; Zhao *et al*., 2005; Gao *et al*., 2006; Lambrecht *et al*., 2007; Li *et al*., 2008; Jadhao *et al*., 2009; Kwon *et al*., 2009; Avellaneda *et al*., 2010; Brahmakshatriya *et al*., 2010; Uttenthal *et al*., 2010; Choi *et al*., 2013; Hemmatzadeh *et al*., 2013
Foot and Mouth Disease Virus (FMDV) *Suids, bovids*	Nak. capsid Pos. ss RNA 8.5 kbp; 1 ORF Picornaviridae	(**1**) Synthetic peptide vaccine, (**2**) Inactivated vaccine, (**3**) Rec. multiple epitope vaccine	(**1**) EITB assay, (**2**) Indirect ELISA, (**3**) NSP - 3ABC Blocking ELISA, (**4**) *E.coli* and baculovirus expressed NSP, (**5**) Antigen-capture NSP ELISA, (**6**) Whole virus blocking ELISA, (**7**) Peptide and recombinant protein-based multiplex assays, (**8**) Mucosal IgA ELISA	Strebel *et al*., 1986; Villinger *et al*., 1989; Bergmann *et al*., 1993; De Diego *et al*., 1997; Mackay *et al*., 1998; Sørensen *et al*., 1998; Chung *et al*., 2002; Clavijo *et al*., 2004; Parida *et al*., 2006; Perkins *et al*., 2007a; b; Shao *et al*., 2011
Classical swine fever virus (CSFV) *Suids*	Env. capsid Pos. ss RNA 12.5 kbp; 1 ORF Flaviviridae	(**1**) Baculovirus expressed rec. subunit E2 B/C neg. vaccine, (**2**) Baculovirus expressed rec. subunit E2 A neg. vaccine, (**3**) Rec. adenovirus vector vaccine expressing CSFV E2 gene, (**4**) Chimeric vaccines containing marker antigens,	(**1**) E^{RNS}-ELISA, (**2**) E^{RNS} sandwich blocking ELISA, (**3**) Recombinant ELISA, ***(4) rRT-PCR***	Van Rijn *et al*., 1996; Bouma *et al*., 1999; Hammond *et al*., 2000; 2001; Van Gennip *et al*., 2001; 2002; De Smit *et al*., 2001; Floegel-Niesmann, 2001; Hahn *et al*., 2001;

		(**5**) Live attenuated , (**6**) E2 glycoprotein subunit vaccine, (**7**) Rec. E2 expression through swinepox vector system, (**8**) Chimeric CSFV/BVDV rec. vaccines expressing E2 or E^{RNS}		Dong *et al*., 2005; Huang *et al*., 2006; Dong and Chen, 2006; Koenig *et al*., 2007; Blome *et al*., 2011; Aebischer *et al*., 2013
Equine influenza A virus (EIV) *Equids*	Env. capsid Neg. ss RNA 8 seg Orthomyxoviridae	(**1**) Inactivated vaccine, (**2**) Canarypox vectored expression of HA	(**1**) *E.coli* expressed NS1 Western blot, (**2**) NP-specific ELISA	Birch-Machin *et al*., 1997; Minke *et al*., 2004
Equine arteritis virus (EAV) *Equids*	Env. capsid Pos. ss RNA 12.7 kbp; 8 ORF Arteriviridae	(**1**) Live G_L neg. live vaccine	(**1**) Synthetic G_L peptide ELISA	Castillo-Olivares *et al*., 2003
Blutongue virus (BTV) *Bovids*	Nak. capsid ss RNA 10 seg Reoviridae	(**1**) Inactivated vaccine	(**1**) *E.coli* expressed rec. NS3 indirect ELISA (**2**) Major core protein VP7 ELISA	Barros *et al*., 2009
Newcastle disease virus (NDV) *Avians*	Env. capsid Neg. ss RNA 15.2 kbp Paramyxoviridae	(**1**) Live rec. NDV with immunodominant NP epitope substitution with MHV S2 GP	(**1**) Synthetic NP immunodominant epitope indirect ELISA, (**2**) Synthetic MHV 5B19 synthetic peptide ELISA	Mebatsion *et al*., 2002
Bovine viral diarrhoea virus (BVDV) *Bovids*	Env. Capsid Pos. ss RNA 12.3 kbp Flaviviridae	(**1**) Chimeric vaccine with heterogenous E^{RNS} GP	(**1**) E^{RNS} GP competitive ELISA with baculovirus rec. E^{RNS}	Luo *et al*., 2012
Peste des petits ruminants virus (PPRV) *Bovids*	Env. capsid Neg.-ss RNA 16 kbp Paramyxoviridae	(**1**) DNA vaccine, (**2**) Sub unit vaccines, (**3**) Rec. H protein through capripox expression, (**4**) Rec. F protein through capripox expression	(**1**) Rec. N ELISA	Diallo *et al*., 2007
Rift valley fever virus (RVFV) *Bovids, humans*	Env. capsid Neg ss RNA 3 seg Bunyaviridae	**(1)** Vectored rec. vaccine expressing GP: gN and gC	(**1**) Multiplex NP and GP	Van der Wal *et al*., 2012

Susceptible hosts are listed in italics under viruses; Env. = enveloped; Nak. = naked; ss = single stranded; kbp = kilo base pairs; seg = segments; ORF = open reading frame; rec. = recombinant; EITB = Enzyme-linked immunoelectrotransfer blot; ELISA = enzyme-linked immunosorbent assay; IFAT = indirect fluorescent antibody technique

Table 1.2 Animal DNA virus DIVA vaccine approaches

Virus	Virion proteome and genome	DIVA approach		References
		Vaccine	Diagnostic assay	
Pseudorabies virus; Suid herpesvirus-1 (PrV; SuHV-1) *Suids*	Env., teg. capsid ds DNA 143.4 kbp; 70+ORF Alphaherpesviridae	(**1**) Live attenuated TK neg. mutant deletion vaccine, (**2**) inactivated deletion vaccine	(**1**) gE protein based indirect ELISA, (**2**) IFAT blocking ELISA, (**3**) Competitive ELISA	Van Oirschot *et al*., 1988; 1990; 1991; 1996; Kinker *et al*., 1997; Van Oirschot, 1999; Klupp *et al*., 2004
Bovine herpesvirus-1 (BoHV-1) *Bovids*	Env., teg capsid ds DNA 135.3 kbp; 73 ORFs Alphaherpesviridae	(**1**) Live attenuated TK. neg., gE deletion mutant vaccine, (**2**) gD neg. eukaryotic expressed subunit vaccine, (**3**) killed gE deletion vaccine, (**4**) gD subunit vaccine, (**5**) gE neg. live attenuated vaccine, (**6**) gE neg. inactivated vaccine	(**1**) Competitive gE blocking ELISA, (**2**) gD/gB indirect ELISA	Flores *et al*., 1993; Kaashoek *et al*., 1994;1995, 1996; 1998; Van Drunen Little-van den Hurk *et al*., 1994; 1997; Bosch *et al*., 1996; Van Oirschot *et al*., 1996; 1997; Van Drunen Little-van den Hurk, 2006; Glazov *et al*., 2010; Zhao and Xi, 2011
Bovine herpesvirus-5 (BoHV-5) *Bovids*	Env., teg. capsid ds DNA 138.4 kbp Alphaherpesviridae	(**1**) Live attenuated TK. neg. mutant deletion vaccine	(**1**) Competitive gE blocking ELISA	Delhon *et al*., 2003; Anziliero *et al*., 2011
Infectious laryngotracheitis virus (ILTV) *Avians*	Env., teg. Capsid ds DNA 152.6 kbp Alphaherpesviridae	(**1**) Rec. gG deleted vaccine	(**1**) Rec. baculovirus/*E.coli* expressed gG ELISA	Fuchs *et al*.., 2007; Lee *et al*., 2011; Shil *et al*., 2012

Susceptible hosts are listed in italics under viruses; Env. = enveloped; Nak. = naked; ss = single stranded; kbp = kilo base pairs; seg = segments; ORF = open reading frame; rec. = recombinant; ELISA = enzyme-linked immunosorbent assay; IFAT = indirect fluorescent antibody technique

1.4.4 Manipulation of antibodies as markers of infection

The DIVA concept was explained in Section 1.4.1, however, it may be argued that since modifying the definition to accommodate all vaccine/diagnostic systems, where a differential antibody response is provoked by the vaccine then subsequently detected by the accompanying diagnostic test, there is no specification of whether the biotechnological development has been towards the vaccine or the diagnostic assay. Since many DIVA approaches utilise conventional, inactivated or live attenuated vaccines, the current criteria for a 'DIVA vaccine' would imply that those conventional vaccines are then DIVA vaccines, which could apply for all vaccines if the relevant antigens could be characterised to differentiate responses to that vaccine. Therefore, 'marker' vaccination should still apply where the focus of development has been on manipulation of the vaccine antigens specifically, and perhaps DIVA applied to the overall system. This section attempts to provide a brief account of some of the many marker vaccines and DIVA approaches, independently, that have been developed against some of the most important RNA (Table 1.1) and DNA (Table 1.2) viral diseases in the livestock and poultry industries.

Exploiting the biological characteristics, i.e. of structural and non-structural proteins of the virus and/or host response to those proteins, is key to detecting differential antibody responses induced by the vaccine and infectious pathogen. Examples are given for six of the most economically important animal RNA (AIV, FMDV, CSFV and PPRV) and DNA (PrV, BoHV-1) viral diseases to highlight the different vaccine and diagnostic methods/strategies utilised, depending on the viral agent.

1.4.4.1 Marker vaccines

(a) Endogenous antigen deletions

Marker vaccines developed for the economically important alphaherpesviruses, PrV and BoHV-1, have been generated as live attenuated vaccines containing a deletion of a non-essential, but immunogenic envelope glycoprotein, gE, and are used effectively as both live and inactivated marker vaccines (Van Oirschot *et al*., 1986; 1996; Vannie *et al*., 1991; Kaashoek *et al*., 1994; 1995; 1996; 1998; Bosch *et al*., 1996; 1997; Brum *et al*., 2010 cited in Anziliero *et al*., 2011). The live marker vaccines are often further attenuated by deletions of the thymidine kinase (TK) gene (Quint *et al*., 1987; Moormann *et al*., 1990; Kalthoff *et al*., 2010; Anziliero *et al*., 2011). As described in Section 1.4.3, this was the first approach for differentiating antibody responses between infected and vaccinated animals. This protein target was particularly suitable as a 'negative marker' as it is not essential for replication or protection (Heffner *et al*., 1993; Van Drunen Little-van den Hurk, 2006). The negative marker approach permits identification of infected animals by detecting specific antibodies to the gE protein that is absent from the vaccine strain, thus vaccinated animals lack antibodies to this protein, but are sero-positive to the other glycoproteins of the virus. Despite other non-essential envelope glycoproteins being identified and characterised as immunogenic from the PrV and BoHV-1 virions (Van Oirschot *et al*., 1996; Jones and Chowdury, 2008; Kramer *et al*., 2011; Zhao and Xi, 2011), only gE has been found to be expressed by all tested field isolates of PrV, making it the most reliable to detect antibody responses to all exposed animals (Van Oirschot *et al*., 1996).

The diagnostic methods for detection of gE-specific antibodies were initially based on a competitive gE ELISA utilising gE-specific MAbs (Flores *et al*., 1993; Van Oirschot *et al*., 1996; 1997; Van Oirschot, 1999). The high sensitivity and specificity of the serology tests for gE has contributed immensely to the success of intensive marker vaccination sero-

surveillance programmes for AD (Pensaert *et al*., 2004). Improvements on the gE ELISA has been attempted using gE epitopes (Jacobs and Kimman, 1994), but concerns over potential antigenic drift (Ben-Porat *et al*., 1986) has limited their application. Recombinant, baculovirus expressed, gE protein ELISAs have proved much more promising and cost-effective (Gómez-Sebastián *et al*., 2008).

Another economically important disease that is likely to greatly benefit from DIVA vaccination strategies, which should pave the way to its global eradication is PPRV. Marker vaccines developed for PPRV have taken advantage of the virus surface proteins, which are highly immunogenic and an epitope mutation in the haemagglutinin (HA) protein has enabled differentiation of infected and vaccinated ruminants as antibodies were induced to the deleted epitope only in infected animals (Buczkowski *et al*., 2012). AIV also expresses immunogenic and protective HA surface proteins, but in contrast to PPRV a live attenuated AIV vaccine was developed focused on epitope deletions of the non-structural protein, NS1, however, only after inactivation was the vaccine strain safe (Brahmakshatriya *et al*., 2010).

Chimeric viruses have proved an effective approach to marker vaccination and have been developed for PPRV and CSFV. By substitution of immunogenic proteins with homologous proteins of a closely related virus, the vaccine can be utilised as a marker vaccine by detecting antibodies of infected animals directed to the protein substituted from the vaccine. The NP protein of PPRV is known to be immunogenic and has been used as a negative marker on a recombinant ELISA after substitution with NP of the closely related RPV (Das *et al*., 2000; Parida *et al*., 2007). The chimeric virus vaccines developed for protection against CSFV were achieved by direct substitution of the analogous sequence of the immunogenic envelope proteins E2 or E^{RNS} with that of BVDV, which have proved highly efficacious marker vaccines and enabled antibody differentiation based on specific antibody detection to the protein absent in the vaccine (Van Gennip *et al*., 2001; 2002;

Koenig *et al*., 2007). The envelope proteins, E2 and E^{RNS}, of this flavivirus, which have high and low neutralising activity, respectively (Hoffmann *et al*., 2005; Huang *et al*., 2006), provide ideal targets for this approach as one immunogenic protein is dispensable. The envelope protein E2 is predominantly protective protein, thus differential antibody responses can be induced by exploiting antibodies that recognise E^{RNS}. However, the problems associated with negative markers in vaccines have been highlighted where the most protective antigen is also the most immunogenic, thus a lack of either one compromises either the diagnostic assay sensitivity or the vaccine efficacy as was the case for recent trials with chimeric marker vaccines for CSFV (Eblé *et al*., 2013). Other biotechnologically advanced approaches to marker vaccination for CSFV, which have proved very successful have included recombinant vectors such as porcine adenovirus, PrV and swinepox virus expressing CSFV E2 gene (Hammond *et al*., 2000; 2001; Hahn *et al*., 2001). CSFV replicon vaccines have been designed to carry CSFV-E2 and CSFV-E^{RNS} deletions (Van Gennip *et al*., 2001; 2002; Stettler *et al*., 2002).

DNA vaccination has not only proven a very promising approach to vaccination in general, but has broadened the potential for marker vaccination. DNA vaccines can be designed to express the immunogenic protein of interest, e.g. E2 of CSFV, and when used in conjunction with companion diagnostic ELISA tests targeting either the non-structural protein, NS3, or envelope protein E^{RNS}, differentiation between infected and vaccinated animals is obtainable when a sufficient antibody response is induced (Andrew *et al*., 2000; Beer *et al*., 2007).

(b) Exogenous antigens additions

Exogenous marker vaccines are developed by either administering ‘foreign’ antigens (i.e. proteins not naturally recognised by the host), within the vaccine formulation (James *et al*.,

2007; 2008) or through insertion of heterologous genes into the vaccine strain genome (Castrucci *et al*., 1992; Walsh *et al*., 2000a; b; Mebatsion *et al*., 2002; Fang *et al*., 2008) in order to induce a detectable differential antibody response. Antibody responses to the additional 'foreign' protein or epitope, detected by a serological test specific for the antigen, indicate that the animal is vaccinated, regardless of infection status. This so-called 'positive marker' (Van Oirschot, 1999) approach is described in detail in Section 3.1.1 and 3.1.2.

1.4.4.2 DIVA strategies

Despite the validity and sensitivity of the gE deletion marker vaccine approach for the alpha-herpesviruses, a subunit vaccine was developed based on an alternative envelope glycoprotein, gD, which is an essential protein of BoHV-1 and is highly protective. This provided a highly cost-effective and safe vaccine that could be used in conjunction with sensitive and specific gE ELISA tests to detect infected animals (Babiuk, 1999; Kowalski *et al*., 1993; Van Drunen Little-van den Hurk *et al*., 1994; 1997). The disadvantage of the subunit vaccines is the delayed antibody response elicited compared to live attenuated vaccines (Van Oirschot, 1999; Van Rijn *et al*., 1996; Bouma *et al*., 1999; De Smit *et al*., 2001; Beer *et al*., 2007) providing a longer window of potential false negative diagnosis.

Highly efficacious vaccines have been available for AIV, FMDV and CSFV, but did not enable infected and vaccinated animals to be serologically differentiated using conventional diagnostics (Suarez, 2005; 2012; Beer *et al*., 2007; Rodriguez and Gay, 2011). This instigated research into diagnostic test development, as opposed to the vaccine specifically, for enabling DIVA strategies for already available subunit or whole inactivated vaccines (Bergmann *et al*., 1993; De Diego *et al*., 1997; Mackay *et al*., 1998; Clavijo *et al*., 2004; Huang *et al*., 2006; Perkins *et al*., 2007a; b; Hema *et al*., 2007; Uttenthal *et al*., 2010; Mohapatra *et al*., 2011). The challenges that are encountered with development of DIVA strategies have varied depending on the viral disease.

DIVA diagnostic test development for whole virus inactivated vaccines against AIV and FMDV has been focused on non-structural proteins, e.g. NS1 of AIV and many of FMDV (Bergmann *et al*., 1993; De Diego *et al*., 1997; Mackay *et al*., 1998; Clavijo *et al*., 2004; Tumpey *et al*., 2005; Hema *et al*., 2007; Kwon *et al*., 2009; Mohapatra *et al*., 2011) as well as structural proteins, e.g. the matrix protein for AIV (Suarez, 2005; 2012; Tumpey *et al*., 2005; Lambrecht *et al*., 2007; Kwon *et al*., 2009; Kim *et al*., 2010; Hemmatzadeh *et al*., 2013). As these proteins are highly abundant when expressed during virus replication within the infected cell they induce antibody responses only in infected animals. They are either absent, or not highly abundant in vaccinated animals as the vaccine strain is not replicating (Clavijo *et al*., 2004; Uttenthal *et al*., 2010). Other characteristics that make the non-structural proteins ideal diagnostic targets is that they are highly conserved amongst serotypes and subtypes of AIV and FMDV (Doel, 2003; Clavijo *et al*., 2004; Uttenthal *et al*., 2010; Shao *et al*., 2011) unlike the vaccines themselves where many serotypes and subtypes exist for AIV and FMDV. However, for CSFV, this actually prevents their application for DIVA diagnostics as the high conservation of non-structural protein NS3 amongst pestiviruses, leads to problems of antibody cross reactivity in the field (Van Gennip *et al*., 2001; Hoffmann *et al*., 2005; Beer *et al*., 2007). This has made subunit vaccine development a preferred approach for CSFV as the envelope proteins can be used instead, i.e. so that the protective E2 protein is used in the vaccine and diagnostic tests are developed for sensitive and specific detection of antibodies to the E^{RNS} protein (Hulst *et al*., 1993; Floegel-Niesmann, 2001; Huang *et al*., 2006). Subunit vaccines have also been developed to the protective HA proteins of AIV (Plotkin, 2011), which are compatible for DIVA strategies using the specific and sensitive diagnostic tests developed to other structural and non-structural proteins.

Multiplex assays have been developed to circumvent problems with variation in immunoreactivity of FMDV non-structural proteins (Perkins *et al*., 2007a; b) and detection of mucosal IgA, which is produced in high levels only in infected animals, has been proposed as an alternative diagnostic to alleviate the issues encountered with serological sensitivity of IgG detection (Parida *et al*., 2006).

Virus-like particles (VLPs) have recently proved an effective approach for DIVA vaccination as they are protective, but lack the internal proteins for which many DIVA approaches are based. This has been achieved for AIV and FMDV with an ELISA based on antibodies to the non-structural proteins (NSPs) (Choi *et al*., 2013; Porta *et al*., 2013). Purer vaccine preparations that lack NSPs of FMDV are also being developed to enhance the reliability of DIVA strategies for FMDV (Wang *et al*., 2002; Li *et al*., 2010). Furthermore, genetically engineered vaccines have been designed with multiple epitopes of FMDV and suitable serotype antigens of AIV to alleviate issues with cross-serotype protection and antigenic drift (Liu *et al*., 2003; Jadhao *et al*., 2009; Shao *et al*., 2011).

More conventional approaches have involved the use of inactivated AIV vaccines with heterologous NA, but homologous HA genes to the field strain, that were applied strategically as DIVA strategies by detection of antibodies to field strain NA to indicate infection (Capua *et al*., 2003; 2004).

However, regardless of the pathogen or vaccine, the ultimate defining factor of DIVA vaccination is the reliable detection of specific antibodies. The lack of sero-conversion and variable antibody initiation and duration continuously reported has emphasised the limitations of DIVA approaches for AIV and FMDV that are based on non-structural proteins (Clavijo *et al*., 2004; Avellaneda *et al*., 2010).

1.4.5 Genetic DIVA strategy

Novel genetic DIVA strategies have also been developed for direct differentiation of the wild-type strain and vaccine strain viral genome within the host. For example, CSFV wild-type strain and attenuated live vaccine strain were differentiated based on nucleic acid sequences using rRT-PCR (Hoffman *et al*., 2005; Beer *et al*., 2007; Blome *et al*., 2011). Genetic DIVA has previously been applied for BoHV-1 by targeting the gE gene, which had been deleted in the vaccine, thus differences between the vaccine strain and wild-type strain sequences can be determined directly (Schynts *et al*., 1999). The genetic DIVA approach could be particularly useful during acute stages of viral infection when antibodies produced to the marker may not yet be detectable, e.g. anti-gE antibodies to PrV are not detectable until 10-17 days post infection (dpi) (Van Oirschot *et al*., 1996).

1.5 Potential of DIVA vaccination for notifiable diseases in aquaculture

The development of DNA vaccines has provided encouraging results for rhabdoviruses VHSV, IHNV and SVCV (Anderson *et al*., 1996a; b; Lorenzen *et al*., 1998; Lorenzen and La Patra, 2005; Sommerset *et al*., 2005a; Emmenegger and Kurath, 2008; Tonheim *et al*., 2008), which may enable a DIVA strategy if immunogenic epitopes of the G protein, for which some have been previously mapped for VHSV (Fernandez-Alonso *et al*., 1998), are absent from the expressed protein in the vaccine. Specific antibody responses to VHSV have been reported to be detectable > 6 months (Lorenzen and La Patra, 1999; Fregeneda-Grandes *et al*., 2008), which would be necessary for DIVA strategies to be implemented for this rhabdovirus. One of the very few reported studies of developing marker vaccines for fish viruses (Enzmann *et al*., 1998; Dhar *et al*., 2010) inserted the G gene of VHSV and IHNV

into pathogenic bacteria (*Aeromonas salmonicida*) as a vector. This induced differential antibody responses from immunised rainbow trout to the vaccine strain and pathogenic virus by western blot (Enzmann *et al*., 1998). In the same study a genetic DIVA vaccine was also constructed by utilising a variable region of the G-gene to develop a differentiating RT-PCR for an attenuated vaccine. Therefore detection of vaccine strain virus can be differentiated from wild-type virus (Enzmann *et al*., 1998). Another example was obtained by successful expression of exogenous foreign marker genes in IHNV, i.e. GFP, following a deletion of the non-structural NV protein gene by reverse genetics (Biaccesi *et al*., 2000). This differential gene expression could be utilised for genetic DIVA approaches.

Another study applied a different approach to positive marker vaccination for another aquatic RNA virus of the birnaviridae family, infectious pancreatic necrosis virus (IPNV). Subviral particles (SVPs) are formed by structural virus proteins self-aggregating to form particles that do not mimic the native virus capsid (Dhar *et al*., 2010). These have been synthesised from infectious pancreatic necrosis virus (IPNV) VP2 protein (Allnutt *et al*., 2007). The subsequent recombinant VP2 (rVP2) particles were also able to carry foreign protein insertions, which reduced IPNV shedding in immunised rainbow trout and elicited specific antibodies to the foreign antigen, *c-myc* (human oncogene) and to VP2 (Dhar *et al*., 2010). If antibodies are also detectable to alternative IPNV proteins only in infected fish, e.g. VP3, then such a vaccine could be utilised for DIVA vaccination.

Previously, an aquatic DNA virus, a member of the alloherpesviridae, channel catfish virus (CCV), has also been investigated for its ability to support the insertion of foreign genes, thus provide an effective vaccine vector (Zhang and Hanson, 1996). The foreign protein was found to induce a specific antibody response in vaccinated catfish, but following infection it would not be possible to indicate fish as uninfected using such a vaccine. A number of vaccines have been developed for KHV, but at present only live attenuated

vaccines have been commercialised (Ronen *et al*., 2003; KV3, KoVax; Cavoy® Novartis). These vaccines do not, however, enable antibodies to be differentiated between infected and vaccinated, although genetic DIVA is available for the KoVax vaccine as a PCR was developed specific for an altered nucleotide sequence in the vaccine strain. This can be differentiated from the wild-type virus by PCR (KoVax).

A recently developed oral subunit vaccine for ISAV is based on the haemagglutinin esterase (HE) protein (Dhar and Allnutt, 2011; Centrovet, Chile). This could potentially enable a DIVA approach by screening for antibodies against the nucleoprotein (NP) that is lacking in the vaccine as only infected fish would respond to this antigen. Indeed the NP protein has been reported as a highly immunogenic antigen (Falk pers. comm. *cited in* Wolf *et al*., 2013) and recombinant proteins developed for the HE protein (Krossøy *et al*., 2001; Müller *et al*., 2008) would enable an indication of vaccine efficacy if coated on ELISA plates.

By using such marker vaccines in conjunction with their companion diagnostic test, it may be possible to implement DIVA strategies using serology whereby all fish within the infected site are destroyed, but all fish in the control and surveillance zones are 'emergency vaccinated' with the marker vaccine. Those populations of fish that are subsequently found to be positive for antibodies to the marker would be destroyed, while those negative may be spared (Fig. 1.2).

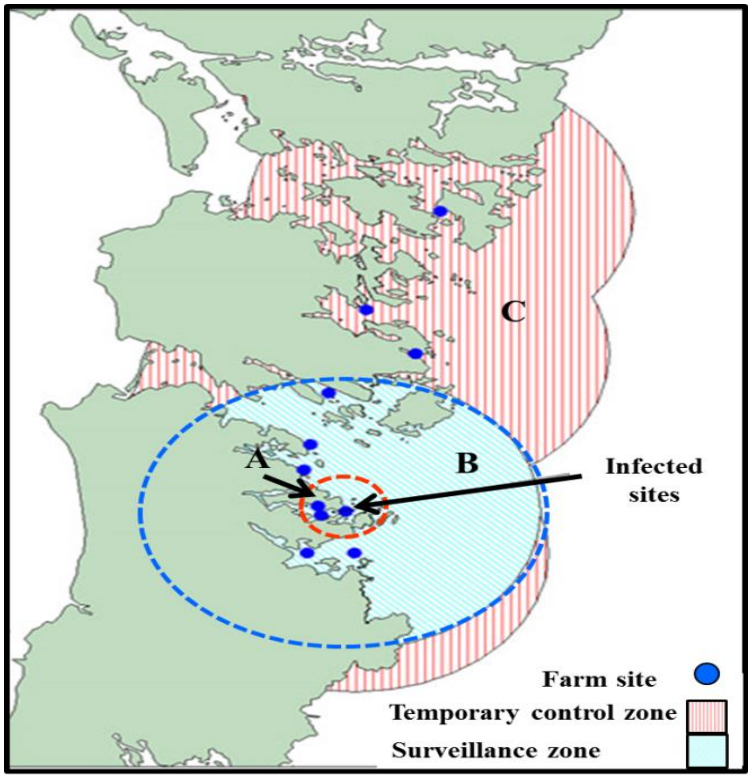


Figure 1.2 Schematic map of hypothetical ISA outbreak in Scotland with control zone and surveillance zones during a DIVA vaccination eradication programme. Implementing a vaccination eradication programme during an outbreak of ISA, all fish at the infected site (A) are culled whilst those fish in farms within a 5-10 km radius of the infected site (Control zone) (B) are emergency vaccinated. Any fish positive for antibodies to the marker are immediately slaughtered, whilst negative fish are spared. Movement of stocks is still restricted within the control zone. All fish are also vaccinated within the surveillance zone (C) and movements are permitted as antibodies to the vaccine can be differentiated from those to infection. *After McGill (2005)*

Dhar *et al.* (2010) pertinently stated that "methods to reduce viral diseases in aquaculture will improve both the quality of life of the animal and make the industry more sustainable". This could be achieved more effectively by DIVA vaccination. However, approaches to marker vaccine development against fish viruses are limited, and the feasibility of DIVA vaccination for fish has not been assessed. Differences in humoral immunity between higher and lower vertebrates must be taken into consideration as well as the DIVA

approach to particular viral pathogens to shed more light on the feasibility of this vaccination strategy for aquaculture.

1.6 Adaptive immunity: The antibody response

The complexity of DIVA/marker vaccination, which is based upon the intrinsic association with viral antigen and host adaptive immunity, was described with examples in Section 1.4.4. Whether the protein targets used for DIVA are based on replication kinetics of the live virus, e.g. screening antibodies against non-structural proteins or matrix proteins in AIV and FMDV, or the absence of protein from the vaccine strain, i.e. in the case of a marker vaccine, e.g. envelope glycoproteins of CSFV, PrV and BoHV-1, the success of DIVA/marker vaccines is ultimately dependent on the adaptive immune response to these antigens. Therefore, for development of DIVA vaccination strategies in lower vertebrates, i.e. for fish, the intricacies of the immune system need to be taken into account. The adaptive immune response, and features that differ in fish compared to mammals, are particularly pertinent in this regard. In evolutionary terms fish are the first group of animals with the basic aspects of the immune system of mammals and there appear to be more similarities than differences (Flajnik, 1996; Sommerset *et al*., 2005a). An account of the adaptive immune response in this section refers primarily from what is known in mammals. Similarities and comparisons to the teleost fish immune system are made throughout. Text referring to Fig. 1.3 is indicated as bold numbers in brackets.

1.6.1 B cell receptors, T cell receptors and complement activity

In general terms, antibodies secreted by B cells bind to antigens of pathogens in the blood stream and at the mucosal surfaces, limiting infection to cells, whereas the T cells are responsible for recognising antigens and killing infected cells (Flint *et al*., 2009). Specific

molecular recognition is mediated by different routes via B cells and T cells (Fig. 1.3). B cells are produced in the bone marrow of mammals (Lydyard *et al*., 2000) and head kidney of teleost fish (Zapata *et al*., 1996; Press and Evensen, 1999) and harbour membrane bound antibody, as an antigen receptor, by the Fc domain of the immunoglobulin (Flint *et al*., 2009). Fc receptors (FcRs) have been described in fish such as carp and catfish (Koumans-van Diepen *et al*., 1994; Fujiki *et al*., 2000; Stafford *et al*., 2006). The head kidney and spleen of teleosts are the main lymphoid organs (Rombout *et al*., 2005). The fish head kidney is also a major producer of antibody as well as melanomacrophage accumulations that are able to retain antigens for long periods of time after vaccination and therefore has a possible role in immunological memory (Press *et al*., 1996; Press and Evensen, 1999).

The T cell precursors are also produced in the bone marrow of mammals, but must migrate to the thymus gland for maturation (Workenhe *et al*., 2010). T cells utilise T cell receptors (TCR) and 1 of 2 glycoprotein oligomers, major histocompatibility complex 1 (MHC I) or 2 (MHC II), that are able to display fragments of internal cellular proteins on the cell surface (Fig. 1.3 (**1)**). While most cells are able to display MHC class I molecules, only the professional antigen presenting cells (APCs), macrophages, neutrophils, B cells and mature dendritic cells, are able to present MHC class II (Flint *et al*., 2009). Following characterisation, these cells have been reported to be functionally and morphologically equivalent to those in fish (Manning and Nakanishi, 1996; Whyte, 2007). Pathogens entering the body are ingested by dendritic cells and macrophages by phagocytosis. This stimulates these cells to mature and migrate to secondary lymphoid tissue where the antigens are presented to naïve lymphocytes, which is critical for the adaptive immune response (Workenhe *et al*., 2010). After binding of FcR to the Fc region of the Ig molecule, a variety of responses are induced. These include a number of cellular responses such as increased phagocytosis and respiratory burst as well as regulation of B cells. Ig binding capacity has

previously been observed in peripheral blood leukocytes of Atlantic salmon and channel catfish (O'Dowd *et al*., 1998; Morrison and Nowak, 2002; Shen *et al*., 2002).

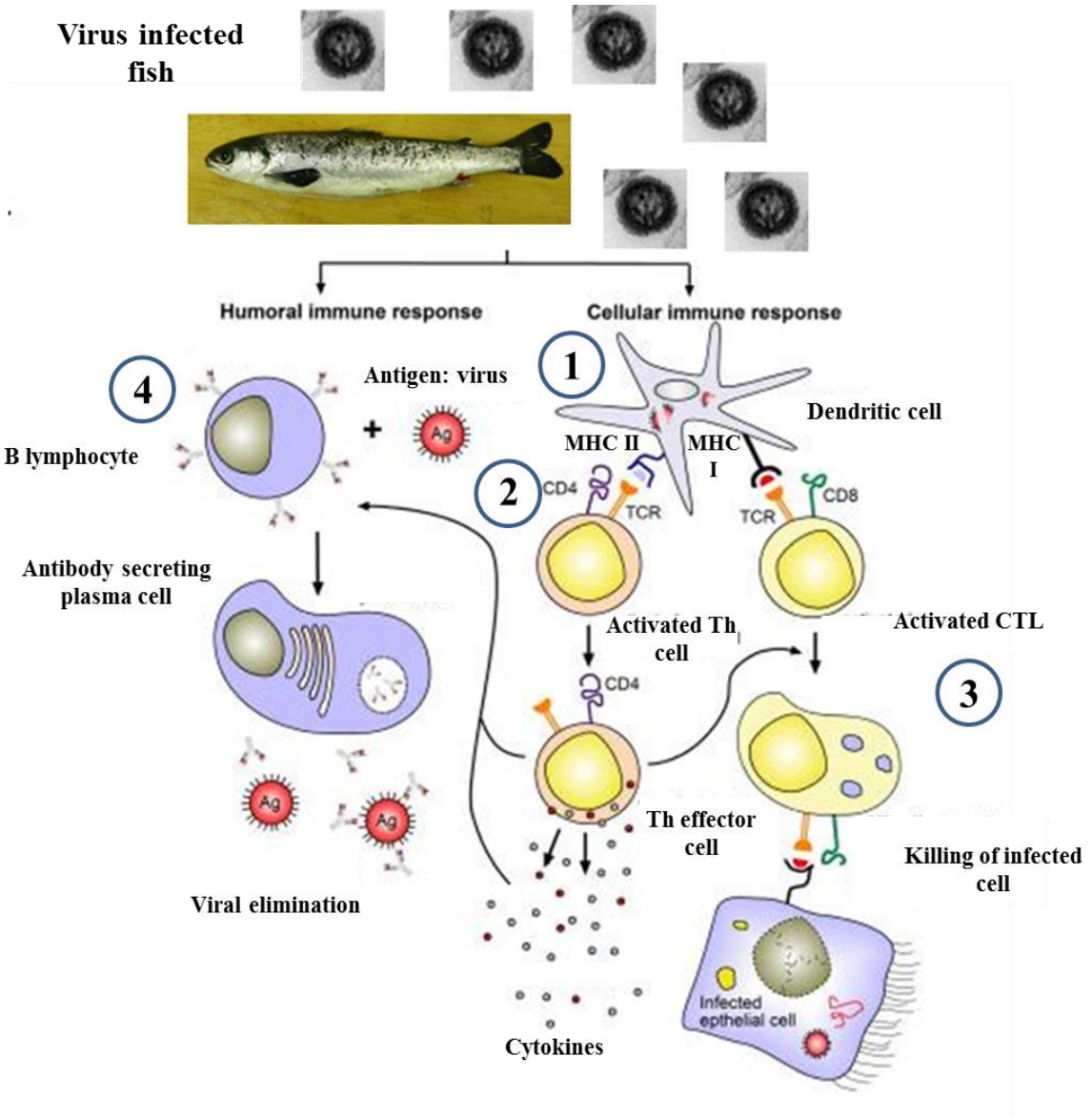


Figure 1.3 Humoral and cellular branches of the adaptive immune response against viral infections. Numbers represent processes described in the text.

After Flint et al. (2009)

Antibody-dependent complement activation is also mediated through the Ig Fc region following antigen complexing. In fish, the complement system constitutes an important facet of defence against microbes. It has been characterised in cyclostomes, elasmobranchs and

teleosts (Yano, 1996) and is considered an adaptation for the lack of a well-developed acquired immune system (Sunyer *et al*., 1998; Nonaka and Smith, 2000). The classical (antibody-dependent), alternative (antibody independent) and lectin complement pathways have been characterised in fish and represent integral aspects of fish immunity (Yano, 1996; Sunyer *et al*., 1998; Nonaka and Smith, 2000; Morrison and Nowak, 2002; Nakao *et al*., 2006). The classical complement pathway is activated, after binding of antibody (IgG or IgM) to C1q (Holland and Lambris, 2002), through a cascade of interactions involving C4, C2, C3, C5, C6, C7, C8 and C9 and the alternative pathway is activated directly through C3 in the presence of factors B and D followed by C5 – C9 (Yano, 1996; Kaattari and Piganelli, 1996; Holland and Lambris, 2002). The lectin complement pathway is initiated by binding of protein complex consisting of mannose-binding lectin (MBL) (Holland and Lambris, 2002). The latter components of the complement pathway (C5-C9) form the membrane attack complex (MAC), which forms a pore within the membrane of virus infected cells, causing lysis of the cell or pathogen, and has been demonstrated in fish (Tomlinson *et al*., 1993; Holland and Lambris, 2002). The MAC has been reported to have effective virucidal activity in salmonids, possibly contributing to resistance to infection (Sakai *et al*., 1994 cited in Yano, 1996). The C1q molecule is an important aspect of the complement cascade and has been identified in fish, e.g. carp and Atlantic salmon (Yano *et al*., 1988 cited in Kaattari and Piganelli, 1996; Arason, 1996). The binding of the first component of complement (C1), requires an intimate association with more than one monomeric Fc, which is considered much more effective with pentameric IgM than monomeric IgG for cross-linking of Fcs with a single C1q molecule (Borsos *et al*., 1981; Winkelhake, 1979 cited in Kaattari and Piganelli, 1996). This may contribute to the efficiency of fish complement activity after binding of the tetrameric IgM, which has been demonstrated in rainbow trout (Elcombe *et al*., 1985).

1.6.2 Lymphocytes: B cells, T cells and natural antibodies

Different antigenic properties determine the specific antibody or T cell response to a particular antigenic determinant. While antibodies can recognise 3-6 amino acids (aa) or 5-6 sugar residues, large molecules are multideterminant (Lydyard *et al*., 2004). Different antigens capable of eliciting an immune response include proteins, carbohydrates, lipids and nucleic acids. Binding of antibody can vary depending on conformational antigenic determinants of the molecule and how it is folded while T cell receptors recognise linear aa sequences (Lydyard *et al*., 2004). B lymphocytes bind directly to discrete epitopes of contiguous sequences or unique conformations of intact proteins. Upon antigen recognition with the membrane bound antibody, the B cell is stimulated to divide and the progeny cells differentiate into antibody secreting plasma cells and a smaller number of memory B cells. The plasma cells are short lived, but continue to secrete clones of the membrane bound antibody of the precursor B cell (Roitt, 1997; Lydyard *et al*., 2004).

During maturation T lymphocytes are selected based on their TCRs and only those that do not possess TCR against self-antigens (1-2%) emerge from the Thymus gland of mammals to differentiate into T helper (Th) cells or cytotoxic T lymphocytes (CTLs). T helper cells are further divided into Th1, Th2 and Th17 cells, which are positive for the cell marker protein, Cluster of differentiation 4 ($CD4^+$). These are able to interact with antigen presentation cells, such as dendritic cells, which have MHC II proteins on their surface (Fig. 1.3 (**2)**). After interaction in lymphoid tissue, the cells mature into either Th1 or Th2 depending on the type of infection being encountered. Following maturation, Th1 cells promote a cell mediated response (Fig. 1.3 (**3)**) by inducing the stimulation of CTLs by releasing cytokines such as interleukin-2 (IL-2) and interferon gamma (IFN-γ), which also leads to inflammatory responses. The release of IL-12 also stimulates immature Th cells to

differentiate into Th1 and also secrete more IFN- γ, which in turn increases the activity of macrophages. The Th2 response is important in antibody production and is stimulated by Il-4. Th2 cells promote the maturation of B cells (Fig. 1.3 (**4)**) as well as activation of macrophages and release of other inflammation associated cytokines such as Il-4, Il-6 and Il-10. The Th2 response is usually more associated with bacterial infections and multicellular parasites, but may also be important for controlling viral blood infections (Flint *et al*., 2009). Differentiation into Th17 cells occurs at interfaces between the internal and external environments, such as in the skin and lining of the gastrointestinal tract. When activated, these cells stimulate strong inflammatory responses, secrete defensins and recruit neutrophils to the site of activation (Flint *et al*., 2009). The immune response to infection must be regulated to prevent immunopathology to the host induced by CTLs. This is achieved by another subset of T cell, regulatory T cells (Treg cells). Stimulated Treg cells divide more quickly than CTLs and through the action of Treg cytokines the CTL response shuts down (Flint *et al*., 2009). Thus, as activated CTLs and Th cells produce Il-2, which is necessary for Treg-cell replication, the pool of Treg cells also diminishes. Ultimately the system returns to an unstimulated state and only a few memory CTL and Treg cells remain, which limits the degree of self-damage incurred to the host when fighting infection (Flint *et al*., 2009). Previously Treg cells have also been identified in puffer fish (*Tetraodon nigroviridis*), which function similar to that of mammals (Wen *et al*., 2011).

Another type of B cell (B1) is the first B lymphocytes produced in mammals in the foetus, which are situated primarily in the peritoneal and pleural cavities of the developed organism. These produce antibodies from unmutated germ-line genes before encountering any external antigen and constitute the natural antibodies, which are thought to represent a form of evolutionary memory in mammals (Roitt, 1997). These antibodies are produced after B1 lymphocytes are stimulated by T independent (TI) antigen interactions, and

characteristically bind with low affinity to commonly encountered pathogenic antigens (Roitt, 1997). These low affinity antibodies are predominantly IgM, which have been shown to be vital for an immediate acute, innate response in mammals, even to the point where some cells differentiate to antibody producing plasma cells immediately following stimulation with antigen, e.g. LPS, whereas others migrate to the spleen to divide prior to differentiation (Yang *et al*., 2007). Natural antibodies are considered to play a particularly important role in lower vertebrate immunity, including fish (Sinyakov *et al*., 2002; 2006; Magnadottir *et al*., 2009; Sinyakov and Avtalion, 2009; Sandmeier *et al*., 2012). It is also considered that the natural antibodies have a role in prevention of B2 cell stimulation of autoantigens as the low affinity, high avidity IgM will block such interactions (Roitt, 1997). The natural antibodies also initiate a first line of cooperation between innate and adaptive immune responses through complement.

1.6.3 Characteristics of IgM – The predominant antibody class of fish

Two of the most important aspects of the immunoglobulin molecule, in terms of antibody-antigen complexes, are the concepts of affinity and avidity. These vary between the immunoglobulin classes, especially considering the differences in conformation between the systemic immunoglobulin classes, IgG and IgM. Affinity is a measure of the binding strength of the antibody binding site on the $F(ab')_2$ fraction of the immunoglobulin to the single determinant of an antigen (Fig. 1.4 C). There are 2 binding sites per monomeric Ig providing a divalent form, thus the multivalence can be calculated by multiplying the number of monomeric subunits by 2 (Denzin and Staak, 2000) (Fig. 1.4). There are five immunoglobulin classes in mammals, IgM, IgD, IgE, IgA and IgG for which IgG is produced after class switching to yield higher affinity binding sites. IgM in mammals lacks high affinity but its high valency, resulting from the pentameric structure, enables cross-linking between multiple epitopes of complex antigens that possess repeating units. This provides an

effective immune strategy during the early stages of an infection (Roitt, 1997) when high affinity IgG is lacking.

Fish lack isotype switching (Workenhe *et al*., 2010) and possess predominantly IgM and to a lesser extent IgD and the recently discovered mucosal immunoglobulins IgT/IgZ (Hansen *et al*., 2005; Tian *et al*., 2009; Tadiso *et al*., 2011). The IgM molecule in mammals is a large pentameric polymer of 5 4-peptide subunits (Roitt, 1997), whereas the form of this differs in fish (Fig 4.1 A), which has led to much debate regarding the specificity of fish antibody binding. Some reports have subsequently challenged the effectiveness of fish diagnostic serology due to the apparent high avidity but low affinity of IgM (Denzin and Staak, 2000). Although the polymeric fish IgM molecule can exist as a pentameric form in elasmobranchs, it is tetrameric in teleosts (Wilson and Warr, 1992) (Fig 1.4 A-B). Monomeric IgM has also been reported in some fish species (Clem and McLean, 1975).

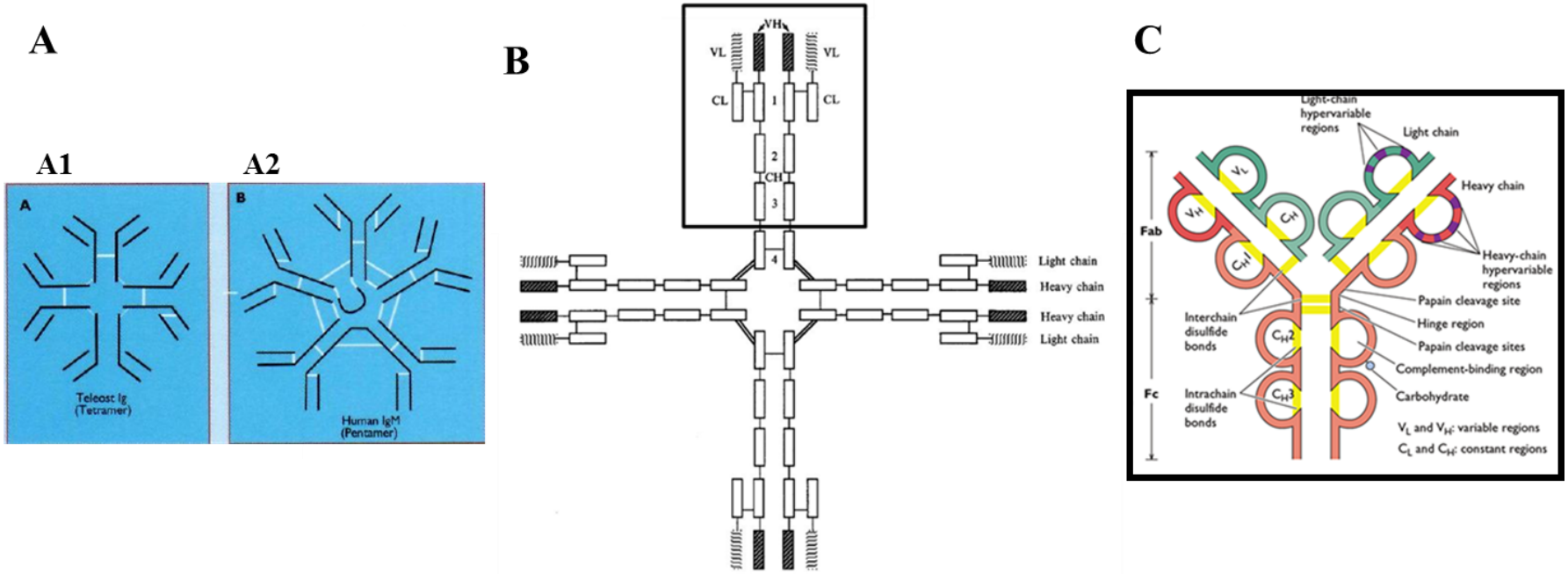


Figure 1.4 Diagrammatic representation of the IgM antibody molecule in teleost fish representing the tetrameric structure (A) The 4 arms of the tetrametic IgM molecule of fish with variable interdomain and interchain disulphide bridging (white lines) (A1) compared to that the 5 arms of the pentameric IgM molecule of mammals with consistent disulphide bridging between domains and chains (A2). The disulphides indicated in fish IgM are potential sites only (Kaattari *et al*., 1998). (B) The tetrametic macroglobulin consists of 8 heavy (μ) polypeptide chains and 8 light (L) polypeptide chains arranged as $(\mu_2L_2)_4$ (where μ= heavy chain and L = light chain (Wilson and War, 1992). (C) The monomeric structure of the Fab and Fc domains of each arm of the IgM showing the interchain disulphide bonds. The variable regions of the heavy (VH) and light (VL) chains are indicated as well as the constant regions, CH and CL. The hypervariable regions and the invariable regions of the Fab and constant region of the Fc are also shown (Flint *et al*., 2009). The example shown is mammalian. Fish lack the consistency of the disulphide bridging seen in mammalian Ig monomers. ***After Kaattari et al. (1998), Wilson and Warr (1992) and Flint et al. (2009)***

The polymerised molecule in fish generally consists of 4 divalent monomer arms attached at the centre, which in mammals is held together by the J chain (Davis *et al*., 1989), however, there is some uncertainty as to the presence of a J chain in fish (Wilson and Warr, 1992; Kaattari *et al*., 1998; Morrison and Nowak, 2002). Variations in the form and functionality of fish IgM have prompted questions regarding its limited immunological diversity. Fish IgM shares common structural and functional features with that of mammalian IgM including high carbohydrate content, similar heavy chain, low affinity but high avidity, lack of logarithmic response following secondary antigenic challenge and limited affinity maturation (Du Pasquier, 1982; Wilson and Warr; 1992; Kaattari *et al*., 1998; Morrison and Nowak, 2002; Cain *et al*., 2002).

The lack of isotypic diversity has been considered a contributing factor to the minimal specificity of antibody responses observed in fish including interesting variation in affinity even between binding sites on the same IgM molecule (Clem and Small, 1970). Furthermore, spectrotypic analysis by isoelectric focusing has revealed that specific antibody heterogeneity is restricted in goldfish (*Carrassius auratus*), tench (*Tinca tinca*) and carp (*Cyprinus carpio*) (Vilain *et al*., 1984; Wetzel and Charlemagne, 1985; Wilson and Warr, 1992).

There are 4 basic chain units of the fish IgM molecule, which comprise of 2 heavy chains (μ) that are held together with 2 associated light chains (L) and are stabilised in various forms by disulphide bridging (Pilström and Bengtén, 1996) (Fig 1.4). The molecule can be divided into domains of approximately 100 aa whereby the most N-terminal domain belongs to the variable (V) domain, and the C-terminal belongs to the smaller constant (C) domain which exist on both heavy and light chains (Pilström and Bengtén, 1996; Morrison and Nowak, 2002). Diversity of the immunoglobulin binding sites is achieved by three regions within the V domain called the complementarity determining region (CDR) (Pilström and Bengtén, 1996).

Diversity among the V domains is an important process during Ig production in order to enable conformational binding to an infinite number of epitopes (Morrison and Nowak, 2002), which is achieved during B cell differentiation, where different segments of DNA are brought together to form the mature genes encoding the 2 Ig chains (Pilström and Bengtén, 1996).

The gene encoding the H chain consists of 3 loci: the variable segment (V), diversity segment (D) and the joining segment (J), whereas the light chain has only V and J segments. Rearrangement of the V, D and J segments in fish bring about diversity, similar to that observed in mammals (Tonegawa, 1983; Wilson and Warr, 1992).

The 3 CDRs are encoded by the V and D segments. The variable heavy chain CDR1 and CDR2 are encoded by the V segment while CDR3 is encoded by the D segment. However, all CDRs of the V light chain are encoded only by the V segment (Pilström and Bengtén, 1996). The affinity for specific antigens is enhanced in mammalian B cell centroblasts by somatic hypermutation, which occurs at the same time as isotype switching and is targeted to the V region (Manis *et al*., 2002; Li *et al*., 2004), often in the CDRs, before cells differentiate into antibody producing plasma cells. This in turn generates a greater diversity between antibodies (Pilström and Bengtén, 1996). However, although it does occur in fish (Du Pasquier *et al*., 1998), somatic mutation, and thus affinity maturation within B cell centroblasts, is more limited in fish (Du Pasquier, 1982). Instead, it is thought that fish may have a greater pressure to express immediate germ-line genes to ensure protection from infectious micro-organisms as the progeny develop outside the mother (Du Pasquier, 1982) in a pathogen-rich aquatic environment. Although Mochida *et al*. (1994) found that immunised Nile Tilapia (*Oreochromis niloticus*) could produce antibodies with increased affinity and specificity similar to higher vertebrates, which were thought to be associated with somatic mutations, it is questionable whether this was the result of affinity maturation as fish lack the

germinal centres that could enable high affinity B cell clones to be selected (Morrison and Nowak, 2002). Alternatively, a minor increase in affinity in fish antibody responses is thought to be of greater significance than would be deemed for mammals, since fish rely predominantly on mulitmeric IgM (Cain *et al*., 2002). It has been suggested that a memory state in fish may result from the expansion of unprimed B lymphocytes (Arkoosh and Kaattari, 1991).

Kaattari *et al*. (1998; 1999) proposed that fish have other means by which to obtain diversified antibody responses. One of the main differences between mammalian IgM and that of fish is the ability to produce structurally heterogeneous IgM by variation in the degree of disulphide polymerisation of monomer and half-mer subunits (Lobb and Clem, 1983). This is thought to occur through the assembly of redox forms of the IgM molecule by post-translational modification resulting in different combinations of intrasubunit disulphide bonding (Kaattari *et al*., 1998; 1999), therefore increasing the diversity of IgM (Fig 1.5 A). The redox structure of antibodies in rainbow trout was also found to vary from different biological fluids, e.g. sera, eggs, ovarian fluid and mucus (Bromage *et al*., 2006). An interesting hypothesis is that fish IgM may benefit over mammalian IgM by increased flexibility as a result of variation in disulphide crosslinking between monomeric subunits (Kaattari *et al*., 1998). Such flexibility may provide a broader latitude for mediating effector functions such as complement fixation via the C1q receptor (complement pattern recognition protein), opsonisation with increased exposure of FcR binding, and an ability to accommodate topologically diverse epitopes (Kaattari *et al*., 1998; 1999) (Fig 1.5 B). In contrast, the rigidity of the uniform covalent disulphide bonded mammalian IgM was suggested to preserve the binding sites for complement (by C1q) during cross-linking of multivalent antigens (Feinstein *et al*., 1986).

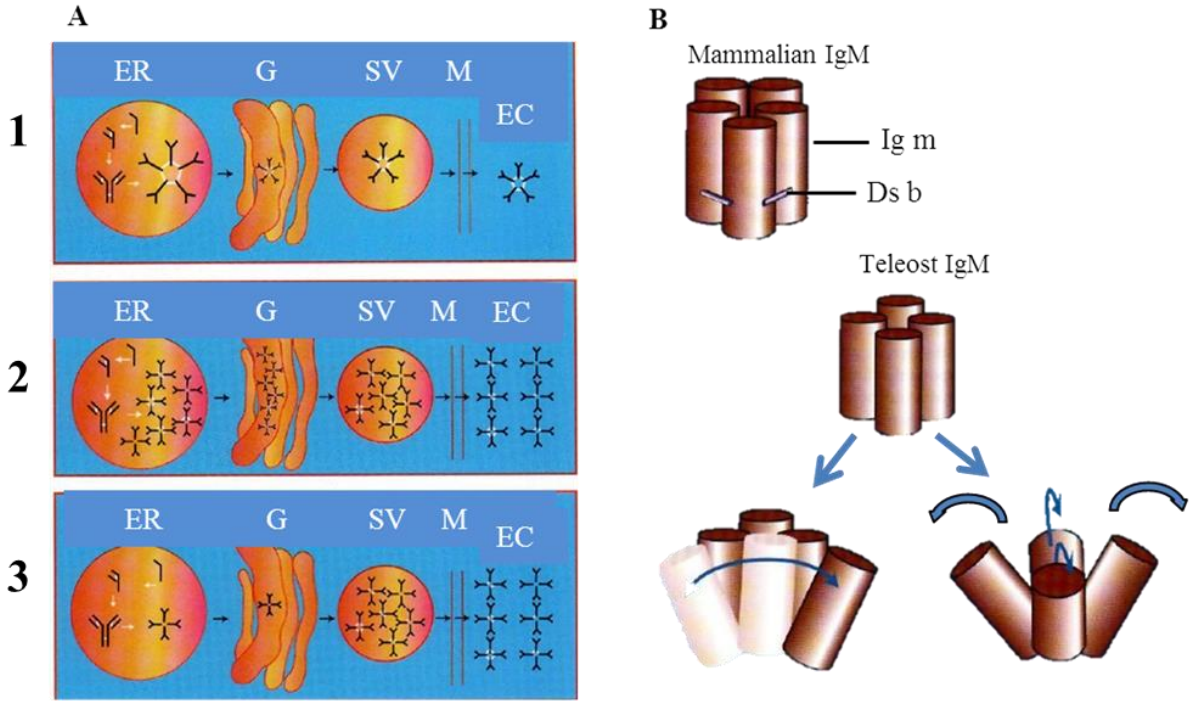


Figure 1.5 Diagrammatic representations of Redox forms of teleost IgM and their possible diverse function (A) The comparison of mammalian IgM pathway during polymerisation of the monomers is shown (1), in relation to 2 possible pathways for fish IgM as proposed by Kaattari *et al*. (1998) (2) and (3). In the mammalian pathway (1) assembly of IgM is via the secretory pathway whereby complete polymerisation of the pentamer occurs in the endoplasmic reticulum. The teleost assembly of IgM may occur through the same pathway as that utilised by mammals (2), however, the diversity of the molecular products secreted by the cell would require all the forms to be already present in the endoplasmic reticulum and subsequently all transported through the secretory pathway in their final form. An alternative possibility is therefore that polymerisation of the secreted molecule is not finalised until late in the secretory pathway (3), whereby the final disulphide linkage does not occur until the immunoglobulin is secreted from the vesicle (Kaattari *et al*., 1998). (B) The rigid, complete monomeric disulphide linked IgM molecule of mammals (1). The variable disulphide linked IgM of teleosts (2). The arrows depict the potential flexibility of the putative fish IgM arrangement with regards to enhanced ability for contact with topologically diverse epitopes as well as latitude for effector functions including complement and opsonisation (Kaattari *et al*., 1998). ER – Endoplasmic reticulum; G – Golgi body; SV – Secretory vesicle; M – Membrane; EC – Extracellular; Ig m – Immunoglobulin molecule; Ds b – Disulphide bridge

After Kaattari et al. (1998)

Despite steric hindrance reducing the valency of mammalian IgM to some antigens, the multivalency potentially allows binding to many epitopes from the single Ig where antigens have several repetitive epitopes (Roitt, 1997). It is generally considered that the high multivalency but low affinity of IgM favours cross-reactions between epitopes, and that the high affinity obtained after isotype switching is a compromise of immunological evolution (Roitt, 1997).

Pilström and Bengtén (1996) suggested that the non-specific defence system including complement and C reactive protein may have improved through evolution for some fish species, which may therefore be of greater importance in the overall immune response against pathogens than specific or adaptive defence mechanisms, thus maybe minimising the necessity for high affinity antibodies.

1.6.4 B cell and T cell co-operation for memory

Immunological memory is generated through the interactions of B cells, T cells and $CD4^+$ helper cells, which together provide the exclusive property of the acquired immune response (Welsh *et al*., 2004). This system is vital for generating protection in mammals following primary infection or immunisation, whereby following clonal expansion and differentiation of B and T cells, a faster secondary immune response can be mounted with higher affinity and at a greater magnitude with increased longevity (Kuby, 1994). However, this enhanced, logarithmic increase characteristic of a secondary response appears restricted to monomeric IgG of mammals, and therefore such a response is more limited for fish multimeric IgM (Kaattari, 1994). Antibodies generated against viral antigen can be crucial for preventing the progression of viral infections by binding to virus and inhibiting its adsorption into host cells (Dimmock, 1984; Abbas *et al*., 2000). T cells also express CD8 surface antigens ($CD8^+$ T lymphocytes) and these cells are important for specific effector

mechanisms, becoming active as CTLs during immunosurveillance against virus-infected or altered cells (Workenhe *et al*., 2010). However, the induction of a B cell response to antigen for the production of specific antibody can vary depending on the type of antigen it encounters. Not all fish are capable of producing specific antibodies, such as Atlantic cod (*Gadus morhua*), which instead appears to produce a much greater yield of non-specific natural antibodies (Magnadottir *et al*., 2009).

The antibody response to an administered antigen may require T cell activity in order to induce the required antibody response for both protection provided by the vaccine antigen, and in the context of marker vaccination and diagnostics, for the induction of specific detectable responses to a marker and/or vaccine antigen (Bly and Clem, 1992; Secombes *et al*., 1996). Antibody production to an antigen depends on its biochemical properties. Polysaccharide antigens tend to induce a B cell antibody response, which is T-independent (TI antigens), whereas proteins will usually induce a T-cell dependent antibody response (TD) (Kaattari and Piganelli, 1996). Some fish have a delayed ability to respond to TD antigens (Etlinger *et al*., 1979; Tatner, 1986), which may result in greater susceptibility at early life stages (Evelyn, 1997). In mammals, B cell responses differ for type 1 T cell independent (TI 1), type 2 T cell independent (TI 2) and TD antigens depending on the requirement for T cell help (Roitt, 1997). Type 1 TI antigens include LPS from bacteria, which can induce a mitogenic polyclonal antibody response via toll-like receptors (TLRs) without being recognised specifically by the B cell hypervariable region surface receptors. B cells are able to focus such antigens on the surface, regardless of concentration, until a high enough concentration is obtained to stimulate its activation, although the antibodies produced are generally of lower affinity (Roitt, 1997). Highly repeated determinants of linear antigen, such as polysaccharides, induce TI 2 responses. These are mediated by macrophages that bind the antigen to specific B cells by cross linking of complementary Ig receptors generating

strong activation signals. However, some antigens are unable to directly stimulate B cells as they are rapidly degraded by phagocytosis, lack mitogenicity or possess univalent specificity of each of their determinants. These antigens require T cell help in order to induce an antibody response. Such antigens are only immunogenic once conjugated to an antigenic carrier protein, after which, if the molecule is large enough, it can be recognised by and endocytosed by the B cell (Roitt, 1997), which has also been demonstrated in various studies on TD and TI antigen immunised fish (Arkoosh and Kaattari, 1991; Jones *et al*., 1999a; Cain *et al*., 2002 Swan *et al*., 2008). The processed peptides of the hapten-carrier conjugate are subsequently presented by the MHC II pathway to the Th2 cell and in return the Th2 cell stimulates the B cell to divide, differentiate and produce anti-hapten antibodies (Roitt, 1997).

1.6.5 Enhanced immunity by adjuvant and carrier molecules and implications on antigenic competition

Adjuvants and carrier proteins can be used for formulating vaccines for TD antigens in order to induce the required specific antibody responses. Adjuvants containing antigens from killed pathogens, i.e. *Mycobacterium* cells of Freunds complete adjuvant (FCL), can induce strong stimulation of both B lymphocyte and T lymphocytes (Anderson, 1992). However, it is often necessary to manipulate the pathogen associated antigens in order to retain potency, but reduce toxicity. Following inactivation of the pathogen, it is not always possible to induce the responses required, as seen with bacterial enterotoxin B subunits, which successfully resulted in strong antibody responses in mice to Hen egg lysozyme (HEL) when co-administered with *Escherichia coli* heat labile enterotoxin (Etx) B subunit, but not the closely related Cholera toxin (Ctx) B subunit (Millar *et al*., 2001). The route of administration, which in fish is usually via ip where a relatively large volume of adjuvant can be inoculated into the peritoneal cavity, can also have a major influence on the antibody production induced to

multiple antigens as inoculation directly into the bloodstream can induce tolerance to the carrier (Serero and Avtalion, 1978).

Hapten-specific antibody responses can, however, be suppressed by booster immunisation resulting in limited production of anti-hapten antibodies especially to immunoglobulin isotypes IgG2 and IgG3 but not to IgM, therefore would hypothetically have little influence on the fish antibody reactivity in terms of DIVA vaccination, i.e. if using conjugated peptide vaccines. After immunisation of fish with hapten-carrier immunogens, Killie and Jorgensen (1994) noted that anti-hapten antibody responses in Atlantic salmon are suppressed when more than one hapten is conjugated to the carrier protein. For example NIP (4-hydroxy-3-iodo-5-nitrophenyl-acetic-acid) suppressed responses to FITC (fluorescein-5-iso-thiocynate), despite antibodies being raised against the carrier molecule *Limulus polyphemus* hemocyanin (LPH). This is considered to be a phenomenon of intra-molecular induced antibody suppression, i.e. antigen induced suppression (AIS) (Pross and Eidinger, 1974; Killie and Jorgensen, 1994). In mammals immunisation with hapten-carrier molecules has resulted in the development of carrier specific T cells and hapten-specific B cells (Mitchison, 1971), although epitope-specific suppression has also been noted where conjugates may provoke the down regulatory effects on the antibody response.

1.6.6 Detection of fish immunoglobulins: The basis of marker/DIVA vaccination

Detection of the specific alternative antibody response to a 'foreign' exogenous marker (positive marker) or absent endogenous marker (negative marker) is achieved by serological testing using ELISA. Only animals inoculated with the vaccine containing the marker antigen will produce a detectable antibody response against the marker, whereas animals responding to epitopes associated with the pathogen indicate that they are infected or have been vaccinated with an alternative vaccine (James *et al*., 2008) (Fig. 1.6 A-B). Although this

approach does not enable a DIVA strategy, it can be applied for DIVA approaches if accompanied with a vaccine that differs sufficiently to the infectious agent to distinguish animals that had been vaccinated prior to becoming infected, which otherwise cannot be serologically identified by negative markers alone. The negative marker approach is achieved by specific detection of antibodies to an antigen absent from the vaccine to indicate infection, but antibodies to alternative antigens of the pathogen indicate vaccination (Fig. 1.6 C-D).

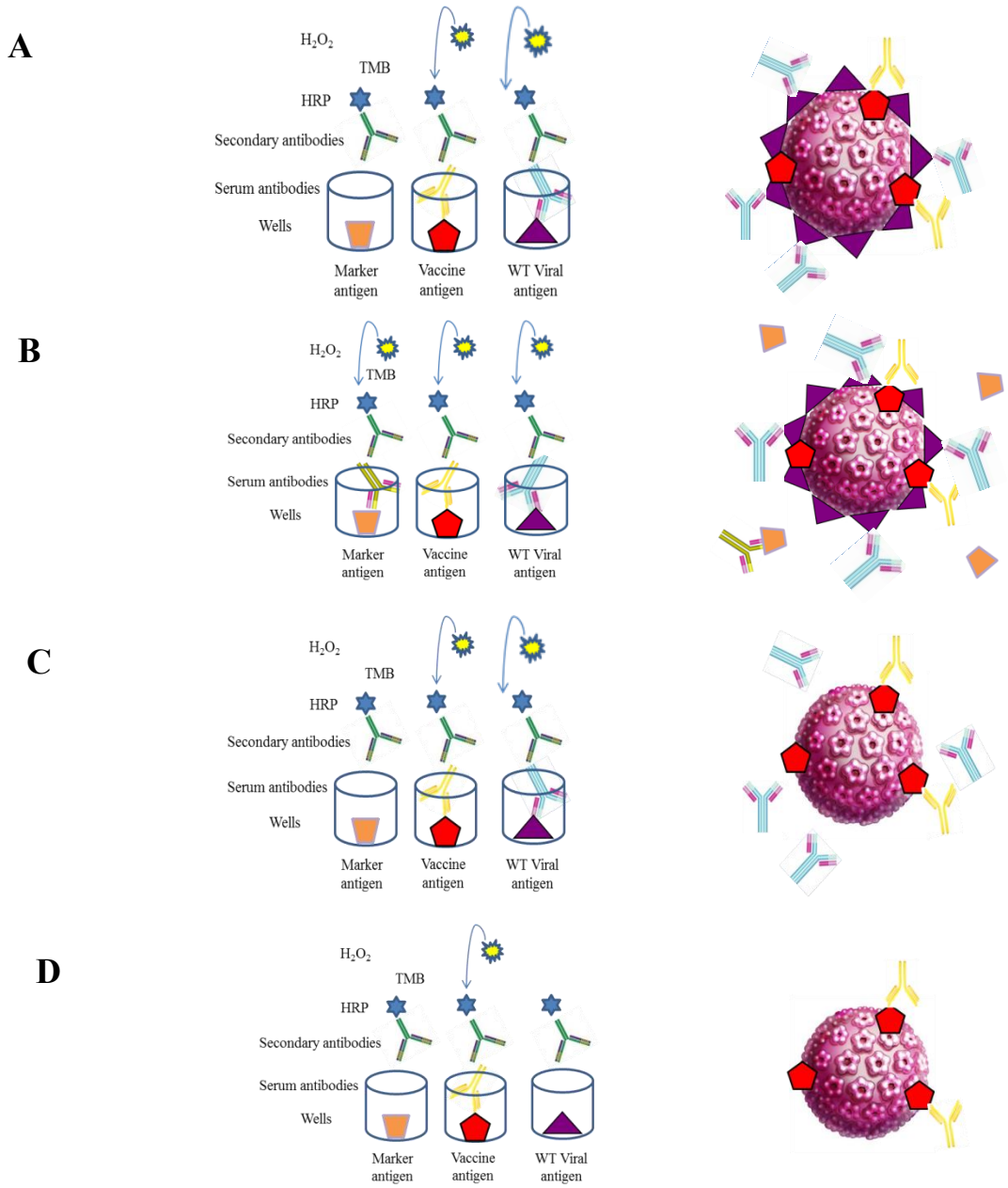


Figure 1.6 Schematic diagram of antigen specific enzyme-linked immunosorbent assay (ELISA) and antibody response to exogenous 'positive' and endogenous 'negative' marker vaccine antigens. A = conventional vaccine and ELISA; B = 'positive exogenous' marker vaccine and ELISA; C and D = 'negative endogenous' marker vaccines and ELISAs. (A) Antibody response to all vaccine antigens. Note no differential response to WT and vaccine; (B) Antibody response induced to vaccine and marker antigens indicating vaccination; (C) Positive signal induced to 2 viral antigens including antigen absent in the vaccine indicating infection; (D) Positive signal only to vaccine antigen present in the vaccine indicating vaccinated, but uninfected. Different coloured immunoglobulins represent specific antibody responses to: Orange = marker, blue = virus, yellow = vaccine. HRP and TMB are enzyme and substrate, respectively, involved in the reaction resulting in a chromogenic signal. WT = Wild type antigen. Shapes represent epitopes of the marker , vaccine and virus antigens.

1.7 Model diseases for the application of marker or DIVA vaccination in aquaculture:
Disease characteristics and aetiological agents

1.7.1 ISA

Thorud and Djupvik (1988) first recognised ISA, which they named Brennes syndrome after the region (Hastings *et al*., 1999; RSE, 2002), as a highly lethal and systemic disease of Atlantic salmon in Norway in 1984. The disease inflicts a broad pathology including lethargy, exophthalmia, ascites, pale gills with filamentous sinus congestion, enlarged haemorrhagic liver with necrosis, splenic congestion, erythrophagocytosis, congestion of the lamina propria in the stomach and foregut, petechiation of visceral fat, haemorrhage of the renal interstitial tissue and tubular nephrosis (Evensen *et al*., 1991; Nylund *et al*., 1993; Jones *et al*., 1999b; Simko *et al*., 2000; Hovland *et al*., 1994; Cipriano and Miller, 2003; Kibenge *et al*., 2004). Mortality rates can be highly variable between 10-100 % both during a disease outbreak and under experimental conditions, which can also be influenced on the genetic background of Atlantic salmon stocks (Nylund *et al*., 1995; RSE, 2002). Although ISA is considered to be a marine disease, and transmission between wild fish is thought to occur during the marine phase of the life cycle, horizontal transmission has been demonstrated in freshwater (Nylund *et al*., 1993; Simko *et al*., 2000). It is well established that the virus is present in the wild (Raynard *et al*., 2001; Snow *et al*., 2003; Plarre *et al*., 2005) with an abundance of potential reservoir hosts including sea trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), herring (*Clupea harengus*) and Arctic char (*Salvelinus alpinus*) (Nylund *et al*., 1994; 1995; 1997; 2002; Nylund and Jakobsen, 1995; Rolland and Nylund, 1999; Devold *et al*., 2000; Snow *et al*., 2001). There is a possibility that these species may

constitute asymptomatic life-long carriers of the virus, which may pose a threat to farmed fish stocks (Murray *et al*., 2010), but this has yet to be clarified (OIE, 2012).

It is possible to isolate and propagate ISAV in cell culture, which has facilitated extensive analysis of the biochemical, physicochemical and morphological features of the pathogen. However, there is a great variation in cell susceptibility and virus titres that are yielded *in vitro* (Dannevig *et al*., 1995; 1997; Sommer and Mennen, 1996; Falk *et al*., 1997; 1998; Kibenge *et al*., 2000; Rolland *et al*., 2003; 2005; Weli *et al*., 2013), which can be detrimental to diagnostics. These variations have been attributed to differential virulence factors in ISAV isolates and strains (Kibenge *et al*., 2000; 2007; Cunningham *et al*., 2002; Cook-Versloot *et al*., 2004), but they may also be associated with different host cell innate immune responses (Joseph *et al*., 2004; Kibenge *et al*., 2005; Schiøtz *et al*., 2009). Ultrastructural and molecular characterisation has revealed the aetiological agent as an enveloped, negative-sense single-stranded RNA virus with a virion of 90-140 nm diameter (Dannevig *et al*., 1995; Koren and Nylund, 1997; Mjaaland *et al*., 1997; Kibenge *et al*., 2004). Further morphological and genomic characterisation of ISAV have led to its classification as an orthomyxovirus (Falk *et al*., 1997; Koren and Nylund, 1997; Mjaaland *et al*., 1997; Krossøy *et al*., 1999) in a newly formed Isavirus genus (Kawaoka *et al*., 2005 cited in OIE, 2012). The genome consists of 8 distinct linear RNA segments that have all been sequenced (Biering *et al*., 2002; Mjaaland *et al*., 1997; Krossøy *et al*., 1999; 2001; Cunningham and Snow, 2000; Rimstad *et al*., 2001; Ritchie *et al*., 2001; 2002; Clouthier *et al*., 2002; Snow *et al*., 2003). The genome segments 7 and 8 have 2 open reading frames (ORFs), while all others have 1 each, similar to influenza virus A and B. The total molecular size of the genome is 14.3 kbp (Clouthier *et al*., 2002) encoding for at least ten proteins including nine structural proteins: the 66 kDa phosphorylated nucleoprotein (NP), 22 kDa matrix protein (M), and 2 glycosylated surface proteins, the 42 kDa haemagglutinin-esterase

protein (HE) and 50 kDa fusion protein (F) (Falk *et al*., 2004; Aspehaug *et al*., 2005), as well as one non-structural protein. The proteins of ISAV have been identified and characterised (Falk *et al*., 2004; Kibenge *et al*., 2004; 2007) and like other orthomyxoviruses e.g. AIV, the haemagglutinin is essential for binding the virus envelope to sialic acid residues on the cell surface and is one of the most important proteins in terms of virulence, but unlike the neuraminidase of AIV, the haemagglutinin of ISAV is accompanied by esterase. The HE protein exhibits haemagglutinating activity of red blood cells as well as receptor destroying activity and the F protein has a role in fusion of the virus to the cell membrane. (Falk *et al*., 1997; Mjaaland *et al*., 1997; Rimstad and Mjaaland, 2002; Kibenge *et al*., 2004). Therefore, although the ISAV haemagglutinin differs remarkably in size from that of AIV, it still conforms to many of the properties of the orthomyxovirus viral heamagglutinins (Rimstad *et al*., 2001). More recently, however, evidence indicates that the 'haemagglutination-infection' differs phenotypically from that of AIV (Workenhe *et al*., 2007) and the lack of elution from infected Atlantic salmon erythrocytes, which is facilitated by acetylesterase activity (Falk *et al*., 1997; Eliassen *et al*., 2000), is an advantage for the virus.

Sequence analysis of the genome has revealed some very important differences, especially with regards to the HE gene, not only in terms of virulence, but also the geographical origin of isolates (Kibenge *et al*., 2004; 2009a; Mjaaland *et al*., 2005; Vike *et al*., 2009). Mutations within the highly polymorphic region (HPR0) of the HE gene, i.e. amino acid deletions, have been associated with virulence (Kibenge *et al*., 2006; 2007).

Genotyping of segment 5 (F protein) of the viral genome revealed that an isolate had originated from Norway in a 'dormant' state before mutations (recombination insertion event) of the F gene occurred resulting in a virulent phenotype leading to disease outbreaks in Chile between 2007-2009 (Kibenge *et al*., 2009a). This highlights the instability of this virus (Kibenge *et al*., 2009a). Therefore, although the HE protein constitutes an important protein,

in terms of virulence, that has rendered it a target for development of a recombinant vaccine (Mikalsen *et al*., 2005; Müller *et al*., 2008; Dhar and Allnutt, 2011; Gomez-Casado *et al*., 2011; Wolf *et al*., 2013), the F protein may also be an effective target regarding its association with virulence, but it too is prone to mutations (Markussen *et al*., 2008).

1.7.2 KHVD

Although the first outbreak of KHVD was described in Germany in 1997 (Bretzinger *et al*., 1999) and the causative agent was identified the following year after disease outbreaks amongst carp and koi in Israel and the U.S.A. (Ariav *et al*., 1999; Hedrick *et al*., 2000), archival evidence and clinical observations suggests that the disease, or at least the virus, may have been prevalent in England in 1996 (Walster, 1999; Haenen *et al*., 2004). This report was based on the detection of KHV DNA in fixed tissues by *in situ* hybridisation (ISH). However, a recent report in South Korea also found viral KHV DNA in archival tissue samples (Lee *et al*., 2012), suggesting that the mass mortalities of common carp experienced there in 1998 was likely to be KHVD-associated. This finding was supported by the detection of herpes-like virus particles in infected common carp tissues by TEM (Choi *et al*., 2004).

Juveniles and younger carp appear more susceptible to KHVD although all age groups succumb to disease (Bretzinger *et al*., 1999; Perelberg *et al*., 2003; Sano *et al*., 2004). Experimental challenges of carp larvae have revealed that prior to maturation (i.e. >3 days post hatch), these fish were resistant to KHVD, however following maturation they succumb to disease (Ito *et al*., 2007a; 2007b *both cited in* Ilouze *et al*., 2011). A number of other factors can influence the manner of disease including stress and population density, especially considering the efficient transmission via faeces and secretions (Perelberg *et al*., 2003; Dishon *et al*., 2005). However, temperature has the most significant impact on KHVD (St-Hilaire *et al*., 2005; 2009; Gilad *et al*., 2003; 2004; Ronen *et al*., 2003; Perelberg *et al*.,

2005; 2008; Uchii *et al*., 2011). Koi herpesvirus disease is a seasonal disease and at optimal temperatures of 18-28ºC mortality rates can reach between 80-100% within 6 – 22 days (Bretzinger *et al*., 1999; Hedrick *et al*., 2000; Perelberg *et al*., 2003) with peak mortalities occurring after 8-12 dpi, whilst morbidity is usually 100% (Perelberg *et al*., 2003). The disease does not manifest above 30ºC due to inhibited viral replication, which has been demonstrated *in vitro* (Dishon *et al*., 2007; Ilouze *et al*., 2012a).

Both behavioural and clinical disease signs of KHVD can initiate rapidly after only 2-3 dpi and vary massively, including lethargy, anorexia, increased respiratory movements, uncoordinated swimming, hyperaemia at the base of the fins and on the abdomen. Fish often gather at water inlets and gasp at the water surface. At these early stages pathology may already be evident in the gills (i.e. after 2 dpi). Gill filaments appear pale, patchy and necrotic with respiratory hyperplasia. Haemorrhaging may also be apparent on the body surface. Eosinophilic intranuclear inclusion bodies have also been observed histologically in the gills and severe inflammation can result in fusion of adjacent lamellae. Hyper-secretion of mucus can result in patches developing on the skin with a sandpaper-like texture. Sunken eyes (enophthalmia) is a common disease sign and is accompanied by emaciated body condition. Neurological signs occur towards the final stages of the disease and the fish swim awkwardly becoming disorientated with a loss of equilibrium. Internally, clinical pathology may include a swollen kidney and spleen with peritubular inflammatory infiltrate and congested blood vessels. (Walster, 1999; Hedrick *et al*., 2000; Haenen *et al*., 2004; Pikarsky *et al*., 2004; Ilouze *et al*., 2006a; Bergmann *et al*., 2007; Miyazaki *et al*., 2008; Michel *et al*., 2010a; Cheng *et al*., 2011). At lower temperatures the disease is notably more protracted (Walster, 1999).

The only susceptible hosts to KHVD are *Cyprinus carpio*: common carp and koi and their hybrids (Gilad *et al*., 2002; Perelberg *et al*., 2003; Hedrick *et al*., 2006; Bergmann *et al*.,

2010b; Ilouze *et al*., 2011; Michel *et al*., 2010a). However, KHV DNA has been detected in a large number of other species that were either experimentally challenged or naturally exposed to the virus, despite these fish being asymptomatic, including grass carp (*Ctenopharyngodon idella*), tench, (*Tinca tinca*), crucian carp, (*Carassius carassius*), Atlantic sturgeon (*Acipenser oxyrinchus*), Russian sturgeon (*Acipenser gueldenstaedtii*), blue back ide (*Leuciscus idus*), *Ancistrus spp*., and goldfish (*Carassisus auratus*) among others (El-Matbouli *et al*., 2007; Sadler *et al*., 2008; Kempter *et al*., 2009; Bergmann *et al*., 2009a; b; 2010b; c; Ilouze *et al*., 2011; El-Matbouli and Soliman, 2011; Fabian *et al*., 2013). Furthermore, other studies have also detected KHV DNA in invertebrates such as the freshwater molluscs, swan mussel (*Anodonta cygnea*) and crustaceans such as scud (*Gammarus pulex*) (Kielpinski *et al*., 2010). These studies emphasise the potential role that abundant and diverse KHV reservoir species may play in the spread and outbreaks of KHVD. Moreover, experimental cohabitations have revealed that goldfish, grass carp and tench are capable of transmitting virulent KHV that has induced mortalities in naïve common carp (Bergmann *et al*. 2007; 2009b; 2010c; Michel *et al*., 2010a; El-Matbouli and Soliman, 2011).

Since its first isolation KHV has been successfully cultured in a number of cyprinid cell lines (Hedrick *et al*., 2000; Neukirch and Kunz, 2001; Oh *et al*., 2001; Ronen *et al*., 2003; Pikarsky *et al*., 2004; Davidovich *et al*., 2007; Dong *et al*., 2011). This has facilitated extensive characterisation of this relatively new virus, which has been crucial for understanding the biology and pathogenesis of this important aquatic pathogen.

Following the isolation of the etiological agent in different research laboratories in both Israel and U.S.A., there was initially some disagreement on the terminology of the virus. The name koi herpesvirus was associated with the morphologic resemblance to viruses of the order herpesvirales (Hedrick *et al*., 2000). Due to its larger genome than other herpesviruses, however, and the associated lesions that resulted from the disease, the virus was referred to as

carp nephritis and gill necrosis virus (CNGNV) in Israel (Perelberg *et al*., 2003; Ronen *et al*., 2003; Pikarsky *et al*., 2004; Hutoran *et al*., 2005). The taxonomic designation of the virus has now been established. Although often still referred to Koi herpesvirus (KHV) the virus is now officially classified as *Cyprinid herpesvirus -3* (CyHV-3) according to the International committee on Taxonomy of Viruses (ICTV) (Waltzek *et al*. 2005). The virus has also recently been classified as a member of the family *Alloherpesviridae* (Waltzek *et al*. 2009), of the newly formed order *Herpesvirales* (Davison *et al*. 2009), based on the complete 295 Kb genome sequence (Aoki *et al*. 2007).

The large enveloped, double stranded DNA virus has an icosahedral capsid measuring 110 nm in diameter, and the mature virion is between 180-230 nm including its glycoprotein envelope (Hedrick *et al*. 2000). Thus KHV has a typical structure for a virus of the Herpesvirales (Mettenleiter *et al*., 2009). The capsid is covered by a proteinaceous matrix that makes up the tegument, which is surrounded by a lipid envelope derived from host cell membranous organelles. The large double stranded linear DNA genome is flanked with left and right repeats (Aoki *et al*., 2007). The virus is closely related with CyHV-1 (carp pox virus; papilloma virus) and CyHV-2 (goldfish haematopoietic necrosis virus; GHNV) and distantly related to IcHV-1 (channel catfish virus CCV) and RaHV-1 (ranid herpesvirus; frog herpesvirus) (Waltzek *et al*., 2005; Ilouze *et al*., 2006b; Davison *et al*., 2009). Strains of KHV from Japan (J), Israel (I) and U.S.A. (U) sequenced by Aoki *et al*. (2007) revealed almost 99% homology despite their geographical distance from each other. Sequence homology between isolates appears to be typical of KHV (Michel *et al*., 2010a). These were divided into 2 lineages: J and U/I, although all were thought to have been introduced into Europe since 2001 (Bigarré *et al*., 2009).

There are 156 potential protein encoding ORFs including 8 ORFs encoded by terminal repeats (Aoki *et al*., 2007) in the KHV genome. The ORF25 family consists of 6

potential membrane glycoprotein encoding ORFs, four of which were detected in mature extracellular virions although the glycoproteins of KHV are not considered to have much similarity with other members of the Herpesviridae (Michel *et al*., 2010a; b). There are a number of other genes that are thought to play a possible role in immune evasion, such as ORF16, which encodes a potential G protein coupled receptor, ORF134, which codes for IL-10 homologue and ORF12 which codes for a tumor necrosis factor receptor homologue. However the majority of the 156 ORFs of KHV (i.e. at least 110) lack any obvious homology with other organisms (Michel *et al*., 2010b). Six of the ORFs encode proteins with the closest relative in the families of Poxviridae and Iridoviridae (Aoki *et al*., 2007), e.g. ORF140 that encodes a thymydylate kinase and ORF 55 that encodes a thymidine kinase (Michel *et al*., 2010b). Some of these proteins have been characterised more intently such as the TK encoded by ORF 55, which is the target of a sensitive PCR method (Bercovier *et al*., 2005), and the IL-10 encoded by ORF 134, which is potentially important for virus immune evasion mechanisms (Van Beurden *et al*., 2011a; Sunarto *et al*., 2012). However, compared with ISAV, very little is known regarding the functionality and complexity of the KHV proteins.

Despite minimal heterogeneity between isolates of KHV (Aoki *et al*., 2007), low diversity molecular markers are being developed to discriminate between 9 genotypes (i.e. 7 from Europe and 2 from Asia) (Kurita *et al*., 2009). Because KHV harbours the largest genome of the order herpesvirales, it has proven to be an ideal model for mutagenesis studies, which are being utilised to generate an infectious bacterial artificial chromosome (BAC) to produce recombinant KHV virus strains and possible live vaccine candidates (Costes *et al*., 2008; Michel *et al*., 2010a). None of the many structural proteins have been studied in detail although the product of ORF81 is thought to represent one of the most immunogenic membrane proteins (Rosenkranz *et al*., 2008; Michel *et al*., 2010a).

1.8 Project aims

The current project was undertaken to assess the feasibility of DIVA vaccination in aquaculture. The goal was to develop a DIVA strategy for vaccinated Atlantic salmon and carp against infectious salmon anaemia and koi herpesvirus disease, respectively, by exploiting fish antibody responses to various vaccine 'markers' using serology. The project was carried out over the course of a number of *in vitro* and *in vivo* experiments:

- Investigate the feasibility of exogenous marker vaccination with whole inactivated vaccines against 2 model notifiable diseases: infectious salmon anaemia in Atlantic salmon and koi herpesvirus disease in common carp. (Chapter 3)

Further studies were then focused on 1 of the models depending on antibody responses to the vaccines. Subsequently, the aims listed below were orientated towards DIVA vaccination for KHVD specifically

- Characterise early pathogenesis with a virulent isolate and its influence on pathogen-directed diagnostic methods prior to the production of pathogen-specific antibodies (Chapter 4)
- Elucidate the most sensitive diagnostic assays for early stage detection (Chapter 4)
- Examine the expression kinetics of virus structural proteins during the course of the infectious cycle *in vivo* and *in vitro*. This could provide useful information regarding their potential application in DIVA diagnostics (Chapters 4 and 5)
- Establish the feasibility of sero-surveillance by screening fish from various case studies in the field and vaccine challenge trials (Chapter 6)
- Develop a highly specific and sensitive serological test that can detect infected /exposed fish reliably (Chapters 3 and 6)

- Utilise recombinant proteins for an endogenous marker approach and evaluate the feasibility of a DIVA strategy for an inactivated vaccine (Chapter 6)
- Extrapolate and characterise any potential targets for DIVA diagnostics (Chapters 5 and 6)

Chapter 2

General Materials and Methods

2.1 Monoclonal antibody production

2.1.1 Hybridoma cell culture

2.1.1.1 Growth and maintenance

Hybridoma cells producing monoclonal antibodies (MAbs) to Koi herpesvirus (KHV) were kindly provided by Dr. Sven Bergmann (Friedrich Loeffler Institut (FLI), Greifswald, Germany). Hybridoma cells producing MAbs to Infectious salmon anaemia virus (ISAV) and rainbow trout (*Oncorhynchus mykiss*) or Atlantic salmon (*Salmo salar*) immunoglobulin M (IgM) were developed at the Aquatic Vaccine Unit, Institute of Aquaculture, University of Stirling, Stirling, Scotland. Cells were cultured in Dulbecco's Minimum Essential Medium plus additive (DMEM+) (Sigma-Aldrich, St. Louis, USA), containing 10% (v/v) foetal calf serum (FCS) (Sigma-Aldrich), 2.5 mL penicillin streptomycin (PenStrep) 1250 units (U) (10,000 U penicillin; 10 mg mL^{-1} streptomycin (Sigma-Aldrich)), 5 mL L-glutamine (200 mM) (Sigma-Aldrich), and 5 mL sodium pyruvate (100 mM) (Sigma-Aldrich). The cells were cultured at 37°C in 5% CO_2. Cells were expanded from 25 cm^2 culture flasks containing approximately 8 mL to 75 cm^2 (30-50 mL) then finally 150 cm^2 flasks (100-125 mL) in order to obtain 1 L of cell supernatant from which the MAbs were concentrated and purified.

Hybridoma cell suspension was harvested when > 90% of cells had died after approximately 10 days of culture. Forty-five mL of lysed cell suspension was aliquoted into 50 mL centrifuge tubes (VWR International, Radnor, USA) and centrifuged at 1912 x *g* in a Sigma 4 K 15 centrifuge for 10 min at 4°C in order to pellet cell debris, and the supernatant containing the MAbs was pooled and retained. The supernatant stocks were kept at either 4°C for short term storage or -20°C for long periods of time.

2.1.1.2 Concentration of MAbs

MAbs were concentrated through either (1) Amicon Ultra centrifugal filters (Millipore, Cork, Ireland) for small scale production, or (2) a Pall LV Centramate for larger scale production according to the manufacturer's instructions.

(1) The 10 Kilodalton (kDa) membrane of the Amicon Ultra centrifugal filters provides a simple method for concentrating MAbs, as high molecular weight immunoglobulins are retained within the filter and concentrated. Approximately 5 mL of hybridoma sample was added to the tubes, which were then centrifuged at 2,000 x *g* for 10 min at 4°C in a Sigma 4 K 15 centrifuge. The eluent was discarded and more sample added. This procedure was repeated to concentrate the MAb samples to a volume of 1-2 mL.

(2) The Pall L V Centramate concentrator consists of a peristaltic pump and membrane system and is more suitable for larger volumes of supernatant. After attachment of the cassettes and gasket to the concentrator, the system was sanitised with 0.1M NaOH at a pressure of 3-5 pounds per square inch (psi), then washed with H_2O prior to equilibration with phosphate buffered saline (PBS), pH 7.2. Approximately 500 mL of hybridoma supernatant was fed through the system at 10 psi providing 25 mL of concentrated supernatant. The system was washed through with 0.5M NaOH pre-heated to 37°C at 25-30 psi between runs with different samples.

2.1.1.3 Purification of MAbs

The concentrated MAb hybridoma supernatant was made up to 50 mL with binding buffer (20mM sodium phosphate, pH 7). The solution was filtered through a 0.66 µm filter step followed by a 0.22 µm nitrocellulose membrane (Millipore). All buffers including binding buffer, elution buffer and Tris-HCl were filtered through a 0.45 µm nitrocellulose membrane

before undertaking the procedure in order to eliminate contaminating particles that could affect the efficiency of the system and purity of the sample.

Purification was undertaken by affinity chromatography through pre-packed 1 mL Affinity Purification High Trap Protein-G columns (GE Healthcare) containing Protein G sepharose for binding Immunoglobulin G. The ÄKTA prime liquid affinity chromatography system (Amersham Biosciences) was used for purification of the MAbs.

After fitting the column to the system, it was washed with ultrapure H_2O and equilibrated with binding buffer prior to use. Filtered samples containing MAbs were passed through the system at a rate of 1 mL min^{-1} to allow IgG to bind to the column. Unbound proteins were washed through the column, monitored by UV spectrophotometry at 280 nm. Once the column was cleared of contaminating proteins, IgG bound to the column was eluted as 1 mL fractions with Glycine-HCl, pH 2.7. The eluted fractions were neutralised with 100 µL Tris-HCl, pH 9. Fractions containing purified MAbs, determined from their absorbance at 280 nm were pooled and dialysed against PBS. Dialysis tubing cut into 20 cm lengths was activated by boiling for 5 min in 5mM EDTA, 200mM sodium bicarbonate then rinsed thoroughly in deionised H_2O. This was repeated before autoclaving the tubing in deionised H_2O. After extensive washing, the dialysis tubing was tied at either end after having added the purified MAb to the tubing. Three x 4 L buffer changes of PBS were carried out during the dialysis procedure before harvesting the sample. The concentration was then determined using a protein assay as described in the section below.

2.1.1.4 Determining the concentration of the purified MAbs

The Pierce BCA protein assay kit (Thermo Scientific, Rockford, USA) was used to determine the concentration of the affinity purified MAbs. The BCA kit is a colorimetric detection and quantification method for proteins, and is based on the reduction of Cu^{+2} to Cu^{+} in the

presence of protein in an alkaline medium, which can then be detected by chelation of molecules of bicinchoninic acid by the cuprous ion (Smith *et al*., 1985). This reaction is spectrophotometrically analysed at 562 nm and the concentration of unknown samples can be determined from a calibration curve of absorbance for known Bovine Serum Albumin (BSA) standards of known concentration. For this, BSA diluted in PBS was used to establish the standard curve according to the manufacturer's instructions. Briefly, 25 µl of standard or unknown samples, in replicate, were mixed with 200 µl (1:8) of working reagent which was composed of 50 parts of solution A to 1 part of solution B and added to 96-well plates (Sterlin, Fisher Scientific, Newport, UK). The plates were covered with foil, shaken for 30 sec at 600 shakes per sec (sps) on a plate shaker (Minishaker IKA) and incubated at 37°C for 30 min. The plate was then read on a spectrophotometer (CECIL CE 2021) at 562 nm. Protein concentrations were calculated from a standard curve of absorbance values from protein standards of BSA ranging from 0-2,000 µg mL^{-1}.

2.2 Fish cell line culture

During the project two salmonid cell lines were used for the production of ISAV and two cyprinid cell lines for the production of KHV. Growth conditions and maintenance varied depending on the type of cell line used. During subculture, volumes, concentrations and types of reagents varied depending on the culture vessels being used and these are summarised in Table 2.1. Unlike hybridoma cell cultures, all of these continuous cell lines are anchorage-dependent requiring trypsinisation for subculture.

Table 2.1 Reagent volumes used during subculturing of cells

Flask size (cm^2)	Dulbecco's PBS (mL $wash^{-1}$)	Trypsin EDTA (mL)	Volume (mL^{-1}) added to new flask
25	5	0.5	5-6
75	10	1.5	18-21
175	20	3	40-45

PBS = phosphate buffered saline; EDTA = ethylenediaminetetraacetic acid

2.2.1 Salmonid cell lines

2.2.1.1 Salmon Head Kidney (SHK-1) cells

SHK-1 cells were kindly provided by Prof. Birgit Dannevig (Norwegian College of Veterinary Medicine, Oslo, Norway). The cell line was originally developed from the head kidney of post-smolt Atlantic salmon and exhibits a fibroblast-like morphology (Dannevig *et al*.,1995), the cells of which have been characterised as leukocytes (Dannevig *et al*., 1997).

The cells were cultured at 20-22°C without CO_2 in Leibovitz's L-15 +GlutaMAX-I medium (Invitrogen, Paisley, UK) containing 5% Australian Foetal Bovine Serum (AFBS) (Gibco, Paisley, Scotland), 4mM L-glutamine (Invitrogen), 40µM 2-mercaptoethanol (Invitrogen) and 50 i.u./µg Pen/Strep (Penicillin/Streptomycin, Invitrogen). Subculturing was undertaken at intervals of 10-14 days at a split ratio of 1:2 or 1:3 depending on the level of monolayer confluence and whether the subculture was for viral culture. A passage between 50-72 was maintained as SHK-1 cells lose their susceptibility to ISAV around passage 80.

Spent media was decanted and the monolayer was washed twice with Dulbecco's PBS (Invitrogen). Trypsin- ethylenediaminetetraacetic acid (EDTA) solution (0.05% w/v trypsin,

0.02% w/v EDTA in PBS) (Invitrogen) was added to the monolayer for 1-2 min. Excess trypsin was decanted off once cells had developed an opaque and rounded appearance. Detached cells were subsequently collected and resuspended in fresh medium containing the necessary supplements, aspirating carefully before transferring to a fresh tissue culture flask and incubating at 20-22°C.

2.2.1.2 Atlantic Salmon Kidney (ASK-2) cells

ASK-2 cells are epithelial-like cells that were isolated from the kidney of a healthy Atlantic salmon for isolation of ISAV (Devold *et al*., 2000). These had originally been purchased from the American Type Culture Collection (ATCC). The culture conditions of these were similar to that of SHK-1 cells with minor modifications in the medium specification and the subculture intervals, which were between 2-3 weeks. The cells were cultured at 20-22°C without CO_2 in Leibovitz's L-15 +GlutaMAX^{TM}-I medium containing 20% FBS (Gibco) and 50 i.u./µg Pen/Strep (Invitrogen) maintained between passage 60-78. These cells were slow growing and could only be split at a ratio of 1:2 following the same protocol described for SHK-1 cells.

2.2.2 Cyprinid cell lines

2.2.2.1 Koi Fin (KF-1) cells

KF-1 cells were developed from epidermal tissue of Koi and are characteristic of fibroblasts (Hedrick *et al*., 2000) (Fig. 2.1 C and D). The cell line was kindly provided by Dr. Keith Way (Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth, UK).

KF-1 cells were cultured in Minimum Essential Medium (MEM) medium containing Eagles's salts (Invitrogen), 10% foetal bovine serum (FBS), 1% Non-Essential amino acids (NEAA, Invitrogen) and 2mM L-glutamine at 22-25°C with 4% CO_2. Cells were subcultured

as described for salmonid cell lines using a 1:3 split ratio at 7-14 day intervals and passages were maintained between 108-144. Noticeable changes in cell morphology occurred towards later passages and the cells became increasingly difficult to maintain.

2.2.2.2 Common Carp Brain (CCB) cells

CCB cells were kindly provided by Dr. Matthias Lenk (Friedrich Loeffler Institut, Greifswald, Germany), which had previously been developed from brain tissue of Common carp (*Cyprinus carpio* L.) (Neukirch *et al*., 1999). The culture conditions for CCB cells were identical to that of KF-1 cells, however a much greater split ratio could be used with CCB cells (i.e. 1:3 to 1:6). One hundred percent confluent monolayers was achieved in just 5-7 days and cell passage was maintained between 69-84, after which cell morphology changed and further subculture was often not possible. These cells also have a fibroblast-like morphology (Fig. 2.1 A and B).

2.2.3 Cell counts

Cell concentrations were required to determine specific seeding densities and multiplicities of infection (moi) for *in vitro* experiments. Cell monolayers were washed twice with DPBS then trypsinised, as described previously in Section 2.2.1.1. After detachment from the flask, 3-6 mL of cell suspension was placed in a universal. One hundred microlitres of cell suspension was combined with 100 µl 0.5% Trypan blue dye in a Bijoux, placed on a haemocytometer (Hawksley, England) and viable cells counted under a compound microscope (Olympus, Japan) at 10x magnification. The mean cell number was determined from the two chambers of the haemocytometer. The average cell count was then multiplied by 2 (as the dilution factor of the cells was 2) giving the total number of cells = n x 10^4 mL^{-1}. This value was then multiplied by the total volume of cell suspension from which the count was made.

2.2.4 Storage of cells in liquid nitrogen

Cells were washed and trypsinised as described in Section 2.2.1.1 and counted as described in Section 2.2.3. The suspended cells were centrifuged (Denley, BR401 centrifuge) at 150 x *g* for 5 min at room temperature (RT) in order to pellet the cells. After removing the medium, the cell pellet was resuspended in fresh medium, containing the optimal serum concentration for the cell line, and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Dorset U.K.) was added. One millilitre ampoules of 3 x 10^6 cells in cryovials were stored at -70°C for 1 h in a polystyrene container, which were then transferred to liquid nitrogen. A similar procedure was undertaken for storage of hybridoma cells except that they were cultured on 24-well tissue culture plates (Nunc, Fisher-Scientific, UK).

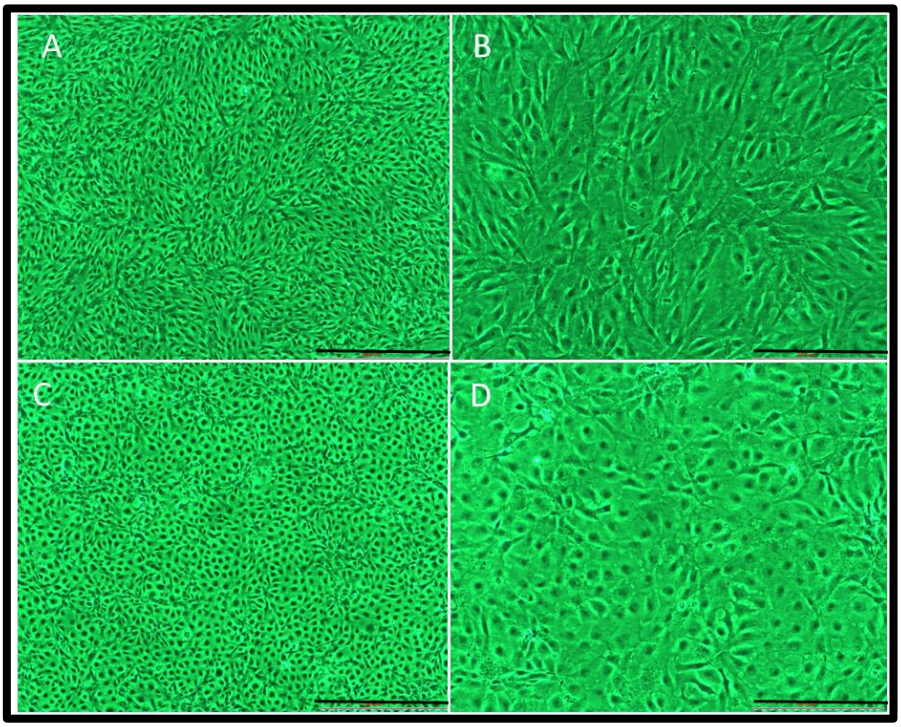


Figure 2.1 Healthy common carp brain (CCB) cells and koi fin (KF-1) cells. Photomicrographs of CCB and KF-1 cell monolayers under phase contrast (A) Uninfected CCB cells 6 days old, x10 mag.; (B) Uninfected CCB cells 6 days old, x25 mag; (C) Uninfected KF-1 cells 6 days old, x10 mag.; (D) Uninfected KF-1 cells 6 days old, x25 mag. Scale bar = 500 µm mag. x10; 200 µm mag. x25

2.2.5 Recovery of cells from liquid nitrogen

Cells were thawed and the 1 mL suspension was mixed with 5 mL of fresh medium containing the required supplements and placed in a 25cm^2 culture flask (Nunc, Denmark). The cells were cultured at the optimal temperature and were checked after 3-5 h for cell attachment. Culture medium was replaced with fresh medium and the cells cultured until confluent.

2.3 Virus Production

2.3.1 Infectious Salmon Anaemia virus (ISAV)

ISAV Scottish isolate V 0431 (Solway Project), obtained at the Virology Unit of the Institute of Aquaculture, University of Stirling, was cultured for use as antigen on ELISA for detecting anti-ISAV antibodies. The isolate was originally isolated from ISA infected Atlantic salmon on 10/02/2009 and was kindly provided by Dr. David Smail (Fisheries Research Services (FRS) Marine Laboratory, Aberdeen, Scotland). ISAV was initially propagated in SHK-1 cells by inoculating a 60-70% confluent monolayer within 48 h of subculturing. Weaker cytopathic effects (CPE) were obtained by increasing the passage number. Therefore, ASK-2 cells were also used as these have been found to be just as susceptible to ISAV as SHK-1 cells (Rolland *et al.*, 2005). The ASK-2 cell line was used at a confluence of 50-60% after 48 h of incubation at 20°C. All virus inoculations were undertaken on pre-formed monolayers.

Culture medium was decanted from cultures to be used for viral propagation, and the monolayer carefully washed with DPBS. One millilitre of ISAV-infected culture supernatant, diluted 1:2 in Hank's Buffered Salt Solution (HBSS, Invitrogen) containing 2% FBS and 1 mL, was applied to the SHK-1 or ASK-2 cell monolayers to allow absorption of the virus.

Cultures were incubated overnight on a rocking platform (Biometra) at 15°C without CO_2 to allow absorption of the virus after which fresh L-15 medium with additives was added and cells cultured as described in Section 2.2.1. The serum concentration added to the ASK-2 cells was reduced from 20% to 5% and the cultures were incubated at 15°C and checked regularly for signs of a cytopathic effect (CPE). Mock infected cells were incubated with HBSS, 2% FBS without virus during the absorption period. Once an obvious CPE and/or lysis was observed, monolayers were freeze/thawed at -70°C, and the cells scraped from the monolayer into suspension using a cell scraper (Nunc, Rochester, New York, U.S.A.). The CPE observed with ISAV in SHK-1 cells rarely developed into a full CPE, however infected medium was passaged every 7-10 days post infection (dpi) onto fresh monolayers. The cell suspension was centrifuged at 2,500 x *g* in an Eppendorf 5804 R centrifuge for 15 min at 4°C. The resulting supernatant was retained and the pellet discarded. The supernatant was aliquoted and stored at -70°C and the viral titre determined by back titration according to the method of Spearman Kärber (Karber, 1931).

2.3.2 Koi Herpesvirus (KHV)

Four isolates of KHV were utilised during the project. An American isolate of KHV, H361, was kindly provided by Dr. Dietner Fichtner (FLI, Germany) via Dr. Keith Way (CEFAS, UK) and was cultured and maintained within the Virology Unit at the Institute of Aquaculture (IOA), University of Stirling and was used for all experimental work undertaken at IOA. This virus had originated from an adult Koi population experiencing mass mortality from koi herpesvirus disease (KHVD) in Eastern USA in 1998 (Hedrick *et al*., 2000). The second KHV isolate (D-182) was isolated from diseased Koi in England and was kindly provided by Dr. Keith Way (CEFAS, UK) and was cultured and maintained in CCB cells at the Friedrich Loeffler Institut, Greifswald, Germany by Miss Irena Werner and Dr. Sven Bergmann. This

isolate was used for the challenge performed in Germany at the FLI with Dr. Sven Bergmann, as part of the study presented in Chapter 4. Attempts to culture an Asian isolate kindly provided by Ms. Yahui Wang (Agri-Food and Veterinary Authority of Singapore (AVA), Lorong Chencharu, Singapore) were unsuccessful as a result of the freeze-dry process used to store the virus stock. However, an Asian isolate was utilised for an experimental challenge undertaken in Singapore by Ms. Yahui Wang and an Israeli isolate was utilised for an experimental challenge undertaken in Israel by Dr. Ofer Ashoulin (Madan, Ma'agan Michael Kibbutz, Israel), as part of the study presented in Chapter 6.

KHV was initially propagated in KF-1 cells (Fig. 2.2 C and D), but susceptibility of the cell line deteriorated at later passages, which has also previously been noted by other researchers (Zhou *et al*., 2005, cited in Dong *et al*., 2011; Gomez *et al*., 2011). The CCB cell line proved to be more stable, grew faster, could be subcultured at a greater split ratio and was highly susceptible to KHV (Fig. 2.2 A and B), thus proving a more suitable alternative to KF-1 cells, particularly because of the need to bulk culture the virus for viral purification. All virus inoculations were undertaken on preformed monolayers.

The cells were subcultured and maintained at 22°C over a period of 24-36 h until a 50% or 70-80% confluence had been obtained in KF-1 and CCB monolayers, respectively. The culture medium containing 10% FBS was removed and the monolayers were carefully washed with DPBS prior to inoculation with KHV. An adsorption period of 1-2 h at 20°C was performed before resupplementing the infected cultures with fresh MEM, which contained a reduced serum content (2%).

Infected cells were maintained at 20°C in 4% CO_2 and CPE was recorded every 2 days. Full CPE was obtained in 7-14 dpi after which the virus was passaged or harvested. Virus was passaged directly from the infected culture medium onto a fresh monolayer and stocks

were retained from each passage of virus in case of attenuation. An aliquot from each viral stock was titrated, stocks of which were kept at -70°C.

Virus was harvested after CPE had reached 90-100% by centrifuging the lysed cell suspension at 3,800 x *g* (Eppendorf 5804 R centrifuge). The clarified supernatant was aliquoted and stored at -70°C.

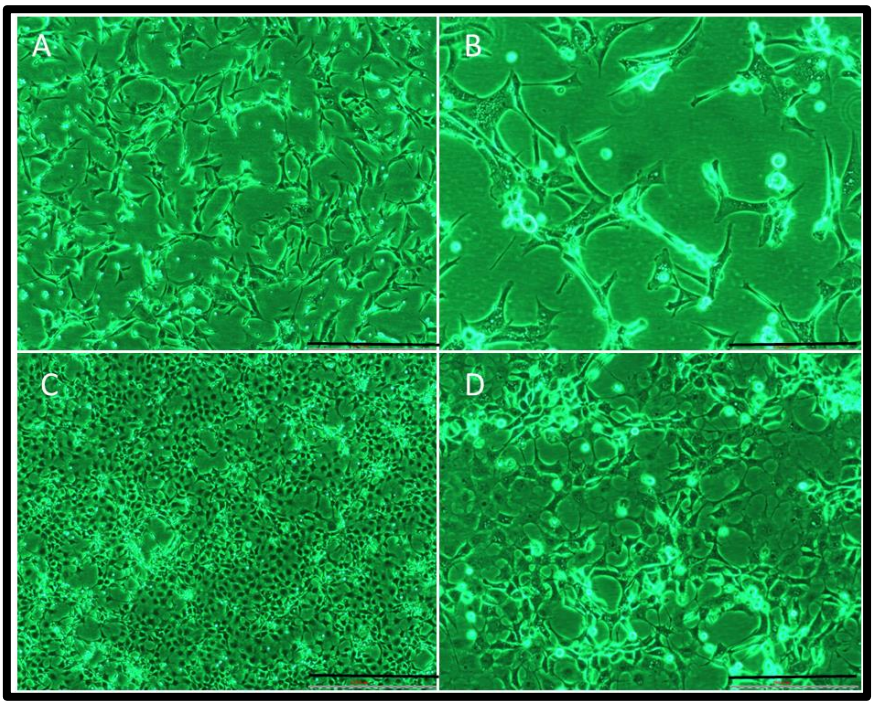


Figure 2.2 Koi herpesvirus (KHV) infected common carp brain (CCB) cells and koi fin (KF-1) cells. Photomicrographs of CCB cell monolayers under phase contrast (A) Infected CCB cells 6 dpi, x10 mag; (B) Infected CCB cells 6 dpi, x25 mag.; (C) Infected KF-1 cells 6 dpi, x10 mag.; (D) Infected KF-1 cells 6 dpi, x25 mag. Scale bar = 500 µm mag. x10; 200µm mag. x25

2.4 Virus quantification

2.4.1 ISAV quantification

2.4.1.1 End-point titration of ISAV from pre-formed cultures

SHK-1 and ASK-2 cells were seeded in 12-well plates to prepare pre-formed monolayers for virus inoculation. Cells were cultured as previously described in Section 2.2.1.2. One medium change was undertaken 24 h post seeding, then once the monolayers had reached a 50-60% confluence after 48 h, after which spent medium was decanted and 0.2 mL of 5-fold serially diluted ISAV in HBSS, 2% FBS was added to the wells. After adsorbing overnight at 15°C the cultures were supplemented with fresh medium, and cells were incubated for 2-3 weeks. Mock infected cells, which received non-infected culture medium, were included in every assay. Cells were scored for CPE after 7, 14 and then finally 21 dpi. Only cultures exhibiting a CPE of at least 50% were scored as positive before determining the $TCID_{50}$ value by the method described in Section 2.4.3.1.

2.4.1.2 End-point titration of ISAV from simultaneous cultures

It proved difficult to undertake a simultaneous inoculation and back titration in 96-well tissue culture plates (Nunc, Denmark) with SHK-1 cells, therefore for simultaneous infection only ASK-2 cells were used. Eighty microlitres of HBSS (2% FBS) were mixed with 20µl of virus culture from Section 2.3.1 from left to right of the 96-well plate to produce a 5-fold dilution series of virus inoculum across the plate. The top and bottom rows of the plate only contained HBSS diluent. Confluent ASK-2 cells from a $25cm^2$ culture flask (Section 2.2.1.2) were trypsinised and 100µl of cell suspension added to every well of the 96-well plate, aspirating after the addition of the cells and incubating at 15°C without CO_2. Cells were scored as

positive or negative depending on the level of CPE after 2 weeks and the end point titre was determined as described in Section 2.4.3.1.

2.4.2 KHV quantification

2.4.2.1 24-well plate end-point titration (pre-formed)

Similarly to the pre-formed titration carried out for ISAV (Section 2.4.1.1), KF-1 and CCB cells were cultured overnight at 22°C in 24 well tissue culture plates (Nunc, Denmark) to form a monolayer. After 50-60 % confluence had been obtained, medium was removed and the cells were inoculated with 100 µL 5-fold serially diluted KHV virus in HBSS, 2% FBS. Mock infected cells received only culture medium with no virus. Absorption of the virus to the plate was undertaken for 1-2 h at 20°C before cells were resupplemented with fresh MEM medium containing 2% FBS. Cells were checked for the development of a CPE after 7 and 14 dpi. $TCID_{50}$ was determined according to the method described in Section 2.4.3.1.

2.4.2.2 96-well plate end-point titration (Simultaneous/pre-formed)

Simultaneous inoculation of 96-well plates was undertaken as that described in Section 2.4.1.2 with KF-1 cells and CCB cells inoculated with 5-fold diluted KHV virus. However, the pre-formed method was preferred for KHV. One hundred microlitres of cell suspension was added to the wells of the 96-well plate (Nunc) and incubated overnight at 22°C in 4% CO_2. The following day, 5-fold dilutions of KHV were prepared and 100 µL of this was added to cells after removing the old culture medium. After 1-2 h adsorption at 20°C, the cells were re-supplemented with fresh MEM medium containing 2% FBS and cultured for 14 days, at which point the titre was determined according to the method described in Section 2.4.3.1.

2.4.2.3 Plaque assay

The plaque assay was predominantly used for confirmation of calculated $TCID_{50}$ values. The plaque assay provides very sensitive, accurate and reliable quantitation of infectious virus particles, with each plaque being derived from a single infectious clone (Burleson *et al*., 1992). The assay was originally developed to determine titres of bacteriophages (Dulbecco and Vogt, 1953) and was applied here to detect KHV (Ronen *et al*., 2003; Hutoran *et al*., 2005). The used protocol was kindly provided by Dr. Maya Ilouze, The Hebrew University-Hadassah, Jerusalem, Israel).

The CCB cells were seeded at 1 x 10^5 cells $well^{-1}$ into a 24-well plate and incubated overnight at 22°C. The following day the old medium was removed and the monolayers were washed twice with DPBS. Five or ten fold serial dilutions of virus were made in HBSS, 2% FBS. Cells were then inoculated with 100µL KHV for 1-2 h at 20°C to allow adsorption of virus on to cells. Mock infected cells received only HBSS, 2% FBS. MEM media was prepared with 8% FBS and the usual supplements of 1% NEAA and 2mM L-glutamine. In some cases when CO_2 was not available for the incubation, 15mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) was added to the medium. The culture media was mixed 1:4 with 1.2% melted sterile agarose (Multi ABgarose, Thermo Scientific, UK) in PBS to form a media overlay containing 0.3% agarose. One millilitre of the MEM/agarose overlay gel was then added to the infected monolayers and the cells were incubated at 20°C with 4% CO_2. After 5-7 dpi, the plaques were fixed with 1 mL 4% formaldehyde for 10 min at RT and the cells were stained with 1.5% Gentian violet Gurr (Certistain®, VWR) in distilled H_20 after careful removal of the overlay. The Gentian violet stains the intact cells of the monolayer allowing the plaques to be easily counted (Fig. 2.3).

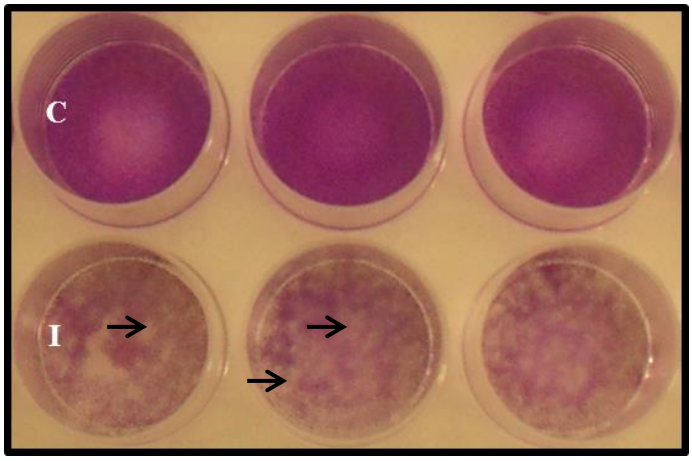


Figure 2.3 Plaques obtained for quantification of koi herpesvirus (KHV) infectious particles on common carp brain (CCB) cells. The cells were fixed and stained 5 dpi and the plaques (arrows) counted. Row C = Control wells, row I = Infected wells.

2.4.3 Quantification of infectious virus particles

2.4.3.1 Spearman-Kärber method for virus titration

$$\textbf{Mean log TCID}_{50}\textbf{ (m) = } X + \frac{1}{2} \times d - d \times \Sigma\left(\frac{r}{n}\right)$$

Where

X = log of the highest reciprocal dilution

d = log of the dilution interval

r = number of test subjects not infected at any dilution.

n = number of test subjects inoculated at any dilution.

After Kärber, 1931

The Spearman-Kärber method (Kärber, 1931) provided a means for determining the dilution of virus (or virus sample) required to infect 50% inoculated cells. This is expressed as tissue culture infectious dose required for 50% infection of cells inoculated (i.e. $TCID_{50}$). The $TCID_{50}$ assay ultimately measures cytocidal virions (Burlesson *et al*., 1992).

After inoculation of cells with serial dilutions of virus the CPE was analysed and an all-or-nothing score was given for the culture: positive for a culture with ≥ 50% CPE of the monolayer and negative for a culture with < 50% CPE. The number of cultures infected at a specific viral dilution were then determined and $TCID_{50}$ calculated using the formula defined above.

2.4.3.2 Plaque quantitation (plaque forming units; PFU)

PFU = Mean plaque number X reciprocal dilution X reciprocal of volume in mL

After Burleson *et al*. (1992).

Only wells with 20-100 plaques were counted at that reciprocal dilution. The number of plaques counted provides an estimate of the total number of infectious virions initiating infection. The infection titre of the plaque assay is expressed as plaque forming units (PFU) per mL and is calculated from the formula defined above.

2.4.3.3 Multiplicity of infection (MOI)

$$m = aN/C$$

Where
a = proportion of viral particles that initiate infection
N = Total number of viral particles
C = Total number of cells

After Dulbecco and Ginsberg, (1988)

The multiplicity of infection is important in order to know the distribution and proportion of cells infected by virus particles which depends on the average number of viral particles per cell. MOI was calculated using the formula defined above.

Therefore, the number of PFU, which is a measure of the proportion of infectious particles, is divided by the total number of cells to give the MOI. Where plaque assays were not performed, $TCID_{50}$ was converted to PFU by multiplying the antilog by 0.69. This takes into account the Poisson distribution which is a measure of the proportion of cells infected by a given number of virus particles (Dulbecco and Ginsberg, 1988), and has also been described elsewhere for determining the MOI for other virus infections (Wang *et al*., 2008; Voronin *et al*., 2009).

2.5 Virus purification

The purification method used differed for the 2 viruses. Caesium chloride gradient ultracentrifugation was used to purify ISAV and sucrose gradient ultracentrifugation for KHV.

2.5.1 ISAV caesium chloride gradient purification

Purification of ISAV was initially attempted using ASK-2 cells for bulk production of particles, as later passages of SHK-1 cells appeared to become more resistant to cell lysis following ISA infection despite permitting replication. However, only low yields of virus were obtained with isopycnic gradient purification from ASK-2 cells, thus a pelleting method of ISAV infected SHK-1 cells was also attempted.

2.5.1.1 Virus harvest and slow speed clarification from infected ASK-2 cells and SHK-1 cells

Cultures of ASK-2 and SHK-1 cells were both used for producing batches of virus cultures for purification, however, purification through a caesium gradient was only attempted for virus grown in ASK-2 cells. Twenty x 175 cm^2 tissue culture flasks were used to culture the virus in both cell

lines. Culture of cells and inoculation with virus was undertaken as described in Section 2.2.1 and 2.3.1. Cells used for antigen production were inoculated with 7 mL flask^{-1} of $10^{5.5}$ $TCID_{50}$ mL^{-1} ISAV adsorbed overnight at 15°C before resupplementing with fresh culture medium as described previously (Section 2.3.1.). Each flask contained a total of 40 mL infected culture media with supplements (as described in Section 2.2.1), which were pooled to provide the final volume necessary for purification/concentration of virus particles, at least 500 mL of infected media. Only ASK-2 cells of passage 60-70 or SHK-1 cells of passage 50-60 were used for virus propagation. After 2 weeks post-infection (wpi), cells were freeze-thawed twice at -70°C and the lysed cell suspension was pooled together for viral purification. Four x 175 cm^2 tissue culture flasks of the mock infected (i.e. uninfected cells) cultures were also harvested in parallel.

All vessels and buffers were RNAse-free by treating with diethyl pyrocarbonate (DEPC, Sigma, USA) prior to initiating the procedure. The ISAV infected cell suspension was aliquoted into 10 mL centrifuge tubes and centrifuged at 2,500 x *g* for 15 min at 4°C. The resulting pellets were resuspended in 1 mL TNE buffer by aspirating then pooled together and dispensed into 4 x 4 mL centrifuge tubes.

The tubes containing the resuspended pellets were placed in an ultrasonicator bath (Kerry) filled with iced water. Four x 30 sec blasts were applied before centrifuging again at 2,000 x *g* for 10 min at 10°C. The pellets were stored at -70°C, in case virus had been retained in lysed cell debris, and the supernatant of ~16 mL was kept at 4°C overnight.

The pooled ISA infected culture media supernatant was dispensed into 6 x 38.5 mL Ultraclear centrifuge tubes (Ultra-Clear, Beckman, UK) and centrifuged for further clarification at 12,000 x *g* for 35 min at 4°C in a SW28 rotor of a Beckman L-80 ultracentrifuge. The supernatants were pooled together and kept at 4°C.

2.5.1.2 High speed clarification and virus pelleting of ISAV

Clarified virus supernatant was then placed into 6 x 13.2 mL Ultraclear ultracentrifuge tubes (Ultra-Clear, Beckman, UK). The tubes were placed in SW41Ti buckets and centrifuged at 100,000 x *g* for 95 min at 4°C on a SW41Ti rotor. The resulting supernatant was discarded and supernatant from the previous step was added to the tubes and the tubes centrifuged again.

Excess media was removed from the pellets immediately following pelleting by inverting the tubes on ethanol wipes. Fifty microlitres of TNE buffer (10mM Tris, 100mM NaCl, 1mM EDTA, pH 7.4) was added to the pellets, which were dissolved at RT for 10 min. For infected SHK-1 cell cultures, the pelleted virus proteins were aliquoted and stored at -70°C as either concentrated or 1:10 stocks. Preparations of non-infected, control SHK-1 cells proteins were also pelleted in the same way to harvest host cell proteins that would also be present in the virus preparation as a negative control. These were also aliquoted and stored at -70°C.

Tubes containing virus pellets from infected ASK-2 cells were covered with Nescofilm and kept at 4°C overnight.

2.5.1.3 Caesium chloride centrifugation and purification of ISAV

A stock solution of 50% w/w caesium chloride was prepared in RNAse-free TNE buffer. Stocks of 40%, 35%, 30%, 25% and 20% caesium chloride were prepared from the 50% stock and a dis-continuous gradient was produced by careful addition of each solution into a clean 13.2 mL Ultraclear centrifuge tube (Beckman) from the most dense to the least dense. The following volumes of caesium chloride solutions were used to make the gradient: 1.5 mL 20%, 2.5 mL 25%, 2.5 mL 30%, 2.5 mL 35% and 2 mL 40%.

One millilitre of pelleted ISAV derived from infected ASK-2 cells was layered on top of the gradient and tubes placed in SW41Ti rotor buckets. The gradients were then centrifuged at 150,000 x *g* for 17 h at 4°C in a SW41Ti rotor. A band was visualised (Fig. 2.4) and collected by puncture of the tube with a 16G needle (Terumo, Leuven, Belgium) attached to a 2 mL syringe (Terumo). The purified virus was diluted in TNE buffer in clean Ultraclear centrifuge tubes (Beckman).

2.5.1.4 Washing and re-pelleting of purified ISAV

The tubes containing diluted ISAV in TNE buffer were centrifuged a final time on a SW41Ti rotor at 100,000 x *g* for 1 h 35 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1 mL fresh TNE buffer. One hundred microlitre aliquots of concentrated virus or 1:10 diluted virus were prepared and stored at -70°C.

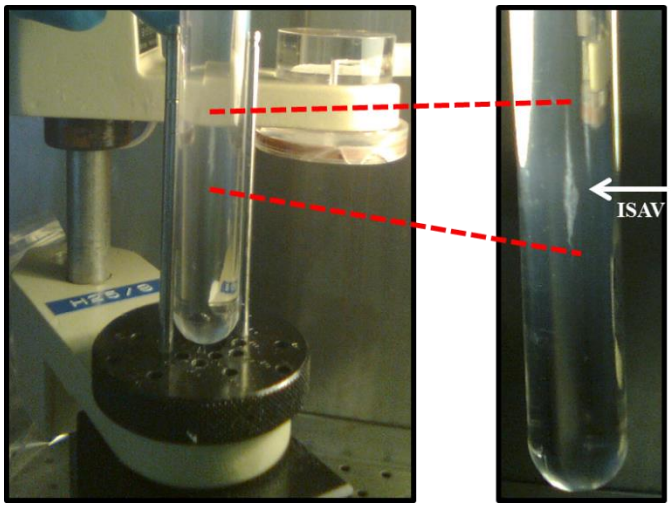


Figure 2.4 The band obtained after CsCl gradient ultracentrifugation of ISAV. This was extracted and the constituents of ISAV particles were characterised by negative staining TEM as shown later in Section 3.3.1.

2.5.2 KHV sucrose gradient purification

Four different protocols were attempted for the purification of KHV, however yields of virus were very low. The most successful approach, similarly to that used for ISAV, was achieved by sonication of the cell pellets prior to ultracentrifugation for separation of intracellular virus from cell debris. Bulk culture of KHV was carried out in CCB cells only as KF-1 cells grew slower and the cell line lacked stability at later passages.

2.5.2.1 Virus harvest and slow speed clarification from infected CCB cells

Purification of KHV was undertaken following procedures similar to those used by Hutoran *et al*., (2005) and Bergmann (pers. comm.) with modifications of the method by Gray *et al*. (2002). Between 500 – 2000 mL of virus culture medium is necessary for a high yield of purified KHV virus therefore CCB cells were bulk cultured in 15-20 x 175cm^2 tissue culture flasks (Nunc) for each purification procedure undertaken. The cells were cultured and split 1:4 as described previously in Section 2.2.2.2. Twenty-four h after seeding, cultures were inoculated with 7 mL $10^{4.4}$ $TCID_{50}$/mL KHV American isolate H361 at a virus passage between 14-17 and cell passage of 73-80. Virus adsorption was allowed for 2 h at 20°C before the addition of fresh media containing 2% FBS. Full CPE was achieved after 11 dpi at which point all monolayers were frozen at -70°C to expose all the cells to freeze/thaw lysis.

All infected media were thawed at RT and the resulting lysed cell suspension was aliquoted into 10 mL centrifuge tubes at approximately the same volume. The solutions were centrifuged at 3000 x *g* for 20 min at 10°C. The resulting supernatant (supernatant 1) was pooled and retained at 4°C. The pellets were kept and incubated with 1 mL TNE buffer (10mM Tris, 10mM NaCl, 3mM EDTA, pH 7.4) for 10 min and dissolved by aspirating. The dissolved pellet solutions were then pooled to yield 4 x 4 mL.

Separation of intracellular KHV virions from cell debris was important for a high yield of purified virus. The same sonication procedure undertaken for ISAV in Section 2.5.1.3 was applied for KHV. The pellets were kept at -70°C, in case of retained KHV protein in the cell debris, and the supernatant (supernatant 2) was kept at 4°C overnight.

2.5.2.2 High speed clarification and virus pelleting of KHV

The supernatants were pooled together and placed into sterilised ultraclear centrifuge tubes (Beckman) and placed into SW41ti rotor buckets on a SW41Ti rotor for ultracentrifugation. The supernatant was centrifuged at 100,000 x *g* for 50 min at 4°C in a Beckman Coulter ultracentrifuge. Pellets were kept and supernatant was discarded. Once all the supernatant had been centrifuged it was discarded immediately to prevent the pellets dissolving and the pellets were subsequently air dried for 5 min. Once the majority of residual supernatant had been removed from the pellets, they were kept at either 4°C overnight or in 0.5 mL TN buffer (10mM Tris, 10mM NaCl, pH7.4) and left to dissolve at RT.

2.5.2.3 Sucrose gradient centrifugation and purification of KHV

Two dis-continuous sucrose gradients were prepared from sucrose dissolved in TN buffer. The gradients were made by the slow addition of 3 mL sucrose solutions of decreasing densities using a 5 mL syringe (Terumo) into a 13.2 mL Ultraclear tube (Beckman) starting from 60% followed by 50%, 40% then finally 20%.

The pellet solutions were made up to a final volume of 4 mL in TN buffer and 2 mL was layered onto each gradient in Ultraclear centrifuge tubes (Beckman). The tubes were placed in SW41Ti buckets on a SW41Ti rotor and centrifuged at 110,000 x *g* for 1 h at 4°C. The gradients were then placed in a fractionator and bands around 40% and 50% were visualised (Fig. 2.5) and collected by puncture of the ultraclear tube with a 16G needle (Terumo)

attached to a 2 mL syringe (Terumo). The bands containing KHV virions were diluted in fresh TN buffer in clean Ultraclear tubes.

2.5.2.4 Washing and re-pelleting of purified KHV

Tubes containing diluted sucrose gradient purified KHV were placed in SW41Ti buckets and centrifuged for a final time at 100,000 x *g* for 60 min at 4°C. The supernatant was decanted and the resulting pellets were dissolved in 1 mL TN buffer at RT. One hundred microlitre aliquots of concentrated and 1:10 diluted purified virus stocks were stored at -70°C until use.

2.5.3 Virus protein quantitation

Purified virus protein was quantified similar to the method used for purified MAbs in Section 2.1.1.4. However, TNE or TN buffer was used as a diluent for BSA standards instead of PBS. The total yield of purified KHV protein was between 0.9 and 1.4mg mL^{-1}.

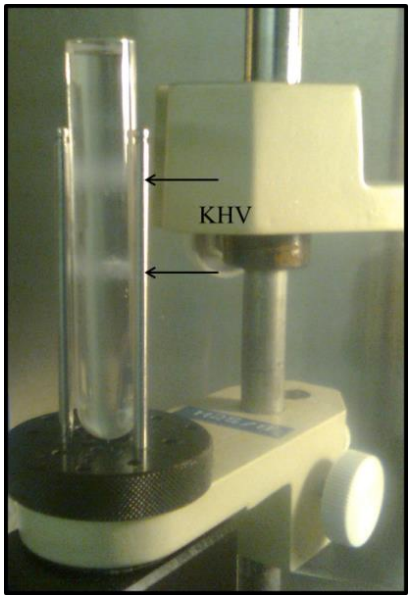


Figure 2.5 Bands obtained following sucrose gradient purification of KHV. The bands were extracted and the constituents of KHV particles were characterised by negative staining TEM as shown later in Section 3.3.5

Chapter 3

Exogenous marker approach - feasibility of marker vaccination for model viral diseases: infectious salmon anaemia and koi herpesvirus disease

3.1 – Introduction

3.1.1 Application of marker vaccination

There has been some debate regarding the use of positive marker vaccines, because it is difficult to differentiate infected carrier animals from vaccinated animals (Van Oirshot, 1999). However, the incorporation of a positive marker can ensure that only authorised vaccines are used during regulated vaccination programmes, which is essential both for trade and surveillance purposes with regards to successful control of notifiable diseases (Suarez, 2005). Positively marked vaccinated animals could then be traded or moved between regions or countries with full awareness of the product the animal has been vaccinated with. The antibody response elicited against the marker identifies the animal as vaccinated, whereas antibodies elicited to the pathogen can indicate that the animal is either infected or vaccinated with unapproved vaccines lacking the marker (James *et al*., 2007). The principle is illustrated schematically in Fig. 1.6 A-B, Section 1.6.6.

3.1.2 Vaccine manipulation to elicit antibodies against foreign antigens

Extensive research has been conducted, although not for fish, for development of vaccines incorporating immunogenic markers (Wigdorovitz *et al*., 1999; Walsh *et al*., 2000a; b; Mebatsion *et al*., 2002; Root-Bernstein, 2005; James *et al*., 2007; 2008; Fang *et al*., 2008) with a view to developing a system with an accompanying serology test that can reliably detect specific antibodies to the respective marker (see Section 1.4.4.1). Two fundamental approaches have been utilised for the meat and poultry industries; (1) addition of an endogenous marker requiring genetic modification of the agent and (2) addition of a foreign

exogenous marker, which can be applied to conventional and commercially available vaccines with ease.

The incorporation of a foreign gene into the genome of a pathogen for endogenous marker vaccination, can prove complicated as the protein expressed must be sufficiently immunogenic. Green fluorescent protein (GFP) derived from the jelly fish, *Aequorea victoria*, is a well characterised, commonly used reporter gene in transfection, microbial pathogenesis and virus mutagenesis studies (Chalfie *et al*., 1994; Ling *et al*., 2000; Geada *et al*., 2001; Engel-Herbert *et al*., 2003; O'Toole *et al*., 2004; Mikalsen *et al*., 2005; Chu and Lu, 2008; Costes *et al*., 2008). When expressed in an anti-tumor vaccine, the protein of the GFP gene was found to be antigenic, inducing a T cell immunogenic response in mice, resulting in increased cytotoxic T cell activity against leukaemic tumor cells (Stripecke *et al*., 1999). The induction of anti-GFP antibodies has also been demonstrated as a possible vaccine marker. For example, Walsh *et al*. (2000a) inserted the gene encoding GFP into the genome of attenuated Rinderpest virus (RPV), which subsequently expressed GFP protein provoking a detectable anti-GFP response. However, the expression system was also found to be vital for sufficient presentation of the GFP antigen to B cell receptors to induce a strong T-cell independent (TI) antibody response, which was conclusively achieved by cell membrane-anchored expression (Walsh *et al*., 2000a; b). Subsequently, antibody responses could be detected to the attenuated vaccine and GFP marker.

Alternatively, a simpler exogenous marker approach conducted by James *et al*. (2007; 2008), successfully induced an alternative antibody response by the inclusion of Tetanus toxoid (TT) in the vaccine formulation of an inactivated Avian influenza (AI) vaccine, intended for the protection of chickens and ducks. Birds responding to TT were subsequently tagged as 'vaccinated'. The production of synthetic peptides has also been proposed as a

potentially feasible approach, focusing on amino acid (aa) sequences associated with antigenic determinants (Root-Bernstein, 2005). This biomarker approach aims to induce an alternative antibody response by the administration of synthetic antigen consisting of sequences that are likely to be antigenic, but differ significantly from all proteins listed in the protein database (Root-Bernstein, 2005). As a result of the dissimilarity to any naturally occurring antigen, only vaccinated animals inoculated with the synthetic antigen will test positive.

3.1.3 Potential for notifiable viral diseases in fish: infectious salmon anaemia (ISA) and koi herpesvirus disease (KHVD)

Many DIVA vaccines use genetic modification to delete immunogenic epitopes of live attenuated vaccines, i.e. so that non-antibody responsive individuals to that antigen can be identified as vaccinated (Van Oirschot *et al*., 1996; Van Oirschot, 1999; Mebatsion *et al*., 2002; Brahmakshatriya *et al*., 2010; see section 1.4.3). However, live attenuated vaccines, particularly genetically modified microorganisms (GMM), are not currently licensed for aquaculture and fish that are immunised with such vaccines are, generally, also regarded as being genetically modified. Increased consumer resistance to using genetically modified organisms for vaccines, particularly in Europe, also makes licensing very difficult (Suarez, 2005; Gomez-Casado *et al*., 2011). Therefore, despite progression in development and commercialisation of vaccines, including DNA vaccines, for notifiable diseases in aquaculture (Anderson *et al*., 1996a; b; Lorenzen and La Patra, 2005; Mikalsen *et al*. 2005; Salonius *et al*., 2007; Gomez-Casado *et al*., 2011; Dhar and Allnutt, 2011; Wolf *et al*., 2013) it is unlikely that such vaccination with 'new generation vaccines' will become feasible for fish, at least in the short term (Lorenzen and Olsen, 1997; Gomez-Casado *et al*., 2011). Although inactivation of the resultant GMM is possible, without affecting its ability to induce a differential antibody response (Kaashoek *et al*., 1995; Van Oirschot *et al*., 1996; Bosch *et*

al., 1997), inactivated whole virus particles will often provide the most protective response possible for an inactivated vaccine as they retain all viral surface antigens and an inactive genomic compartment (Dhar and Allnutt, 2011). Positive marker vaccines can also be developed by insertion of genes encoding immunogenic protein antigens into the genome of the respective pathogen to produce positive marker GMMs. Previous research has, however, found that certain positive markers require the vaccine to be live for sufficient presentation of antigen (Walsh *et al*., 2000a; b). Whilst a number of marker vaccines have been successfully developed for notifiable avian and mammalian diseases (Walsh *et al*., 2000a; b; James *et al*., 2007; 2008; Fang *et al*., 2008), only one positive marker approach has previously been attempted for fish (Dhar *et al*., 2010) using a non-notifiable disease model, infectious pancreatic necrosis (IPN) in rainbow trout (Section 1.5), which would have limited application in the field. Considering that effective inactivated vaccines are often available for notifiable fish diseases (Dhar and Allnutt, 2011; Gomez-Casado *et al*., 2011), but OIE and EU regulations prevent their use, partly because of the inability to differentiate infected and vaccinated hosts, an attractive marker approach is the addition of immunogenic foreign proteins to commercially available vaccines.

To successfully implement marker vaccination programmes, specific antibodies to the marker and vaccine must be detectable at all stages of the fish life cycle in order for control strategies, i.e. ring vaccination, to be effective in the event of an outbreak. This is particularly important for salmonid vaccines that are administered during the early stages (Eggset *et al*., 1999; Mikalsen *et al*. 2005; Tobar *et al*., 2010; Wolf *et al*., 2013), thus, the response to marker vaccination for ISA would need to be detectable throughout the production cycle. Sero-surveillance may be easier for diseases of non-anadromous fish like KHVD.

Previous studies have demonstrated both the challenges and potential of successful vaccination against ISA using predominantly inactivated vaccines, but also subunit and DNA

vaccines (Jones *et al*., 1999b; Brown *et al*., 2000; Mikalsen *et al*. 2005; Tobar *et al*., 2010; Gomez-Casado *et al*., 2011; Lauscher *et al*., 2011; Wolf *et al*., 2013), however, the application of vaccination for ISA remains the subject of debate with regards to OIE legislation and the impacts associated with carrier fish is still a concern following vaccination (Section 1.3). However, Tobar *et al*. (2010) and Lauscher *et al*. (2011) did report successful protection of salmon following oral vaccination and ip vaccination, respectively, of experimentally ISAV challenged fish. If vaccinated fish are capable of clearing infectious virus without harbouring a carrier status then exogenous marker vaccination may be a highly desirable approach for implementing vaccination strategies. A contrasting case stands for KHVD where highly efficacious vaccines are available (Ronen *et al*., 2003), but as these are live, licensing is difficult. Such a strategy could enable those fish 'marked' to be diagnosed as antibody positive for ISA or KHV vaccination as opposed to antibodies produced as a result of infection. The other challenge lies in the reliability of the accompanying serological diagnostic test, for which antibody enzyme linked immunosorbent assay (ELISA) will not be an accepted diagnostic method for ISAV or KHV until such assays can be standardised (OIE, 2012).

3.1.4 Effects of smoltification and temperature on humoral response

Studies have previously been undertaken to determine if smoltification affects efficacy of vaccination in Atlantic salmon (Melingen *et al*., 1995a; b; Eggset *et al*., 1997a; b; 1999). Vaccination is performed either in March-May, only a few weeks prior to sea water transfer, August-October, six months prior to sea water transfer or in the autumn (i.e. August-October) by manipulation of light and temperature regimes (Eggset *et al*., 1999). During the smoltification period, the immunological defensive system of fish is reduced and there is an increase in plasma cortisol levels (Langhorne and Simpson, 1981; Specker and Schreck, 1982), which is indicated by reduced splenic and circulating lymphocytes (Maule *et al*., 1987;

Zapata *et al*., 1992). Timing of vaccination may therefore prove pivotal for the detection of specific antibodies to marker antigens administered with an ISA vaccine. The vaccination procedure itself is deemed to have an impact on smoltification development of salmon when performed at the onset of smoltification when light and temperature regimes are manipulated (Eggset *et al*., 1999) and lower antibody values have been reported when fish are vaccinated during smoltification (Melingen *et al*., 1995a). Any adverse affects on specific antibody production would compromise serology and thus marker vaccination strategies.

Temperature has also been noted as a major factor influencing differences in the immune response of ectotherms compared to endotherms (Paterson and Fryer, 1974; Bly and Clem, 1992; Le Morvan *et al*., 1998). Higher environmental temperatures generally result in faster antibody production and of a greater titre (Rijkers *et al*., 1980; Ellis, 1982), which of course may have a major effect on serological diagnostic testing for specific antibodies, e.g. to marker antigens. The temperature limits for establishing good immune responses depends on the physiological temperature range of the fish. Non-permissive temperatures for carp, have been suggested at <14°C, whereas for salmonids this is in the region of around 4°C (Bly and Clem, 1992). Lillehaug *et al*. (1993) demonstrated that temperatures as low as 2°C were sufficient to vaccinate Atlantic salmon against cold water vibriosis, but where fish are immunised at temperatures below that permitting an immune response, defined by Bly and Clem (1992) as the 'Non permissible temperature limit' will no longer elicit a primary immune response. Killie (1987) (Cited in Eggset *et al*., 1997a) showed this lack of immune-responsiveness in Atlantic salmon immunised with hapten antigens and held at 1-2°C. The differences of fish humoral immunity to that of warm blooded mammals and birds must therefore, in the context of marker vaccination and diagnostics, also be taken into account.

3.1.5 Aims

The aim of this Chapter was to develop a marker vaccine system for fish by administering 'foreign' immunogenic antigens with inactivated vaccines so that fish could be identified as vaccinated using serology. The feasibility of positive marker vaccination was assessed for ISA in Atlantic salmon and KHVD in Common/Koi carp. The implications of antigenic competition, i.e. inter-molecular and intra-molecular antigen induced antibody suppression (AIS), within the vaccine strain of marker vaccinated fish, was also investigated, particularly with regards to detection of specific antibodies to the marker and vaccine antigens, but also the potential interference on the protective effects of the vaccine antigen- induced antibodies. A number of antigens that were likely to be immunologically foreign to Atlantic salmon (tetanus toxoid (TT), keyhole limpet hemocyanin (KLH) and fluorescein isothiocyanate (FITC)) and carp (green fluorescent protein (GFP) were applied as candidate marker antigens.

The impacts of smoltification on serological detection of ISA vaccinated salmon has not previously been investigated, thus the current study also assessed these factors from pre-smolt to post-smolt salmon cultured under 12 hour light: 12 hour dark photoperiod as this may have major implications on the feasibility of marker vaccination against ISA. Furthermore, a marker antigen administered with a KHV vaccine was also assessed for this approach in carp. The influence of temperature was taken into consideration during assessment of specific antibody responses to the potential markers and vaccines.

3.2 – Materials and Methods

3.2.1 ISAV production

3.2.1.1 ISAV production

Salmonid cell lines were cultured and maintained as described in Section 2.2.1. and ISAV was cultured as explained in Section 2.3.1.

3.2.1.2 Reverse transcription polymerase chain reaction (RT-PCR) for ISAV

(a) RNA extraction

RNA was extracted directly from salmon head kidney (SHK-1) (passage; P. 53) cell supernatant infected with ISAV (Scottish Isolate, P. +5) after 20 days post infection (dpi) at 15°C once an obvious cytopathic effect (CPE) was obtained. RNA extraction was undertaken using the NucleoSpin® RNA Virus, Viral RNA Isolation kit (Machery-Nagel, Germany) according to the manufacturer's instructions. The protocol for cell-free biological fluids with Nucleospin RNA virus (Protocol 5.1, Nucleospin® RNA virus User manual) was used.

(b) RT-PCR

RT-PCR was undertaken using Ready-To-Go™ RT-PCR beads (Amersham Biosciences, UK). For each 50 µL reaction the beads consisted of ~2.0 Units (U) Taq DNA polymerase, 10mM Tris-HCl, 60mM KCl, 1.5mM $MgCl_2$, 200µM of each dNTP, Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (FPLC *pure*™), RNA guard™ Ribonuclease Inhibitor (porcine) and stabilisers, including RNase/DNase free BSA. RT-PCR was undertaken according to Mjaaland *et al.* (1997; 2002) cited in OIE: Manual of diagnostic tests for aquatic animals (OIE, 2012).

One Ready-To-Go RT PCR bead was dissolved in 43 µL of RNase-free H_2O which was briefly mixed and centrifuged before the addition of 1 µL of 12.5pmol forward primer

ISA-F-5'-GGCTATCTACCATGAACGAATC-3' and 1 µL of 12.5pmol reverse primer ISA-R-5'-GCCAAGTGTAAGTAGCACTCC-3'. Five microlitres of template RNA was added to make a final volume of 50 µL and reactions were performed on a thermocycler (Biometra®). Samples for RT-PCR included 1 positive RNA control (ISA virus RNA), 1 negative RNA control (Irrelevant, non-ISA virus (infectious pancreatic necrosis virus; IPNV) RNA), 1 negative sample (uninfected SHK-1 cell supernatant), 1 test sample (suspected ISA virus positive infected SHK-1 cell supernatant) and a no template control (RNase-free H_2O).

Reaction conditions were 42°C for 30 min for 1 cycle to make cDNA then 95°C for 5 min for 1 cycle for initial denaturation followed by 32 cycles of 95°C for 1 min, 55°C for 1 min for annealing, 72°C for 1 min for extension then a final cycle at 72°C for 7 min. Final PCR products were stored at 4°C until visualised in a gel.

A 1% agarose gel was prepared with 0.5 g agarose (Multi ABgarose, Thermo Scientific, UK) dissolved in 50 mL TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8). The agarose was melted for 1 min in a microwave and 1 µL of ethidium bromide was added to the solution. Once the gel had set after 30 min incubation at room temperature (RT) in a caster containing a multi-well comb, the gel was placed into an electrophoresis unit (Pharmacia, Sweden). The unit was filled with 1x TAE buffer and the gel was loaded with 5 µL of 100 bp ladder and 5 µL of PCR products which had been mixed with 1 µL loading buffer. A current of 85 volts for 40 min was passed through the gel and the products were visualised under UV light using a trans illuminator (Syngene, BioImaging).

3.2.1.3 Transmission electron microscopy (TEM) characterisation of ISAV

(a) TEM of cultured cells

A Scottish isolate of ISAV with a titre of $10^{5.3}$ $TCID_{50}$/mL was cultured in a 75 cm^2 culture flask of Atlantic salmon kidney cells (ASK-2) (p.77) as described in Section 2.3.1. The stock

was diluted 1:2 in Hanks buffered salt solution (HBSS), 2% foetal bovine serum (FBS) prior to inoculation. Another culture of ASK-2 cells was inoculated with diluent only (HBSS, 2% FBS) to serve as a negative control. After 3 dpi when first signs of CPE were evident the inoculated monolayer was harvested by removal of old media and monolayers were washed twice with 10 mL Dulbecco's phosphate buffered saline (DPBS, Invitrogen). Cells were fixed with 6 mL 2.5% gluteraldehyde (Sigma-Aldrich, UK) by completely submerging the monolayer with fixative, scraping the cells into suspension using a cell scraper (Nunc, Rochester, New York, U.S.A.) then immediately centrifuging 3 mL of the suspension in 2 x 12 mL centrifuge tubes at 2000 x *g* for 10 min at 4°C to fix cells into a pellet. Slow speed centrifugation was vital to the procedure to prevent rupture to the cells and thus loss of ultrastructural architecture when visualised by TEM. Excess gluteraldehyde was decanted off and the fixed pellets were retained. Fresh 2.5% gluteraldehyde was added to the pellets which were subsequently fixed for 2-4 h or overnight at 4°C. The fixative was removed and 2 mL cacodylate buffer rinse was added to the pellets which were flicked into suspension using a wooden applicator to ensure the pelleted cells had fixed. Fixed pellets were stored at 4°C until processing.

Gluteraldehyde fixed cell pellets were post-fixed in 1 % osmium in cacodylate buffer in closed vials for 1 h at RT. The pellets were then washed for 3 x 10 min in distilled H_2O. Thorough rinsing of cacodylate buffer was important at this stage as 'En-bloc' staining was undertaken with uranyl acetate and sodium cacodylate is incompatible with uranyl salts. 'En-bloc' staining of pellets was undertaken with 2 % uranyl acetate in 30 % acetone in the dark for 1 h then the pellets were dehydrated through an acetone series of ascending concentrations. Dehydration was carried out in 60 % acetone for 30 min, 90 % for 30 min, 100 % for 30 min then incubation in fresh 100 % acetone for 1 h.

Pellets were then infiltrated with agar low viscosity resin (ALVR) on a rotator (Taab, UK). The pellets were first incubated with ALVR diluted 1:1 in acetone for 45 min followed by 100% ALVR for 1 h and then into fresh ALVR for another h. The pellets were finally embedded in green or blue block moulds and polymerised in an oven at 60°C overnight. One hundred micron thick ultra-thin sections were prepared from the resin blocks using a microtome (Reichert Ultracut E, Leica, UK) with a diamond knife (Diatome, US) and placed on 200 µm mesh Formvar-coated copper grids.

(b) TEM negative staining

A drop of caesium chloride gradient purified ISAV in TNE buffer (10mM Tris, 100mM NaCl, 1mM EDTA, pH 7.4) was placed on a 3 mm Formvar Carbon 200 µm mesh film coated copper grid for 2 min at RT. Excess buffer was blotted off with filter paper and a drop of PBS was added to the grid which was then incubated for 2 min at RT. Excess PBS was then blotted off with filter paper before staining the preparation. Staining was undertaken with 2% Phosphotungstic acid adjusted to pH 7.2 with 10M KOH and left to incubate for 2 min at RT before blotting again and allowing the grid to air dry. The grid was then analysed for virus particles under an FEI Tecnai Spirit GR Bio Twin Transmission electron microscope.

3.2.1.4 ISAV purification

ISA virus antigen required for serological screening of ISA vaccinated fish was cultured in SHK-1 and ASK-2 cells and purified by caesium chloride gradient ultracentrifugation as described in Section 2.5.1.

3.2.2 KHV production

3.2.2.1 KHV virus production

Cyprinid cell lines derived from Common carp brain (CCB) and Koi fin (KF-1) tissues were cultured as described in Section 2.2.2. and KHV was cultured in both Cyprinid cell lines as explained in Section 2.3.2.

3.2.2.2 PCR for KHV

(a)DNA extraction

DNA was extracted directly from KF-1 cell supernatant infected with KHV (American isolate, H361, P.17 (St-Hilaire *et al*., 2009)) after 8 dpi at 20°C. DNA extraction was undertaken similar to the RNA extraction procedure undertaken for ISAV using the NucleoSpin® RNA Virus, Viral RNA Isolation kit (Machery-Nagel, Germany) according to the manufacturer's instructions. The protocol for cell-free biological fluids with Nucleospin RNA virus was also used here with modifications for DNA extraction, which includes the addition of proteinase K (Machery-Nagal, Germany).

(b) PCR

PCR was conducted using the 2 X Reddy Mix PCR Master Mix (1.5mM $MgCl_2$) (Thermo-Scientific, UK). The Master Mix consisted of 1.25 Units Thermoprime Plus DNA Polymerase (*Thermus Aquaticus*; *Taq*), 75mM Tris-HCl (pH 8.8 at 25ºC, 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01% (v/v) Tween® 20, 0.2mM each of dATP, dCTP, dGTP and dTTP and Precipitant and Red dye for electrophoresis. PCR was carried out according to Gilad *et al*. (2002).

A total reaction mix of 50 µL was made up from 25 µL of 2 X Reddy Mix PCR master mix, 0.5 µL of 30 pmol forward primer, Gil-F-5'-GACGACGCCGGAGACCTTGTG-3' and 0.5 µL of 30pmol reverse primer, Gil-R-5'-

CACAAGTTCAGTCTGTTCCTCAAC-3', 2.5 µL of template DNA and 21.5 µL RNAse/DNAse-free H_2O. Samples that were tested consisted of uninfected KF-1 cell supernatant, KHV infected KF-1 cell supernatant and no-template control. A positive and negative KHV DNA control was not available at the time of screening.

Reaction conditions were one cycle at 95°C for 5 min for denaturation followed by 40 cycles of 94°C for 1 min, 68°C for 1 min for annealing and 72°C for 30 sec for extension with a final cycle of 72°C for 7 min. Final PCR products were stored at 4°C until visualised in an ethidium bromide stained gel as described in Section 3.2.1.2(b)

3.2.2.3 TEM characterisation of KHV

(a) TEM of cultured cells

The American isolate of KHV (H361) from a stock with a titre of $10^{3.8}$ $TCID_{50}$ mL^{-1} was cultured in a $75cm^2$ tissue culture flask of CCB cells (P.86) as described in Section 2.3.2. The stock was diluted 1:5 in HBSS, 2% FBS prior to inoculation and another culture of CCB cells was inoculated with diluent only to serve as a negative control. Once plaque formation and CPE was extensive after 8 dpi, the monolayer was harvested as described for ISAV in Section 3.2.1.3(a) and prepared for TEM analysis.

(b) TEM negative staining

A drop of sucrose gradient purified KHV in TN buffer (10mM Tris, 10mM NaCl, pH7.4) was placed on a 3 mm Formvar Carbon 200 µm mesh film coated copper grid and processed as described in Section 3.2.1.3(b) for visualisation of negative stained KHV virions.

3.2.2.4 KHV purification

KHV antigen to be used for serological screening of KHV vaccinated fish was cultured in CCB cells and purified through ultracentrifugation in a sucrose gradient as described in Section 2.5.2.

3.2.3 Anti-salmon IgM MAb production and screening

In order to ensure that an appropriate monoclonal antibody (MAb) against salmon immunoglobulin was used to measure the fish antibody response in the ELISA, a variety of existing MAbs raised against rainbow trout immunoglobulin M (IgM) were screened to establish which cross-reacted with Atlantic salmon IgM using an ELISA.

3.2.3.1 MAb production and purification

The panel of hybridoma cell lines producing MAbs raised against Rainbow trout/Atlantic salmon IgM, developed in the Aquatic Vaccine Unit (Institute of Aquaculture, University of Stirling, Scotland), were cultured as described in Section 2.1.1. Monoclonal antibodies were harvested, concentrated, purified and quantified also as described in Section 2.1.1.

3.2.3.2 Screening anti-salmon MAbs against Atlantic salmon IgM by ELISA

Serum was prepared from the fish outlined below in Section 3.2.4.2. Atlantic salmon or Rainbow trout sera (1 mL) was mixed with sodium sulphate (14% w/v) and incubated in a 25°C water bath (Grant Instruments, Cambridge, UK) until the sodium sulfate (Na_2SO_4) had dissolved. The resulting solution was centrifuged at 17,000 x *g* in a microfuge (Microlite) for 15 min at RT. The supernatant was discarded, the pellet resuspended in PBS and washed twice by centrifuging the redissolved pellet at 17,000 x *g* for a further 15 min. The final pellet was resuspended in approximately 1 mL PBS and the absorbance read spectrophotometrically at 280 nm in a 1 cm^3 path length quartz cuvette using a Spectrophotometer (CECIL CE 2021). An optical density (OD) of 1.4 gave an equivalent of approximately 1 mg mL^{-1} fish IgM.

Sodium sulphate precipitated Atlantic salmon IgM (14.9 mg mL^{-1}) and Rainbow trout IgM (11.5 mg mL^{-1}) were diluted in PBS to 20 µg mL^{-1} in coating buffer (0.05M carbonate-bicarbonate (Sigma-Aldrich, St.Louis, UK)) and 100 µL added to the wells of an Immulon-4

HBX 96-well microtitre plate (Thermo Fisher Scientific, Germany). PBS was also added to empty wells as a blank background control. The plates were incubated overnight at 4°C before washing 3 x with low salt wash buffer (LSWB: 0.02 M Trizma base, 0.38 M NaCl, 0.05 % Tween-20, pH 7.3). The plates were then post-coated with 250 µL $well^{-1}$ 3% w/v casein (Marvel Original, Dublin, Republic of Ireland) for 2 h at RT in H_2O in order to block non-specific binding sites. The plate was washed again 3 x with LSWB after which 100 µL $well^{-1}$ of purified anti-trout/salmon MAbs (from a panel of 8) (mouse IgG) were added to the plates. This included the commercially available anti-trout/salmon IgM MAbs from Aquatic Diagnostics Ltd., Stirling, Scotland, which were also added to the plate after diluting 1/33 in antibody buffer (0.1% w/v BSA in PBS) according to the manufacturer's instructions. The MAbs were incubated for 1 h at RT and then plates were washed again 5 x with high salt wash buffer (HSWB: 0.02 M Trizma base, 0.5 M NaCl, 0.1% Tween-20, pH 7.7) with a 5 min incubation on the last wash. One hundred microlitres $well^{-1}$ horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG MAbs diluted 1/4000 in conjugate buffer (Sigma-Aldrich, St.Louis, UK) were then added to the wells. After a further 1 h incubation at RT the plate was washed a final time with HSWB with 5 min incubation on the final wash before the addition of 100 µL $well^{-1}$ chromogen (42 mM tetramethylbenzidine dihydrochloride in 1 part acetic acid to 2 parts distilled water) diluted 1:100 in substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4, 0.033% H_2O_2) and incubated for 10-15 min at RT to allow colour development. The reaction was stopped after adding 50 µL $well^{-1}$ of 2M H_2SO_4 and plates read at 450 nm using the Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, USA).

3.2.4 FITC-KLH ISA marker vaccination of Atlantic salmon, *Salmo salar*

3.2.4.1 Antigen formulation

Initial exogenous marker vaccination trials were undertaken unsuccessfully with a tetanus toxin (TT) vaccine (TT concentrated formaldehyde-inactivated commercial vaccine containing 150 International Units (I.U.) of purified tetanus toxoid mL^{-1} absorbed to aluminium hydroxide, Intervet Schering-Plough, Milton Keynes, UK) and accompanying ELISA using TT antigen (Calbiochem®, Darmstadt, Germany) derived from formaldehyde inactivated TT. Therefore alternative foreign antigens KLH and FITC were used for further ISA marker vaccine trials. The antigens were prepared independently and as a conjugated antigen in order to assess intra-specific as well as inter-specific antibody competition.

(a) KLH

Keyhole Limpet Hemocyanin (Calbiochem, Darmstadt, Germany) was reconstituted in PBS, mixed gently to dissolve and aliquoted as 5 mg mL^{-1} stocks, which were stored at 4°C until used. It was important not to vortex the solution of KLH vigorously as it is a large protein complex, which can form aggregates and precipitate from solution. Antigen formulations for salmon were prepared by diluting with PBS into 4 mL stocks of 1.67 mg mL^{-1} prior to mixing 30:70 with water in polymer adjuvant, Montanide^{TM} ISA 760 VG to a final concentration of 0.5 mg mL^{-1}. The solution was vortexed gently for 5 min to homogenise into an emulsion prior to inoculating fish with a final dose of 50 µg $fish^{-1}$.

(b) FITC

Fluorescein isothiocyanate (Calbiochem, Darmstadt, Germany) was reconstituted in PBS, mixed vigorously to dissolve, and aliquoted as 5 mg mL^{-1} stocks, which were stored at -20°C until used. Antigen formulations were prepared by diluting with PBS into 4 mL stocks of

1.67 mg mL^{-1} prior to mixing 30:70 with water in Montanide^{TM} ISA 760 VG to a final concentration of 0.5 mg mL^{-1}. The solution was also vortexed for 5 min to homogenise into an emulsion before fish were inoculated with a dose of 50 µg $fish^{-1}$.

(c) FITC-KLH conjugation

Two methods were used for conjugation of FITC (hapten) to KLH (carrier protein). The first approach was undertaken using a FITC-labelling kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 10 mg mL^{-1} of KLH protein was dialysed overnight in carbonate buffer concentrate (supplied in the kit). Dialysis was conducted in tubing prepared similar to that described for purified MAbs in Section 2.1.1.4. The protein was removed from dialysis tubing and diluted to 2 mg mL^{-1}. At least 1 mg mL^{-1} of KLH protein was kept aside in order to measure the approximate concentration of the final conjugate. One vial of FITC supplied in the kit was added to 850 µL Solvent reagent (also supplied in the kit) to make a concentration of 1 mg mL^{-1} and mixed well by vortexing for 1-2 min. Subsequently, 500 µL of FITC solution was added to the KLH solution and vortexed providing an approximate conjugation ratio of 200 µg FITC to 1 mg KLH. This solution was mixed end-over-end on a blood tube rotator (SBI, Stuart Scientific, UK) for 2 h at RT and the container was sealed with aluminium foil to block out ambient light. The final solution was then dialysed in 1 L PBS overnight at 4°C with two buffer changes.

The FITC/protein molar ratio and the final conjugate concentration was determined spectrophotometrically using a CECIL CE 2021 Spectrophotometer with the following formulae:

F/K Molar Ratio

Molar F/K = Mole FITC/Moles KLH

$$\text{Molar F/K} = \frac{2.77 \times A_{495}}{A_{280} - (0.32 \times A_{495})}$$

Concentration of FITC-KLH

$$\text{Protein x (mg/mL)} = \frac{A_{280} - (0.32 \times A_{495})}{E^{0.1\%}}$$

Where $E^{0.1\%}$ is the A_{280} reading of a 1mg mL^{-1} solution of the unconjugated KLH protein, as measured in a cuvette of $1cm^3$ path length.

The concentration of conjugated protein using this method was between 0.45 mg mL^{-1} and 0.76 mg mL^{-1} with approximate molar ratios varying from 1.5:1 FITC:KLH and 12:1 FITC:KLH.

For larger scale immunisation trials more conjugated antigen was required, therefore a second method was employed similar to that described by Jones *et al*. (1999a). This method was much simpler but the conjugation procedure was not as efficient as the kit. Two milligrams KLH was mixed with 1 mg FITC in 2 mL 0.05M carbonate-bicarbonate (Sigma-Aldrich, St.Louis, UK) on an end over end rotator for 2 h at RT. The conjugated protein was dialysed into PBS as described above and the FITC/protein molar ratio and the final conjugate concentration was determined spectrophotometrically as described in Section 3.2.4.1(c).

Concentration of conjugated antigens was undertaken in 10 kDa centrifuge filter units (Amicon Ultra, Ultra centrifugal filters, Millipore, Cork, Ireland) by centrifugation at 2,000 x

g for 6-8 min in a (Sigma 4K15 centrifuge (Germany) at 4°C until a final volume of ~1 mL of concentrated antigen remained. The flow through was discarded. Filter centrifugation was also used for removal of unbound FITC where only a small volume of conjugated antigen was required (as conjugated FITC to KLH would be retained in the solution of larger (>10 kDa) proteins). Final doses for the vaccination trial were prepared in PBS after concentrating the conjugate.

(d) FITC-BSA conjugation

In order to assess specific antibody responses to FITC on the FITC-KLH conjugate, the hapten was also conjugated to BSA and this antigen used to coat ELISA plates. The method used was similar to that used for FITC-KLH conjugation described in Section 3.2.4.1(c). Briefly, 2 mg BSA was mixed with 1 mg FITC in 2 mL 0.05M carbonate-bicarbonate in an end over end rotator for 2 h at RT. After dialysis, as described in Section 3.2.4.1.(c), the FITC/protein molar ratio and the final conjugate concentration was determined spectrophotometrically.

(e) ISAV

An inactivated compact oil adjuvanted monovalent ISA vaccine (Intervet Schering-Plough, Milton Keynes, UK, January 2009) was used for inoculation of salmon with 0.1 mL per dose. The vaccine had been developed by formaldehyde-inactivation of whole ISAV cultured through a cell line. The vaccine was also vortexed to produce a homogenised emulsion prior to inoculating fish.

(f) PBS control

A formulation of 30:70 PBS in Montanide ISA 760 VG was prepared as an injection control.

3.2.4.2 Experimental design

(a)Pre-smolt fish

Pre-smolt Atlantic salmon from a stock of fish reared for commercial smolt production at the Fresh Water Research Unit, Buckieburn, were used in the study that had never been vaccinated against or exposed to ISA. Fish with an average weight of 30.7 g ± 5.2 were not fed for at least 24 h prior to initiating the trial. Vaccination was undertaken at this time to simulate vaccination regimes used for commercial aquaculture production. Ten fish were pre-bled, to ensure that fish lacked antibodies against all of the antigens being investigated, after euthanasia with an overdose of anaesthetic (8 mL benzocaine (10% w/v in ethanol) in 5 L of rearing water) from the caudal vein using a 25G needle (Terumo, Leuven, Belgium) attached to a 1 mL syringe (Terumo, Leuven, Belgium). Blood samples were dispensed into eppendorf tubes after removal of the needle and allowed to clot overnight at 4°C. The following day the samples were centrifuged (Microlite, Thermo IEC, USA) at 850 x *g* for 5 min to pellet red blood cells. The serum was carefully removed, aliquoted (20 µl per aliquot) then stored at -20 or -70°C until analysis.

Atlantic salmon (*n* = 555) were injected on the 18th November, 2010 with 0.1 mL of nine different vaccine formulations as indicated in Table 3.1. All injections were administered ip after lightly anaesthetising fish by immersing into 10 L of rearing water containing 4 mL benzocaine (10% benzocaine (Sigma-Aldrich, St. Louis, USA)) w/v in ethanol (Fisher Scientific, Loughborough, UK). Dual injections were administered to some groups, one either side of the ventral line, as indicated in Table 3.1. After immunisation, each of the nine groups receiving different vaccine formulations were allocated to two replicate 300 L tanks supplied with flow through, gravity fed freshwater from the Buckieburn reservoir at a temperature of 6°C ± 1 and a flow rate of 25 L min^{-1}. The number of fish immunised with

each antigen and the tank that those fish were allocated to is indicated in Table 3.1. The fish were kept on a simulated natural photoperiod under 28W fluorescent tubes (3800°K). Feeding was resumed 2-3 days post vaccination (dpv) *ad lib* on a commercial pellet diet (Nutra Olympic 25, Skretting, Stavanger, Norway) and fish were checked twice daily for signs of distress or mortality. Temperature was monitored and recorded daily.

Six pre-smolt fish were sampled from each tank 98 dpv (24th February, 2011) by overdosing with anaesthetic and bleeding as described above. Each sampled fish was then weighed and measured to calculate its body condition factor (CF) and its smoltification score according to Sigholt *et al.* (1995), and modified by Dr. John Taylor, Institute of Aquaculture, University of Stirling (pers. comm.) (Fig. 3.1).

The peritoneal cavity was also checked for the presence of adjuvant. CF was calculated using the following formula according to Fulton's condition factor (K) (Rikardsen and Elliot, 2000; Reid *et al*., 2005):

$$K = \frac{100 \text{ X } W}{L^3}$$

Where

K = Condition factor/coefficient of condition

W = Weight of fish in grams (g)

L = Length of fish in millimetres (mm) from rear edge of fork, centre of the caudal fin to the tip of the snout. (Length^3 because growth in W of salmon is proportional to growth in volume).

(b) Smolt fish

Following sampling, all pre-smolt fish were then transferred to larger tanks of 800 L on the same flow-through system. Two randomly netted fish from each tank were scored for

smoltification 29 days post-transfer (dpt), after lightly anaesthetising as previously described (Section 3.2.4.2. (a)), to ensure fish were smolting prior to the next sampling point. Ten days later (39 dpt; 137 dpv; 14th April, 2011) 6 smolts from each tank were sampled as described previously for pre-smolts (Section 3.2.4.2.(a)).

Table 3.1. Immunisation groups of pre-smolt fish used in infectious salmon anaemia marker vaccine trial

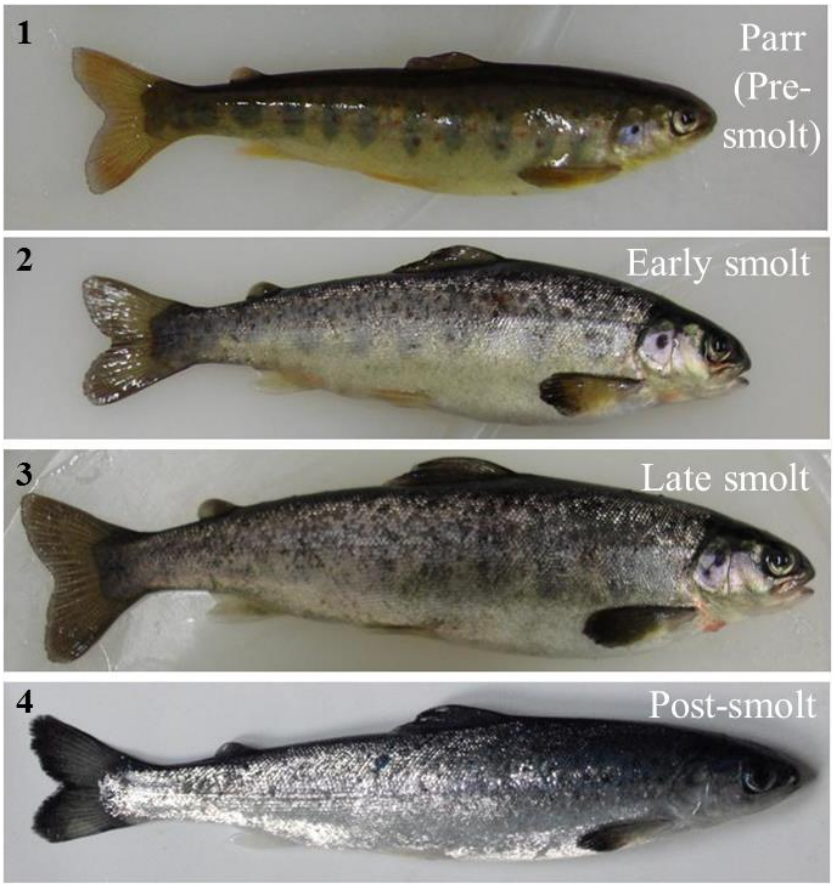
Treatment group	Fish group abbreviations	No. injections	Tank	Number of fish
PBS	C	1	H11	36
PBS		1	H6	35
FITC	F	1	H12	35
FITC		1	H15	35
FITC + ISA vaccine	FI	2	H7	35
FITC + ISA vaccine		2	H2	35
ISA vaccine only	I	1	H1	35
ISA vaccine only		1	H8	35
KLH	K	1	H16	35
KLH		1	H14	35
ISA vaccine + KLH	IK	2	H9	35
ISA vaccine + KLH		2	H13	35
FITC-KLH conjugate	FK	1	H17	35
FITC-KLH conjugate		1	H10	35
FITC-KLH + ISA vaccine conjugate	IFK	2	H18	32∗
FITC-KLH + ISA vaccine conjugate	IFK	2	H19	32∗

∗Less fish inoculated due to insufficient antigen
PBS = phosphate buffered saline; FITC = fluorescein isothiocyanate; ISA = Infectious salmon anaemia vaccine; KLH = keyhole limpet hemocyanin

(c) Post-smolt fish

Two randomly netted fish from each tank were scored again (20th April), 6 days since the last sampling point, for smoltification as described previously (Section 3.2.4.2.(b)) in order to assess the level of silvering prior to transferring fish to salt water. Salt water tolerance was further assessed with a salt water challenge undertaken on 10 randomly netted fish. The fish were held in a 100 L, aerated tank of static salt water (34 ppt), similar to the salt water challenge test used by Sigholt *et al*. (1995), for at least 48 h. Following a survival rate of 90%, the remainder of fish were considered ready for transfer to salt water.

A



B

SMOLT INDEX SCORE SHEET

1. SILVER COLOUR

INDEX	NONE	WEAK	VISIBLE	SILVER
SCORE	1	2	3	4

2. PARR MARKS

INDEX	STRONG	WEAK	VISIBLE	SILVER
SCORE	1	2	3	4

3. DARK FIN EDGE

INDEX	NONE	WEAK	VISIBLE	BLACK
SCORE	1	2	3	4

Figure 3.1 Smolt index scoring used for determining advancement of smoltification in the infectious salmon anaemia marker vaccine trial. *After Sigholt et al. (1995) with modifications by Dr. John Taylor (pers.comm.).*(A) Changes in appearance of salmon during smoltification from immature parr (1) to mature post smolt (4). (B) Numerical scoring system based on appearance of smolting salmon including silvering, parr marks and fin edges.

A temporary salt water recirculation system was established using filter pumps (Fluval Fx5, Hagen, UK) running water through each of 4 x 300 L tanks at a flow rate of 10 L min^{-1}. A total of 40.8 Kg sea salt (Tropic Marin, Wartenberg, Germany) was dissolved in 1200 L of freshwater, from the same reservoir inlet as used previously, for 24 h before transferring fish. During smoltification, a fungal infection with *Saprolegnia* had caused some mortalities in all tanks, resulting in a reduction of fish numbers. In addition to this, it was not possible to transfer all of the fish to salt water due to insufficient salt water for the complete recirculation system. Therefore only 4 vaccine groups (the ones considered to provide the most informative results), were transferred to the salt water system 162 dpv (29th April, 2010).

The 2 replicate tanks of the 4 groups were pooled together and transferred into 4 separate tanks on the same saltwater recirculation system with a total of 12 fish from the control group, 16 fish from the FITC-KLH vaccinated (FK) group, 18 fish from the ISA vaccinated (I) group and 15 fish from the FITC-KLH and ISA vaccinated (FKI) group. Feeding was resumed after 2-3 dpt and fish were monitored daily for signs of distress. All mortalities were removed and recorded.

All fish were finally sampled after 176 dpv (13th May, 2010) by overdosing with anaesthetic and bleeding as described previously (Section 3.2.4.2 (a)). Due to poor fish body condition and technical problems, a number of mortalities resulted in low fish numbers surviving to the final sampling point.

3.2.4.3 ELISA to measure antibody responses against the FITC-KLH ISA marker vaccine

(a) ELISA optimisation

ELISA optimisation was based on previous publications with modifications, through a checkerboard design with variations in antigen concentration; blocking reagents, e.g. 1%

BSA, 3% casein; serum concentration; primary antibody (anti-trout IgG) concentration; secondary antibody conjugate (anti-mouse IgG) concentration. Additionally, Roti block (Roth, Karlsruhe, Germany) was used as a blocker and casein added to the serum to reduce non-specific IgM activity as described by Kim *et al*. (2007a).

The final protocol used for screening salmon sera was established based on the principle of an ELISA absorbance with anti-FITC, anti-KLH or anti-ISAV MAbs signal of $OD_{450nm} \geq 1$ with negative sera below the sensitivity threshold. Assay optimisation was carried out using sera from a preliminary immunisation trial, which was used to indicate the level of antigenicity of marker antigens prior to initiating the large scale marker vaccine trial (not shown).

(b)KLH and FITC marker antigen ELISAs

The KLH and FITC ELISAs were carried out using a similar method to that described by Jones *et al*. (1999a) for rainbow trout with modifications. Briefly, 20 µg mL^{-1} of KLH diluted in coating buffer (carbonate-bicarbonate, pH 9.6) was coated onto all wells of an Immulon-4 HBX 96-well microtitre plate, while only half of a plate was coated with 20 µg mL^{-1} of FITC-BSA conjugate. The other half of the FITC-BSA plates were coated with BSA only. The plates were incubated overnight at 4°C. The plates were then washed 3 x with LSWB and post-coated with 3% casein in H_2O for 5 h at RT to block non-specific binding. The plates were then washed again 3 x with LSWB and 100 µL Atlantic salmon serum diluted 1/50, 1/40 or 1/20 in PBS containing 1% casein (w/v), was added. Serum from each fish was applied to replicate wells of whole KLH ELISA coated plates or on the two halves of FITC-BSA and BSA coated plates in order to determine FITC-specific antibody responses. One hundred microlitres of anti-FITC mouse ascites fluid (Sigma-Aldrich, St.Louis, USA) diluted 1/2000 or anti-KLH mouse ascites fluid (Sigma-Aldrich, St.Louis, USA) diluted

1/500 in PBS were added to replicate wells of the FITC-BSA and KLH ELISA plates, respectively, instead of sera, to act as positive controls for ensuring antigen adherence to the plate and for plate to plate comparisons. Phosphate buffered saline was also added 100 µL $well^{-1}$ in duplicate, instead of sera, as a blank negative control. After overnight incubation at 4°C the plates were washed 5 x HSWB with a 5 min incubation on the last wash. One hundred microlitres anti-rainbow trout/Atlantic salmon MAbs (Aquatic Diagnostics Ltd., Stirling, Scotland) diluted 1/33 in antibody buffer according to the manufacturer's instructions or neat from hybridoma cell culture supernatant of anti-trout MAb cell line 6 were added to the wells of the plates. After 1 h incubation the plates were washed again 5 x HSWB with 5 min incubation on the final wash and the remainder of the procedure was carried out as described in section 3.2.3.2. The sensitivity threshold of the assay was determined as 3x the absorbance value of wells containing PBS. Any sample above this value was considered positive for specific antibodies to the respective antigen.

(c) ISAV ELISA

Immulon-4 HBX 96-well microtitre plates were coated with 100 µL 20 µg mL^{-1} of concentrated/purified ISAV (described in Section 2.5.1) in carbonate-bicarbonate buffer on one half of the plate and 100 µL of 20 µg mL^{-1} pelleted uninfected SHK-1 cell antigen in carbonate-bicarbonate on the other half of the same plate in order to determine antibody responses specific to the virus. The plate was incubated overnight at 4°C then the following day the plate was washed 3 x with LSWB and 250 µL $well^{-1}$ 3% w/v casein in H_2O was added to the wells and incubated for 2 h at RT. The plate was washed again 3 x with LSWB and 100 µL salmon serum samples diluted 1/50 (or 1/20 and 1/40 for the final assay) in PBS containing 1% v/v casein were added to the wells. Additionally, 100 µL PBS only was added to replicate wells to serve as background controls and 100 µL $well^{-1}$ of anti-ISAV MAb hybridoma supernatant (MAb 3-3), developed at the Institute of Aquaculture, University of

Stirling) were added to replicate wells to serve as positive antigen controls. It was not possible to obtain positive sera to ISAV during the study, therefore anti-ISAV MAbs were utilised for initial optimisation of the assays. PBS was used in place of negative control sera. After overnight incubation at 4°C the plate was washed 5 x with HSWB and the remainder of the procedure was also performed the same way as that described for FITC and KLH ELISAs (Section 3.2.4.3 (b)).

3.2.5 GFP KHV marker vaccination in *Cyprinus carpio*

3.2.5.1. Antigen formulation

(a) GFP

Purified recombinant GFP (Millipore, Temecula, USA) from a solution of 300 µg in PBS (20 % glycerol) was diluted to stocks of 50 µg 100 μL^{-1} (0.5 mg mL^{-1}) in PBS. After mixing the protein 30:70 in Montanide^{TM} ISA 760 VG the final dose was 0.15 mg mL^{-1}. The solution was emulsified by vortexing for 4-5 min prior to inoculating carp with a dose of 15 µg $fish^{-1}$.

(b) KHV

An inactivated KHV vaccine was kindly provided by Dr. Ian Pardoe (Henderson Morley PLc., Birmingham, England - Jan 2009), which was developed through formalin inactivation of purified virus. The total dose of vaccine was 1.6 mg mL^{-1} purified KHV in aluminium hydroxide adjuvant.

3.2.5.2 Experimental design

An immunisation trial was undertaken to assess the feasibility of utilising GFP as an exogenous marker antigen in Mirror carp (*Cyprinus carpio* L.), which would not interfere with antibody responses induced by the KHV component in the vaccine. Mirror carp (n = 50) weighing between 30 – 40 g were obtained from a carp farm in Hampshire, England

(Hampshire Carp Hatcheries, Hampshire, England) with no history of KHV. The carp were maintained in fresh water at the Aquatic Research Facility (ARF) at the Institute of Aquaculture, University of Stirling, Stirling, Scotland in 100 L tanks of fresh dechlorinated (Elga Piltramat AC4) mains water on a flow through system at 14°C ± 2 with a flow rate of 1 L min^{-1} oxygenated through air stones (Betta, J and K Aquatics Ltd., UK). The fish were acclimated to these conditions for at least 4 weeks prior to commencing any experimental work. Five fish were pre-bled to screen for anti-KHV antibodies by antibody ELISA. For the vaccination experiment, 36 of the remaining fish were randomly divided into three groups of 8 and one group of 10 and vaccinated as described in Section 3.2.4.2 (a). One group of 8 carp were injected ip with 0.1 mL $fish^{-1}$ of adjuvanted recombinant GFP. The second group of 8 fish were injected ip with 0.1 mL of inactivated KHV vaccine. A third group of 8 fish received two ip injections of 0.1 mL adjuvanted GFP and 0.1 mL KHV vaccine, one injection either side of the ventral line. The final group of 10 fish were inoculated ip with 0.1 mL of adjuvanted PBS to serve as a negative control. Following vaccination the fish were transferred to a recovery tank of rearing water before being replaced into their respective holding tanks. The fish were fed *ad lib* on a commercial pellet diet (Skretting, Norway) and checked twice a day for any adverse reactions. After 6 wpv, fish were sampled for serological analysis as described for Atlantic salmon in section 3.2.4.2 (a).

3.2.5.3 GFP KHV marker vaccine ELISA

(a) ELISA optimisation

The GFP ELISA was optimised using a similar approach to that taken for the FITC and KLH ELISAs to screen salmon serum as described in Section 3.2.4.3.(a). The protocol used for screening carp sera for anti-GFP antibodies was considered optimised once the absorbance

for the anti-GFP MAbs had an absorbance $OD_{450nm} \geq 1$ and negative sera was below the sensitivity threshold.

Optimisation of the KHV ELISA was undertaken based on published protocols according to Adkison *et al*. (2005) and St-Hilaire *et al*. (2009) and the protocol developed by Dr. Sven Bergmann (pers. comm.) with modifications. Intensive blocking appeared critical as carp sera tended to produce a high level of non-specific binding, thought to be associated with natural antibodies. As positive and negative control sera was available, it was possible to perform checkerboard assays with sera using various blockers including casein, BSA and a synthetic blocker, Roti-block (Roth), until the minimal ODs from negative sera were obtained without affecting the endpoint titre of the positive control of 1/1600 (i.e. until the concentrations that generated the maximum positive to negative (P/N) ratio).

(b) GFP ELISA

Immulon-4 HBX 96-well microtitre plates were coated with 100 µL of 20 µg mL^{-1} of purified recombinant GFP (Millipore) $well^{-1}$. The plate was incubated at 4°C overnight then washed 3 x with LSWB and post-coated to block non-specific binding. Blocking methods differed for GFP protein and KHV antigen as signals from carp sera on the GFP ELISA were always much lower compared to that of KHV coated plates during optimisation. The plate was blocked with 5% casein in H_2O for 3 h at RT then washed 3 x with LSWB. One hundred microliters per well of 2-fold dilutions of carp sera from 1/100 to 1/1600 diluted in PBS was added to the plate in duplicate and incubated overnight at 4°C. Positive antigen control wells received 100 µL of 4 µg mL^{-1} in duplicate of anti-GFP MAbs (Roche, Mannheim, Germany) diluted in PBS. Negative control blank wells received 100 µL PBS instead of carp sera. The following day the plate was washed 5 x with HSWB and 100 µL $well^{-1}$ anti-carp IgM mouse IgG MAbs (Aquatic Diagnostics, Stirling, UK) diluted 1/55 were added to the plate and

incubated at RT for 1 h. The plate was washed again 5 x HSWB with a 5 min incubation on the final wash and the remainder of the assay was undertaken identically to that used for the detection of anti-FITC, anti-KLH and anti-ISAV antibodies (Section 3.2.4.3).

(c) KHV ELISA

Immulon-4 HBX 96-well microtitre plates were coated with 50 µL of sucrose purified KHV (Section 2.5.2) or BSA at 0.3 µg $well^{-1}$ in 0.05M carbonate-bicarbonate buffer (Sigma-Aldrich) and incubated overnight at 4^{o}C, followed by three washes with LSWB. Non-specific binding sites on wells were blocked with 250 µL of 10 % casein (w/v) in distilled water for 5 h at RT, before being washed with LSWB and adding 50 µL of either two-fold serial dilutions of vaccinated Mirror carp serum samples from 1/200–1/3200 or positive and negative control serum diluted in 5 % casein in PBS, as well as 5 % casein in PBS to two duplicate wells on each plate as a blank negative control. Pre-bled Mirror carp prior to vaccination were used for negative sera controls and anti-sera from infected koi from an experimental challenge undertaken at the Centre for Environment, Fisheries and Aquaculture science (CEFAS) with an antibody titre of 1/1600 (kindly provided by Dr. Peter Dixon) was used as a positive control. After incubating the sera on the ELISA plate overnight at 4^{o}C, wells were washed 5 x HSWB with 5 min incubation on the last wash. Fifty microlitres of mouse anti-carp IgM diluted 1:73.3 in 0.1% BSA in PBS was added to each well and incubated for 1 h. Wells were washed with HSWB and incubated with 50 µL of goat anti-mouse IgG-HRP Conjugate (Sigma-Aldrich) diluted 1:4000 in 0.1% BSA in LSWB. Following the wash step with HSWB, 100 µL chromogen (42 mM TMB in 1 part acetic acid to 2 parts distilled water) diluted 1:100 in substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4, 0.033% H_2O_2) was added to each well. The reaction was stopped after 12 min by the addition of 50 µL of 2M H_2SO_4 $well^{-1}$ and plates read at 450 nm using the Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, USA).

3.2.6 Analysis and statistics

Data was tested for normality using the Anderson-Darling test and tested for homogeneity of variance using the Levene's test. Where data was normally distributed and equal variance could be assumed, univariate General Linear Model (GLM) was used to compare the analysis of variance between means followed by post-hoc testing between groups using the Fisher Least significant difference (LSD) test. Where data was not normally distributed, attempts were made to transform the data by Log10 and Square root. When this failed to normalise data, non-parametric testing was undertaken using Kruskal-Wallis one-way ANOVA by ranks and median test and Mann Whitney-U pairwise comparison test. SPSS (IBM® SPSS® Statistics 19) and Minitab (Minitab 16) statistical software packages were used for all statistical analyses.

3.3 – Results

3.3.1. Confirmation and characterisation of purified ISAV antigen for anti-ISAV antibody screening

The RNA extracted from ISAV was successfully amplified in the RT-PCR from infected SHK-1 cells supernatant (Fig. 3.2 C). ISAV virus particles were also detected after 3 dpi in fixed infected ASK-2 cells (Fig. 3.2 A- B) by TEM confirming the virus had been successfully cultured. Negative staining and positive anti-ISAV MAb controls on ELISA further confirmed the successful purification of ISAV after ultracentrifugation (Fig. 3.2 D).

3.3.2 Screening anti-salmon IgM MAbs by ELISA

Of the eight anti-rainbow trout IgM MAbs tested, affinity purified MAb from hybridoma cell line 6 produced the highest ODs to sodium sulphate precipitated Atlantic salmon IgM when ELISA plates were coated with 10 µg mL^{-1} of the precipitated fish IgM (Fig. 3.3). Therefore, this cell line was used to detect salmon IgM, as well as the commercial anti-salmon MAb

produced by Aquatic Diagnostics Ltd., in the ELISAs for detecting antibody responses to vaccine and marker antigens.

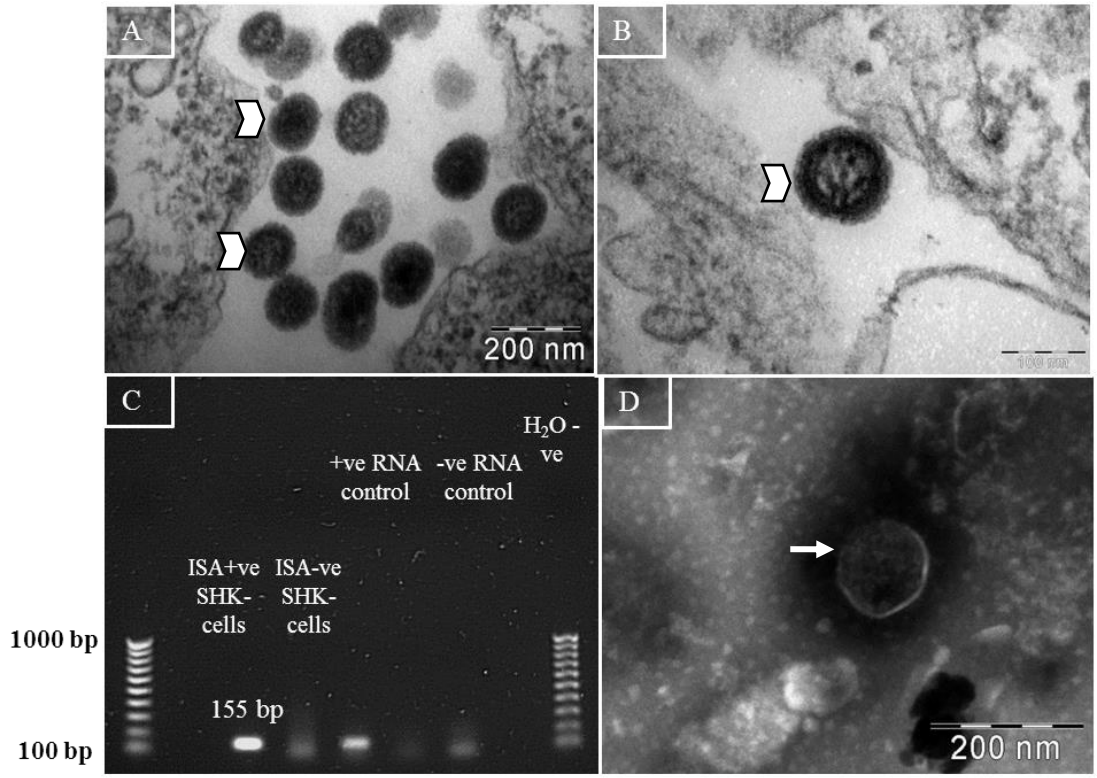


Figure 3.2 Molecular and morphogenic characterisation of infectious salmon anaemia virus (ISAV) from infected salmon head kidney (SHK-1) and Atlantic salmon kidney (ASK-2) cells by Reverse transcription polymerase chain reaction (RT-PCR) and transmission electron microscopy (TEM). (A) TEM micrograph of ISAV infected ASK-2 cells with many extracellular virions; (B) TEM micrograph of ISAV infected ASK-2 cells showing an intracellular virion; (C) Stained 1% agarose gel showing products of single round RT-PCR according to Mjaaland *et al*. (2002) using RNA extracted from ISAV infected and non-infected SHK-1 cells. Note expected molecular weight bands from positive samples of 155 bp; (D) TEM micrograph of negative stained preparation of ISAV virion, which appears to be a non-enveloped capsid. Arrow heads = enveloped virions; Arrow = purified unenveloped virus capsid.

3.3.3 FITC-KLH ISA marker vaccine trial in Atlantic salmon

3.3.3.1 Antibody response to markers and vaccine throughout the course of smoltification

Since initial screening of salmon from a pre-trial resulted in low antibody responses to any antigen at serum dilutions ≥ 1/100 using the optimised protocol, sera from the main trial were

only screened using serum dilutions of 1/20 and 1/40, and differences of antibody responses were based on absorbances OD_{450nm} at these dilutions, i.e. antibody values, obtained by ELISA.

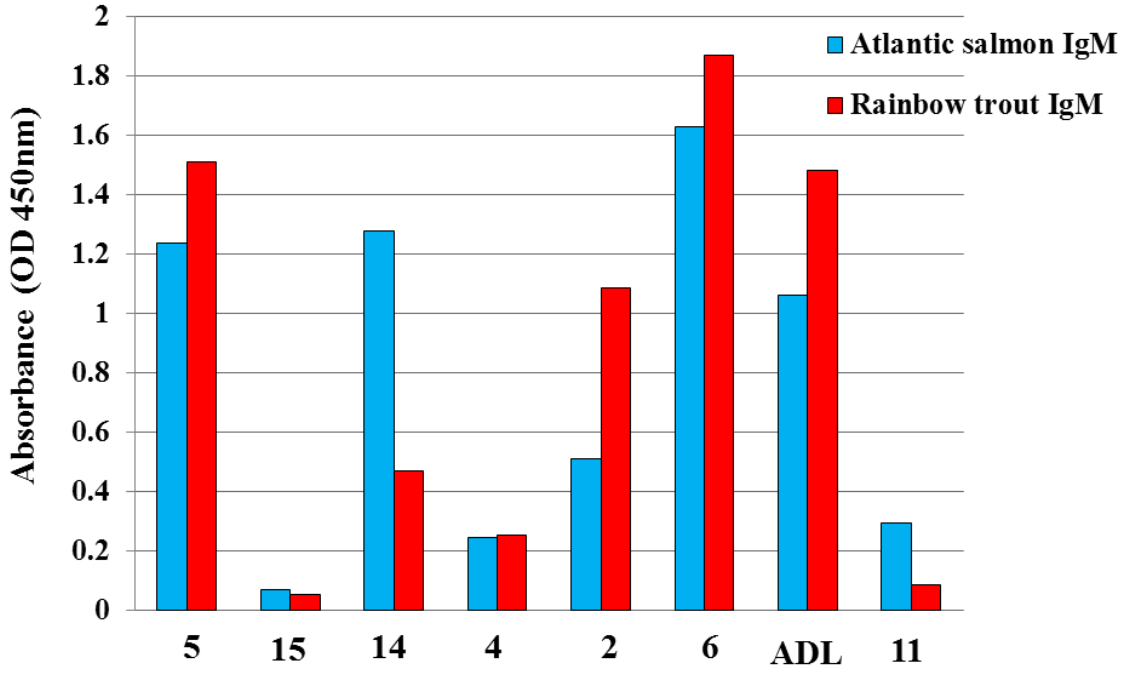


Figure 3.3 Detection of fish immunoglobulin (IgM) by anti-rainbow trout/Atlantic salmon IgM mouse IgG MAbs from different hybridoma cell lines. The different anti-fish IgM hybridoma cell line reference number is shown on the *x*-axis. Bars indicate Mean absorbance OD_{450nm} from replicate wells.

Responses were generally very low against all vaccine formulations administered to fish during the trial, and very few vaccinated fish produced a positive antibody response at a dilution of 1/40 (results not shown). However, significant differences were noted between vaccine groups at a dilution of 1/20 and there appeared to be trends between fish antibody responses to the different antigens, within and between groups, and stages of smoltification. Poor antibody responses were observed in the various ELISAs in spite of the high values obtained from the positive control MAbs (i.e. $OD_{450nm} \geq 1.0$) used, indicating that the low

antibody responses in fish were not associated with low antigen concentrations on the plate (results not shown). It should also be noted that all positive control MAbs were also diluted with internal blocking diluent, i.e. 1% casein.

The CF had also been determined for vaccinated fish, sampled for serology, and the expected negative correlation between mean CF and period of smoltification was evident as previously reported for smolting salmon (Sigholt *et al*., 1995), in all vaccine groups (Table 3.2; Fig. 3.4). This was supported by the fact that there were significantly lower CF of smolt salmon than pre-smolt salmon ($p < 0.0001$) in April and significantly lower CF of early post-smolt salmon to smolt salmon ($p < 0.0001$) in May (Fig. 3.4). No significant differences in CF were noted between vaccine groups at each sampling point (not shown), thus all fish were considered to be of a similar physiological state and different antigens did not affect CF. The reduced CF corresponded well with the smolt index scoring (Fig. 3.1) indicating that sampling points were representative of pre-smolt, smolt and post-smolt fish.

(a)Variation of antibody response in pre-smolts

At the pre-smolt stage, despite significantly higher antibody values in fish immunised with FITC, from the FI and FK vaccine groups, compared to controls ($p < 0.05$), with no significant differences to BSA, the very low absorption values (OD_{450nm}) obtained at low serum dilutions of 1/20 suggest that there was no specific antibodies induced to FITC despite apparent absorbances (OD_{450nm}) above the sensitivity threshold (Table 3.2; Fig. 3.5; Fig. 3.6 A).

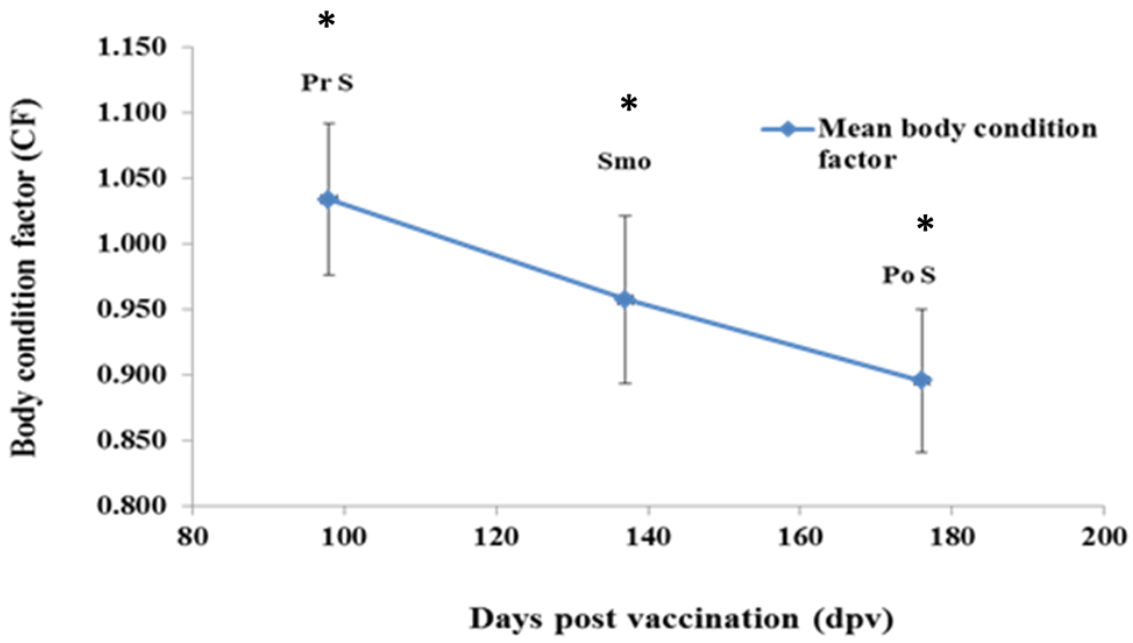


Figure 3.4 Body condition factor (CF) of sampled Atlantic salmon from infectious salmon anaemia (ISA) marker vaccine trial during the course of smoltification. Different letters above SE bars indicate a different sampling point during the trial. Prs = Pre-smolt salmon, Mean ± SE (n = 96); Smo = Smolt salmon, Mean ± SE (n = 96); PoS = Post-smolt salmon, Mean ± SE (n = 22). Stars indicate significant differences of CF between smolt stages (Mann Whitney-U pairwise comparison test; $p < 0.05$).

At the pre-smolt stage some significantly higher antibody responses were obtained against ISAV in the sera of ISA vaccinated fish (IK, p=0.03; IF, p=0.028) compared to controls (Fig. 3.5). Very few fish were deemed positive for anti-ISAV antibodies at this stage (Fig. 3.6 B) with most positive responders (4/12) observed from the IK vaccine group (Table 3.2). Despite the apparent cross reactive antibodies of immunised fish with viral and non-viral antigen, all control pre-smolt fish were negative for ISA, FITC, BSA and SHK-1 antigens (Table 3.2).

Table 3.2 Number of fish with a positive antibody response (i.e. with an absorbance at 450 nm greater than the sensitivity threshold at 1/20 sera dilution)

Fish group	Mean condition factor	ELISA antigen				
		FITC	**BSA**	**ISA**	**SHK-1cells**	**KLH**
			Pre-smolt			
C	1.03	0/12	0/12	0/12	0/12	2/12
F	1.028	**0/12**	1/12	-	-	-
K	1.035	-	-	-	-	**7/12**
I	1.029	-	-	**2/12**	1/12	-
IF	1.029	**0/12**	0/12	**1/12**	1/12	-
IK	1.017	-	-	**4/12**	3/12	**3/12**
FK	1.044	**1/12**	1/12	-	-	**5/12**
IFK	1.058	**2/12**	3/12	**3/12**	1/12	**8/12**
			Smolt			
C	0.939	2/12	1/12	1/12	0/12	4/12
F	0.981	**0/12**	0/12	-	-	-
K	0.942	-	-	-	-	**5/12**
I	0.962	-	-	**5/12**	2/12	-
IF	0.93	**0/12**	0/12	**3/12**	1/12	-
IK	0.963	-	-	**4/12**	4/12	**6/12**
FK	0.992	**1/12**	2/12	-	-	**5/12**
IFK	0.952	**0/12**	0/12	**2/12**	0/12	**3/12**
			Post-smolt			
C	0.86	0/3	0/3	2/3	1/3	0/3
I	0.932	-	-	**2/7**	0/7	-
FK	0.863	**0/6**	0/6	-	-	**1/6**
IFK	0.91	**0/6**	0/6	**2/6**	0/6	**2/6**

Results in bold indicate fish expected to have specific antibodies to the respective antigen.
Underlined results are false-positive results from negative control fish or immunised fish to non-specific antigen.
Dashes (-) indicate tests not undertaken as the antigen on the ELISA had not been administered to the fish. Letters indicate the following antigens: C (Control), F (FITC), K (KLH), I (ISA), IF (ISA+FITC), IK (ISA+KLH), FK (FITC+KLH), IFK (ISA+FITC+KLH)

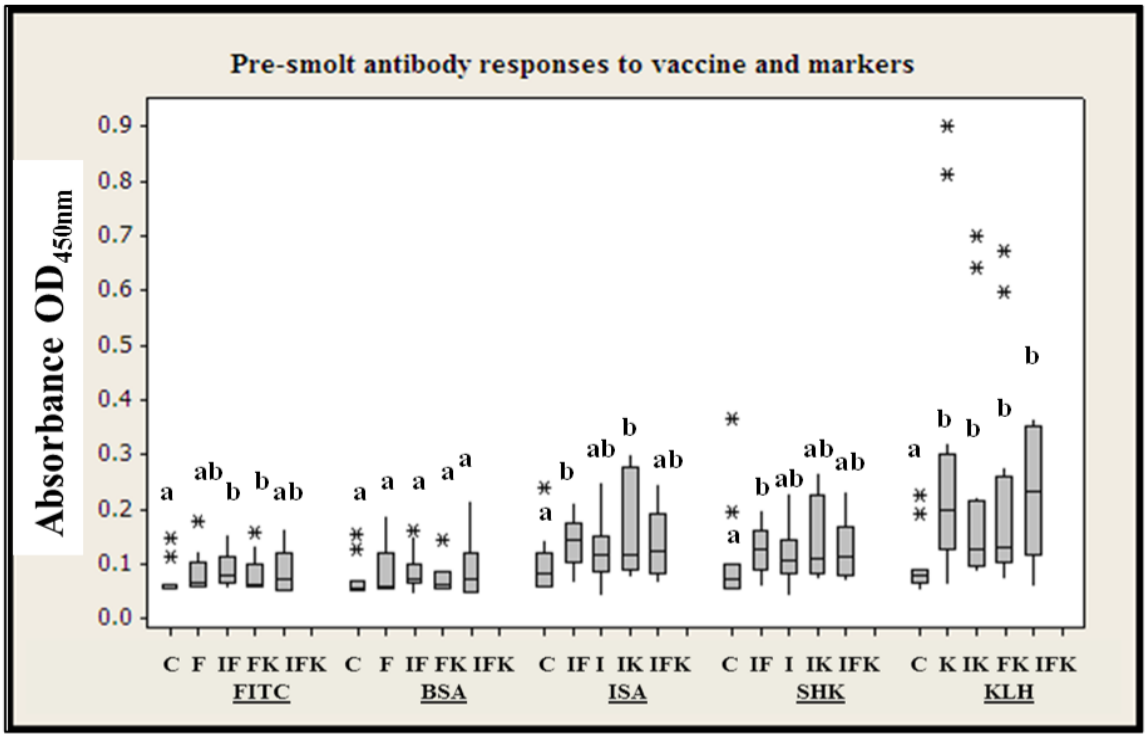


Figure 3.5 Box plot of antibody responses of infectious salmon anaemia (ISA) marker vaccinated pre-smolt salmon at a serum dilution of 1/20. Letters on the *x* axis represent vaccine groups: C = Control, F = FITC only, IF = FITC + ISA vaccine, FK = FITC conjugated to KLH only, K = KLH only, I = ISA vaccine only, IK = KLH + ISA vaccine, IFK = FITC conjugated to KLH + ISA vaccine. Abbreviations at foot of the *x* axis indicate ELISA antigen that sera is screened against. ∗ = outliers. Different letters indicate significant differences within groups for each antigen. Data are medians of each fish vaccine group (n = 12). (Mann Whitney-U pairwise comparison test; $p < 0.05$).

All KLH immunised groups of fish were positive by KLH ELISA and produced significantly higher antibody responses to KLH compared to controls ($p<0.01$) (Fig. 3.5) with 7/12 and 8/12 fish from the K and IFK vaccine groups positive, respectively, and a total of 23/48 (48%) positive of all the KLH immunised fish (Table 3.2; Fig. 3.6 C). There was a great variation of antibody values between individual positive fish from an OD of 0.15 to 0.9, which resulted in a number of extreme outliers in the group, which may have been particularly strong responders to the antigen (Fig. 3.5).

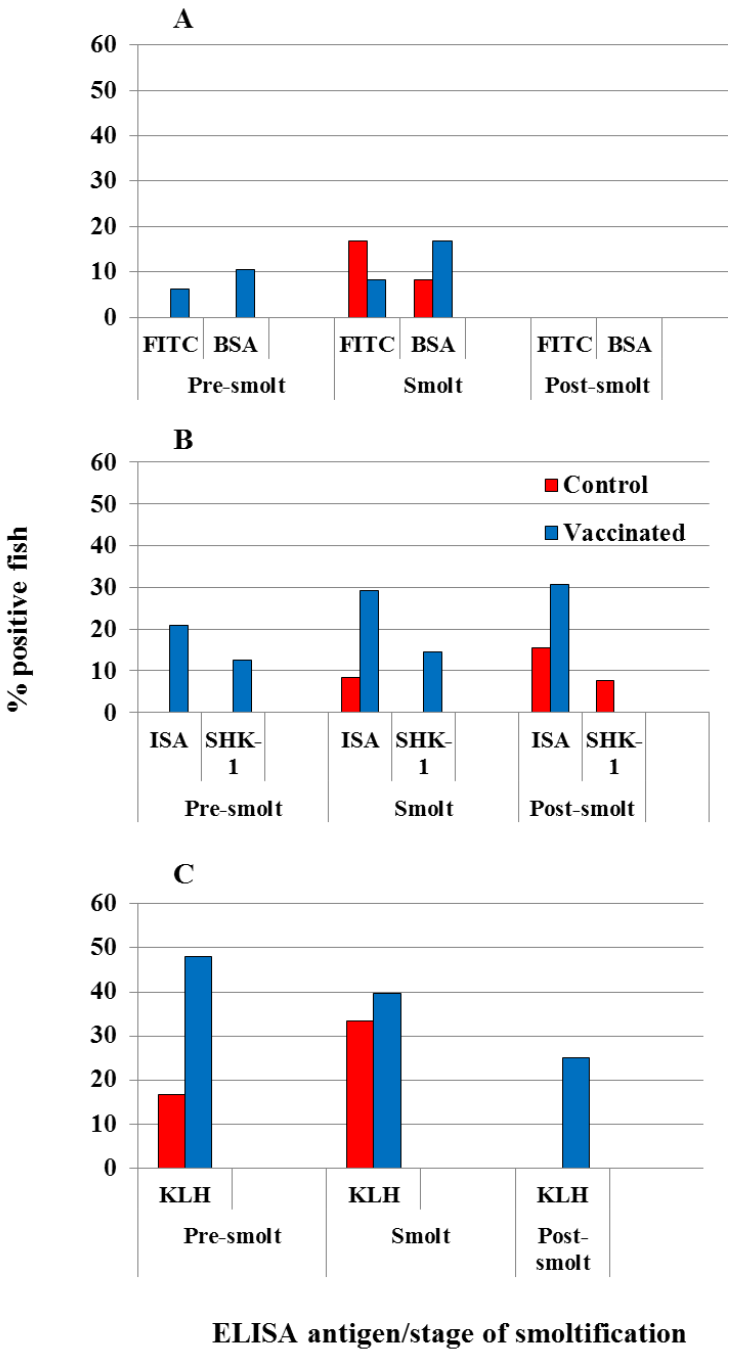


Figure 3.6 The total percentage of immunised fish with positive antibody values (OD_{450nm}> sensitivity threshold) to fluorescein isothiocyanate (FITC), infectious salmon anaemia virus (ISAV) and keyhole limpet hemocyanin (KLH) by enzyme-linked immunosorbent assay (ELISA). (A) Positive antibody values of fish immunised with FITC, FITC-KLH, FITC-ISA and FITC-KLH-ISA; (B) Positive antibody values of fish immunised with ISA, FITC-ISA, KLH-ISA, FITC-KLH-ISA; (C) Positive antibody values of fish immunised with KLH, FITC-KLH, ISA-KLH and FITC-KLH-ISA. Pre-smolt control n = 12, immunised n = 48; Smolt control n = 12; immunised n = 48; Post-smolt control n = 3, immunised n = 12 – 13.

Even where high antibody values were observed at a dilution of 1/20, a 2 to 3-fold decrease in OD occurred subsequent to serial dilution of sera to 1/40 (results not shown). There were 2 control fish that were also sero-positive for KLH, however these same fish produced relatively high antibody values of OD_{450nm} of 0.195 and 0.395 to unrelated antigens such as uninfected SHK-1 cells, and represented the only control group outliers, thus such fish may harbour higher levels of natural antibodies leading to non-specific reactions.

(b) Variation of antibody response in smolts

During smoltification, the majority of antibody responses were still very low with many non-responders to vaccination. There were no antibody responses to FITC and in spite of a few strong individual responders to KLH from KLH immunised fish (i.e. with OD_{450nm} 0.56 – 1.05), the response to KLH was not significantly greater in these fish compared to control fish (Fig. 3.7), which is in contrast to pre-smolt fish. Although 19/48 (40%) of fish immunised with KLH antigen were positive by ELISA, 4/12 (33%) control fish also had antibody values above the sensitivity threshold for KLH (Fig. 3.6 C; Table 3.2).

Antibody responses of ISA vaccinated smolts to ISAV antigen were still inconclusive, however, significantly higher antibody values were noted between ISA vaccinated (I, p=0.008) and control fish (Fig. 3.7). There were also significantly higher responses from the I vaccine group compared to IFK vaccine group (p=0.009). A total of 14/48 (29%) ISA vaccinated fish were deemed positive for ISAV antibodies (Fig. 3.6 B) and, although positive results were also observed to uninfected SHK-1 cells, 7 of these fish were only positive to ISAV antigen (Table 3.2).

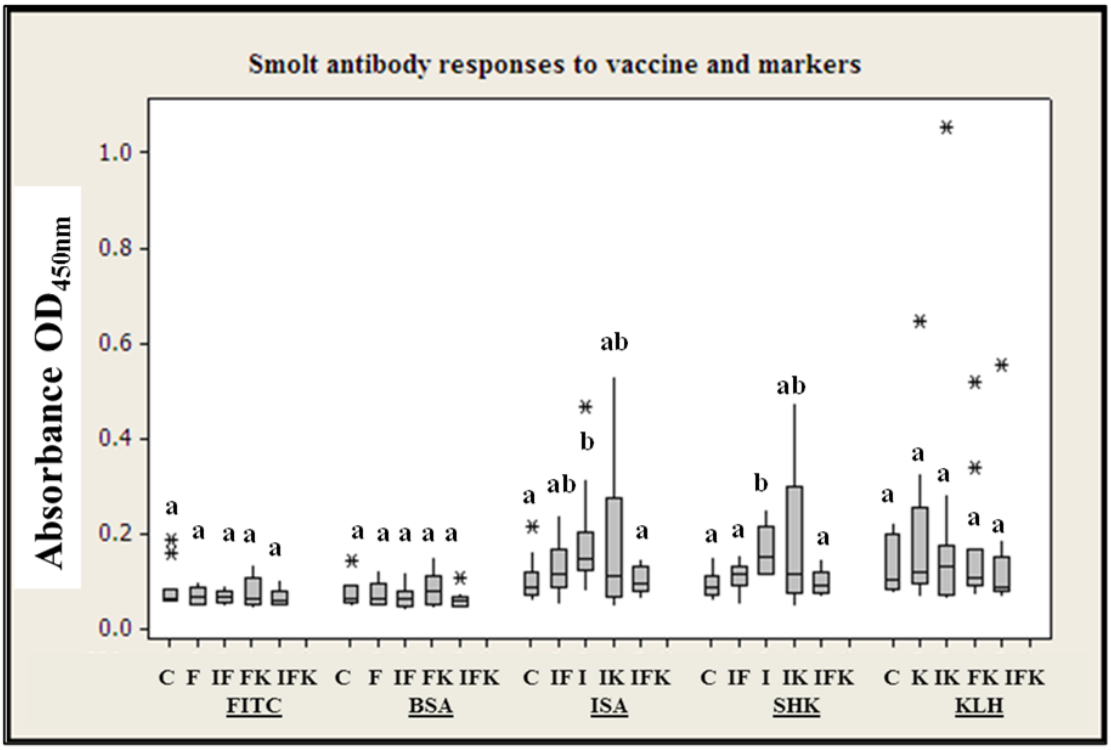


Figure 3.7 Box plot of antibody responses of infectious salmon anaemia (ISA) marker vaccinated smolt salmon at a serum dilution of 1/20. Letters on the *x* axis represent vaccine groups: C = Control, F = FITC only, IF = FITC + ISA vaccine, FK = FITC conjugated to KLH only, K = KLH only, I = ISA vaccine only, IK = KLH + ISA vaccine, IFK = FITC conjugated to KLH + ISA vaccine. Abbreviations at foot of the *x* axis indicate ELISA antigen that sera is screened against. ∗ = outliers. Different letters indicate significant differences within groups for each antigen. Data are medians of each fish vaccine group (n = 12). (Mann Whitney-U pairwise comparison test; $p < 0.05$).

(c)Variation of antibody response in post-smolts

As a result of fungal infections affecting smolting fish, a number of fish from the study died, prior to, or following transfer to salt water. Therefore, only a limited number of vaccine groups were analysed at the final time point: the I vaccine group, FK vaccine group, IFK vaccine group and controls. There were no positive responses observed in the FITC-ELISA from any fish and only 3/12 (25%) fish were positive for KLH antigen (Fig. 3.6 C), 2 from the IFK vaccine group and 1 from the FK vaccine group (Table 3.2), which were also significantly different from each other ($P < 0.05$) (Fig. 3.8). All control post-smolt fish were

negative to these antigens (Fig. 3.6 A & C). There were no significant differences in antibody values between vaccinated fish and controls. Four of the thirteen fish from either the I vaccine group or IFK vaccine group were positive for anti-ISAV antibodies, although no responses from any of the ISA vaccinated groups were significantly different from controls (Fig. 3.8). Despite 2/3 of the control fish producing antibody values above the sensitivity threshold (Table 3.2; Fig. 3.6 B), these were much lower than those produced by vaccinated fish (Fig. 3.8) An interesting point to note from these fish was that significantly higher antibody values to ISAV antigen compared to SHK-1 cell antigen ($P < 0.05$) was observed for the first time in the study.

(d) Variation of antibody responses between Atlantic salmon life stages

Variations in mean antibody responses to ISAV and KLH occurred at the different salmon life stages, but not to FITC (Fig. 3.9), however mean antibody values were very low (OD_{450nm} <0.3) due to the high number of negative fish as noted previously (Table 3.2).

After pooling data from all ISA vaccinated salmon, a significant increase in response to ISAV from smolt to post-smolt was noted (p=0.02). There was no significant difference in the responses to SHK-1 cell antigen (p=0.34) suggesting a specific anti-ISAV antibody response from the vaccinated fish. There was also no significant difference in response of the control group between the smolt to post-smolt life stages (ISA p=0.19; SHK-1 p=0.43) although the number of post-smolt fish analysed was very low. Responses to KLH were in contrast from those to ISAV antigen with a general decrease of antibody values from pre-smolt to smolt fish from all KLH immunised groups (Fig. 3.9). Interestingly, antibody values from the control group increased when fish were smolting (not shown).

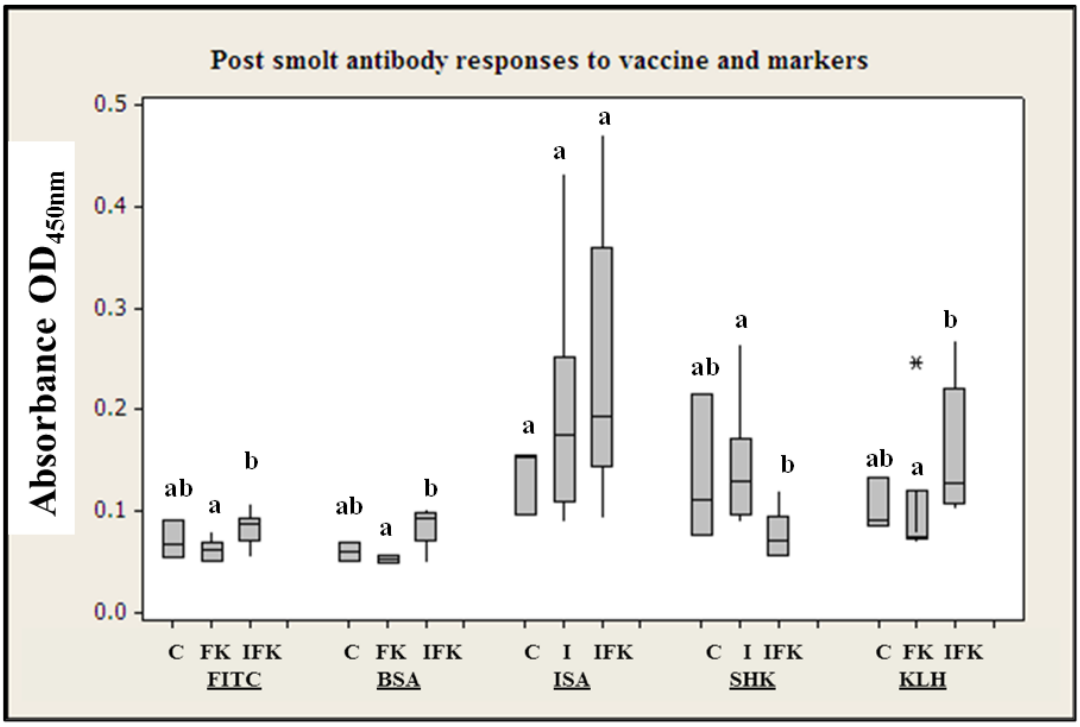


Figure 3.8 Box plot of antibody responses of infectious salmon anaemia (ISA) marker vaccinated post-smolt salmon at a 1/20 sera dilution. Letters on the *x* axis represent vaccine groups: C = Control, F = FITC only, IF = FITC + ISA vaccine, FK = FITC conjugated to KLH only, K = KLH only, I = ISA vaccine only, IK = KLH + ISA vaccine, IFK = FITC conjugated to KLH + ISA vaccine. Abbreviations at foot of the *x* axis indicate ELISA antigen that sera is screened against. ∗ = outliers. Different letters indicate significant differences within groups for each antigen. Data are medians of each fish vaccine group (C: *n* = 3; FK: *n* = 6; I: *n*=7; IFK: *n* = 6) (Mann Whitney-U pairwise comparison test; $p<0.05$).

After pooling of data from KLH immunised salmon, there was a significant decrease in response to KLH ($p=0.01$) from pre-smolt to smolt, but a significant increase ($p=0.01$) from controls. There were also lower mean antibody values from pooled data of KLH immunised post-smolt fish compared to smolt fish, however this was not statistically significant ($p=0.55$) (Fig. 3.9). The temperature regime is also indicated in Fig. 3.9, which was notably low from December to March and may have influenced the antibody responses observed over the course of the trial.

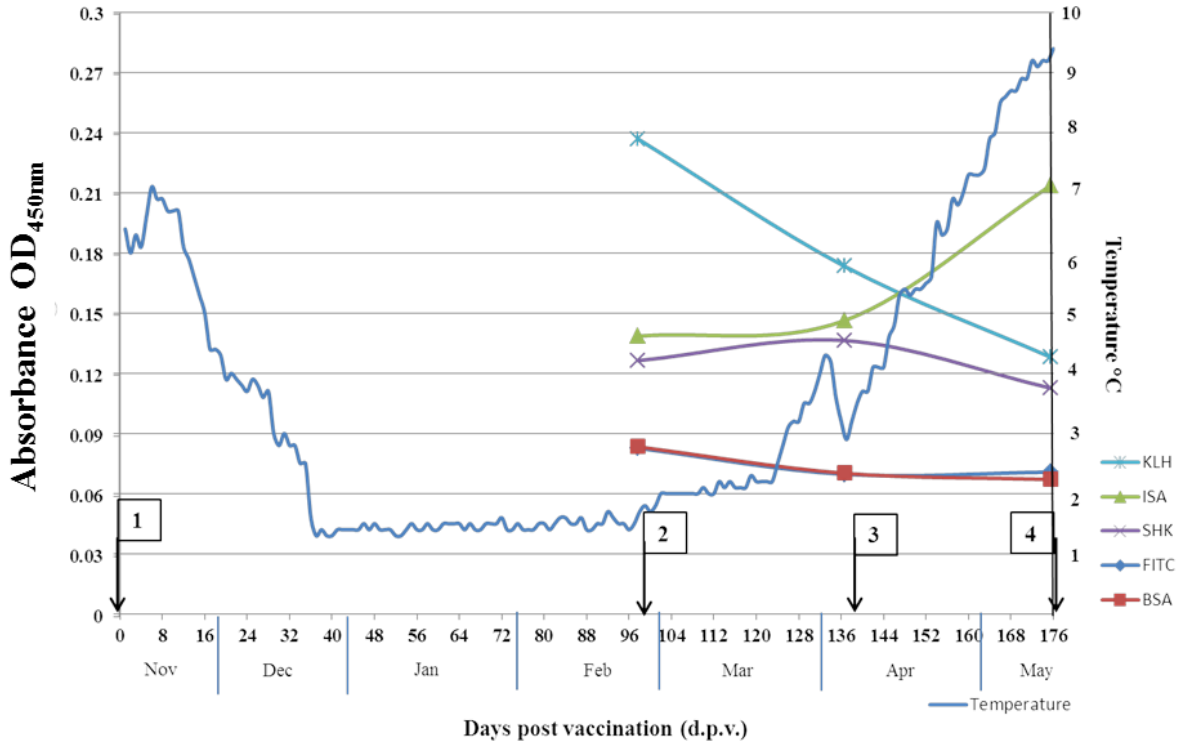


Figure 3.9 Sampling points for Atlantic salmon antibody analysis of infectious salmon anaemia (ISA) marker vaccinated fish during the course of smoltification. The graph shows the temperature regime throughout the course of the study and the months and days following vaccination in which sera was collected for analysis. The Blue line graph from 0 – 176 dpv shows the water temperature (°C) which is indicated on the second *y*-axis. The mean antibody responses for each time point, that the sera was analysed, are indicated in the legend. The antibody OD_{450nm} indicated for responses to each antigen are the mean of pooled results from all vaccinated groups immunised with ISA (*n* = 48, 48, 13), FITC (*n* = 48, 48, 12) or KLH (*n* = 48, 48, 12). BSA and SHK are control antigens for FITC and ISA ELISA, respectively. Results of control fish are not shown. Numbers in boxes indicate initiation of experiment and sampling points at different salmon life stages: (1) Vaccination of salmon with ISA, FITC and KLH antigen formulations, (2) Sampling of pre-smolt salmon 98 dpv, (3) sampling of smolt salmon 137 dpv and (4) sampling of post-salmon 176 dpv and termination of experiment. All sera diluted 1/20.

3.3.4. GFP KHV marker vaccine trial in carp

3.3.4.1 Purification of KHV antigen for anti-KHV antibody screening

The DNA from KHV was successfully amplified in the PCR using infected KF-1 cell supernatant (Fig. 3.10 A), and KHV particles were also detected after 8 dpi in fixed infected KF-1 cells by TEM (Fig. 3.10 B), confirming that the virus had been successfully cultured to utilise as antigen for serology. Further confirmation was obtained by negative staining of purified KHV antigen following ultracentrifugation, which appeared as 2 bands on the gradient (Section 2.5.2). TEM analysis revealed that both enveloped particles (Fig. 3.10 C) and unenveloped capsids (Fig. 3.10 D) were present in the whole virus antigen sample.

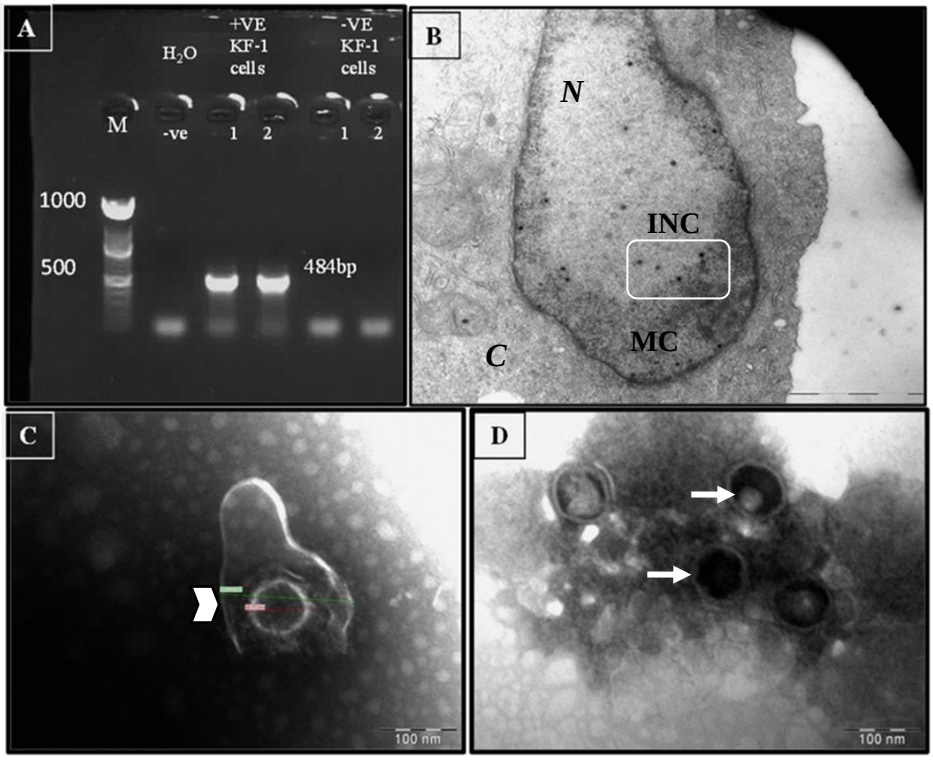


Figure 3.10 Molecular and morphological identification of koi herpesvirus (KHV) from infected koi fin (KF-1) cells. (A) Single round PCR according to Gilad *et al*. (2002) with expected molecular weight bands from positive samples of 484 bp, (B) TEM micrograph KHV infected KF-1 cell with marginated chromatin (MC) and nucleocapsids in the nucleus (INC), (C) TEM micrograph of negative stained preparation of enveloped KHV virion, (D) TEM micrograph of negative stained preparation of disrupted KHV virion with unenveloped capsid. *N* = Nucleus; *C* = Cytoplasm; Arrow head = enveloped virion; Arrows = unenveloped capsids.

3.3.5 Carp antibody responses to GFP and inactivated KHV vaccine

3.3.5.1 Optimisation of the GFP-ELISA

No conclusive positive anti-GFP antibody responses were detected, despite numerous attempts, mainly due to high background levels from control fish resulting in very high cut off ODs (i.e. >0.7) (Fig. 3.11 A & B). However, greater antibody values were detected in the GK vaccine group compared to all other groups (Fig 3.11 A), and although K vaccine group fish also produced high antibody values to rGFP, this group were also positive to BSA antigen suggesting a greater presence of cross-reacting non-specific antibodies (Fig 3.11 B). Interestingly, the GK vaccinated group were still the strongest responders with a positive antibody titre of 1/256 during optimisation involving intensive blocking, although there were only minimal differences compared to responses from non-GFP immunised fish in the K vaccine group and controls, which were also always higher than the sensitivity threshold of the assay (results not shown), thus producing false positive results.

3.3.5.2 Anti-GFP antibody screening from GFP + KHV marker vaccine trial

All fish from the trial were finally screened using internal sera blocking with 1% casein as a diluent, which minimised the non-specific binding, but revealed a lack of GFP-specific antibodies in any of the vaccinated fish in this trial (results no shown) suggesting the results observed during optimisation may have been associated with natural antibody responses.

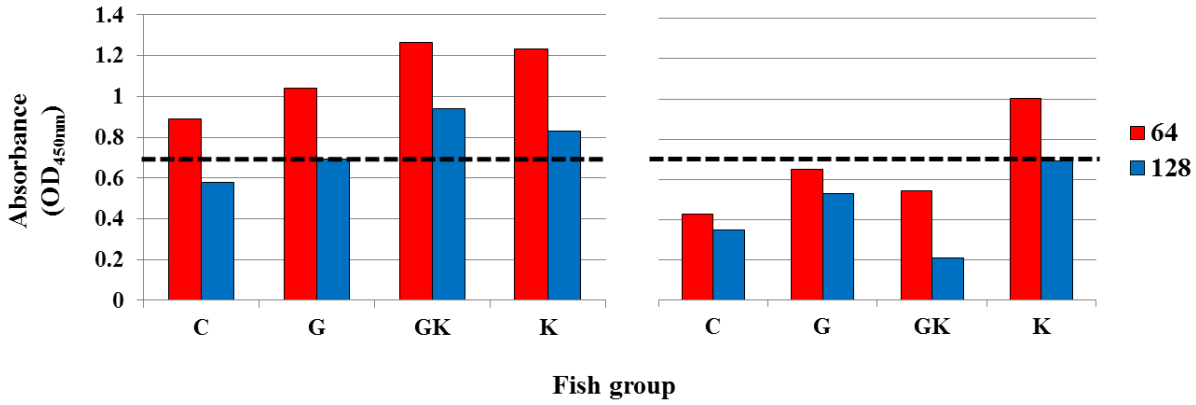


Figure 3.11 Optimisation of green fluorescent protein (GFP) enzyme linked immunosorbent assay (ELISA) with sera from carp immunised with GFP. (A) ELISA plate coated with 3µg mL^{-1} recombinant GFP and screened with pooled sera from each vaccine group (n=2 – 4) from a GFP + KHV marker vaccine trial. (B) ELISA plate coated with 3µg mL^{-1} BSA and screened with the same serum samples as 'A'. Colour coded legend indicates the dilution of serum used for screening: red = 1/64; blue = 1/128. C = Control, G = GFP inoculated only, GK = GFP + KHV vaccinated, K = KHV vaccinated only. Black line indicates cut-off.

3.3.5.3 Optimisation of KHV ELISA

In contrast to responses observed to GFP antigen, there were strong antibody responses to KHV, up to titres of 1/3200, from pooled sera of all fish in KHV vaccinated groups (results not shown). However, there were also very high backgrounds noted with negative control sera, especially at lower dilutions (1/100), whereby pooled fish sera from the G vaccinated group and control group produced ODs as high as 0.59 resulting in false positive results (results no shown). Therefore further optimisation was carried out and 10% casein proved to be the most effective blocking reagent for post-coating ELISA plates, similar to that utilised by St-Hilaire *et al*. (2009) for KHV serology. Intensive blocking using 5% casein within the sera, that was similar to the method employed by Kim *et al*. (2007a), but more stringent than that applied in the KHV ELISA by Adkison *et al*. (2005), reduced absorbance ODs in both negative and positive control sera, however, while the negative control sera remained negative at a 1/200 dilution, the end point antibody titre remained at 1/1600 for positive

control sera (results not shown). Therefore reduced ODs from blocking of positive sera was not detrimental to the assay as the highest affinity IgM remained bound to the antigen, even at higher dilutions.

Other ELISA parameters that were varied in an attempt to optimise the assay included altering the concentration of the conjugate, anti-carp MAb and the KHV coating antigen by screening with 2-fold dilutions of carp sera. Variation in antigen concentration had the biggest impact on improving the assay, which resulted in reduction in absorbance of approximately OD_{450nm} 0.1 per 0.1 µg $well^{-1}$ KHV protein. Due to the limited amount of purified KHV and the difficulty in generating purified virus, as described in Section 2.5.2, a final working assay concentration of 0.4 µg mL^{-1} was utilised for carp antibody screening, whereby an OD_{450nm} of 0.6 could still be obtained at a dilution of 1/800. Limiting the concentration of anti-carp MAbs also resulted in little variation of positive signal. Controls were consistently negative at all dilutions with all assay variations attempted during optimisation, further verifying the effectiveness of the intensive serum blocking.

3.3.5.4 Anti-KHV antibody screening from GFP + KHV marker vaccine trial

The mean response from KHV vaccinated fish was significantly higher (p = 0.01) than controls at titres from 1/200 to 1/3200 (Fig. 3.12 A). There was no significant difference between the GK vaccine group and the K vaccine group (p = >0.05), although the mean response of the K vaccine group was consistently higher than GK group. However, 6/8 carp from the control group (sham vaccinated) were also sero-positive for KHV at a 1/200 sera dilution and 1/8 were positive at a 1/400 dilution (Fig. 3.12 B). The naïve carp sera (negative control) remained negative and the positive control (pooled anti-KHV sera from experimentally challenged carp, CEFAS) (Fig 3.12 A) was consistently positive. Six

vaccinated fish were positive at a titre of 1/800 from both the K vaccine group and GK vaccine group and 5 of these fish produced even higher titres (Fig. 3.12).

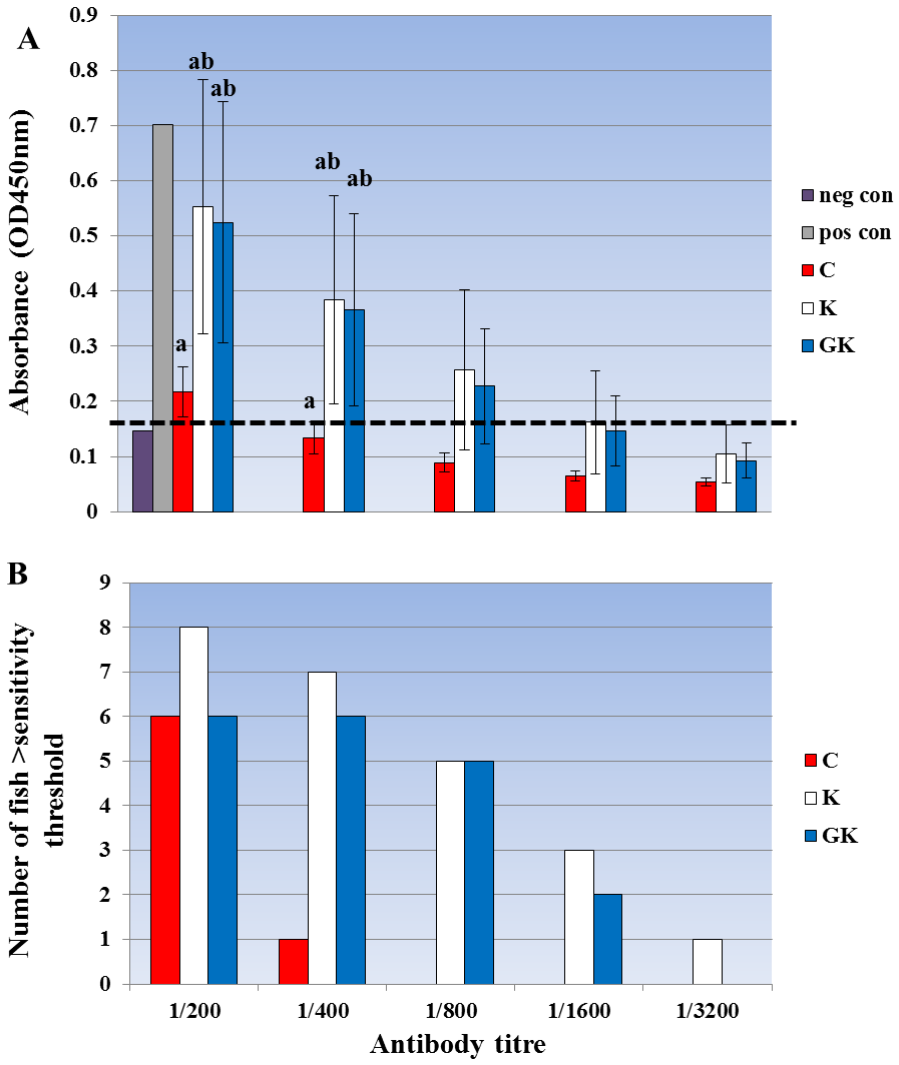


Figure 3.12 Optimised koi herpesvirus (KHV) enzyme linked immunosorbent assay (ELISA): Final screening of carp sera from KHV marker vaccine trial, 6 weeks post vaccination (wpv). ELISA was undertaken with the optimised protocol for KHV antibody screening. (A) Values are mean ± SE (n = 7 - 8) of antibody responses from each vaccine group. Colour coded legend indicates the different vaccine groups; C = Control, K = KHV vaccinated only, GK = GFP + KHV vaccinated. Note GFP only group was not tested against KHV antigen. Different letters indicate significant differences within groups for each antigen (Mann Whitney-U pairwise comparison test; p<0.05); (B) Number of serologically positive fish from from each vaccine group at each titre above the cut-off. Note the high number of false positive fish from C group at 1/200 dilution despite protocol optimisation. Black broken line indicates cut-off from background controls.

3.4 Discussion

Vaccines containing positive markers, foreign to the host, have been shown to enable differentiation between vaccinated and non-vaccinated mammals and birds using serology (Walsh *et al*., 2000a; b; Mebatsion *et al*., 2002; James *et al*., 2007; 2008, Fang *et al*., 2008). Marker vaccination approaches like these may enable the implementation of control and eradication programmes for notifiable diseases.

A model exogenous marker approach was undertaken in this study using inactivated vaccines for ISA and KHVD by administering foreign antigens found previously to be immunogenic in mammals, avians and fish (Hodgins *et al*., 1967; Gonzalez *et al*., 1988; Killie and Jørgensen, 1994; Jones *et al*., 1999a; Cain *et al*., 2002; Companjen *et al*., 2006; James *et al*., 2007; Swan *et al*., 2008; Lu *et al*., 2009; Valdenegro-Vega *et al*., 2013). The principle objective was to determine if an antibody response to the marker antigens could be produced and detected by serology using ELISA, thus enabling the distinction between vaccinated and unvaccinated Atlantic salmon and Mirror carp to ISAV and KHV, respectively.

3.4.1 Exogenous marker vaccination for infectious salmon anaemia in Atlantic salmon

Tetanus toxoid, the inactivated form of tetanus toxin produced from *Clostridium tetani*, was previously utilised by James *et al*. (2007; 2008) to develop an exogenous marker vaccine for AI, which could be used to differentiate between vaccinated and infected ducks and chickens. The vaccine used was an inactivated preparation of subtype H6N2 containing TT and the presence of antibodies to TT indicated that animals had been vaccinated while non-vaccinated birds lacked anti-TT antibodies. As TT is likely to be immunologically foreign to

fish, it has the potential as a very useful marker antigen to use in aquaculture vaccines, especially since it is already registered for applications in food animal vaccines (James *et al*., 2007). Only a few studies have examined the serological responses of fish to TT, in which the 14S macroglobulin fraction of IgM, from the catfish, *Ictalurus punctatus*, was found to successfully neutralise tetanus toxin (Ourth, 1982). An exogenous marker like TT added to an ISA vaccine could potentially pave the way to DIVA vaccination if a standardised ELISA could be developed for ISA, as proposed by James *et al*. (2008) for AI. Recent ISA infection could then perhaps be indicated by elevated antibody titres compared to non-infected, vaccinated fish. However, the TT marker vaccination did not induce a strong detectable specific anti-TT antibody response in Atlantic salmon with the doses administered (results not shown). This was despite anti-TT MAbs always producing antibody values of absorbance OD_{450nm} >1.0 and anti-salmon IgM MAbs binding strongly to precipitated Atlantic salmon IgM during optimisation, thus the TT assay was considered to be performing well (results not shown). The lack of response may have been associated with insufficient doses of TT administered to the fish or poor immunogenicity of TT in Atlantic salmon, which corroborates previous immunisation studies of rainbow trout to a closely related bacterial antigen, *Diphtheria toxoid* (Eide *et al*., 1994), thus suggesting that this antigen is not sufficiently immunogenic as a marker in fish. These preliminary results highlighted the challenges facing exogenous marker vaccination, especially with regards to cost for vaccine and serological test development, but also potential implications of multiple antigen administration, thus alternative antigens were utilised as model markers.

Keyhole limpet hemocyanin and fluorescein isothiocyanate, carrier protein and hapten respectively, were investigated for their potential as exogenous markers as they have been utilised previously for immunisation studies in fish and their immunogenicity in salmonids has been demonstrated (Hodgins *et al*., 1967; Killie and Jørgensen, 1994; 1995; Jones *et al*.,

1999a; Alcorn and Pascho, 2002; Cain *et al*., 2002; Swan *et al*., 2008; Valdenegro-Vega *et al*., 2013). Furthermore, these antigens are highly accessible and cost-effective with regards to vaccine formulation and serological test development. The conjugation of FITC-KLH was also intended to provide an insight into the potential complications of antigenic competition between simultaneously administered antigens, a phenomenon well characterised in fish (Avtalion and Milgrom, 1976; Killie and Jørgensen, 1994; 1995), which is required for marker vaccination.

A stronger antibody response to KLH than to FITC was observed in pre-smolt fish from all groups vaccinated with KLH. The stronger response to KLH than to FITC observed in Atlantic salmon vaccinated with the FITC-KLH conjugate, is in contrast to the findings of Jones *et al*. (1999a) in immunised rainbow trout. Jones *et al*. (1999a) did however, also note strong antibody responses to KLH in trout immunised ip with either conjugated FITC-KLH or monovalent KLH. Thus, the relatively strong responses of both pre-smolt salmon and rainbow trout indicated that KLH was able to induce a T-cell independent (TI) antibody response, which was detectable to a similar degree in both the Jones *et al*. (1999a) study and the current study, up to 14 wpv. This differs to the findings and of Killie and Jørgensen (1994; 1995), who proposed that AIS results from the antigenic competition of B-cell T-dependent (TD) antibody responses to the hapten, i.e. FITC, suppressing the TI antibody response to the carrier, which in that study was *Limulus polyphemus* hemocyanin (LPH). In the current study, there appeared to be no responses to FITC, with the highest antibody value of $OD_{450nm} < 0.18$ at a 1/20 serum dilution. This non-responsiveness to FITC may have been associated with low FITC:KLH ratios following conjugation, but this is unlikely as hapten:carrier protein conjugate ratio has been reported previously not to be influential on the antibody response to the 2 antigens in fish (Killie and Jørgensen, 1994). In fact, Avtalion and Milgrom (1976) found that carp immunised with heavily substituted haptenic antigens were

non-responsive to the carrier. They suggested that the heavily substituted carrier, in this case BSA, loses its ability to stimulate B cells, but not the T-cell directed antigenic determinants. Furthermore, the lack of anti-FITC response was surprising as it has also been found that there is a hapten - carrier effect in fish, whereby an antibody response to the hapten by B cells requires carrier-specific and thus putative, T-cell cooperation, which should have been provided by KLH in FITC-KLH inoculated fish (Stolen and Mäkelä, 1975; Avtalion and Milgrom, 1976; Ruben *et al*., 1977). Perhaps a secondary response may have been elicited following a booster immunisation as amnestic responses to TD antigens conjugated to KLH have previously been demonstrated in rainbow trout (Arkoosh and Kaattari, 1991). Conjugation to KLH previously induced a minor increase in antibody affinity to the hapten dinitrophenyl (DNP) (Cossarini-Dunier *et al*., 1986), whereas the affinity and titre of the antibody response to FITC in FITC-KLH immunised rainbow trout was found to increase significantly over the course of 4-6 weeks (Cain *et al*., 2002; Swan *et al*., 2008). The unexpected lack of FITC response accompanied with a stronger KLH response may have been solely a result of the low temperatures (6ºC during immunisation) leading to poor T cell stimulation. T cells are typically sensitive to low temperatures (Bly and Clem, 1992; Secombes *et al*., 1996; Le Morvan *et al*., 1998) therefore the carrier protein may have induced a TI B cell response without B cell antigen presentation to Th2 cells in order for the hapten to be processed via the MHC II pathway. This is a pertinent point with regards to immunisation regimes for marker vaccination in fish as specific antibody responses may be suppressed to the marker antigen at low temperatures.

Low level antibody responses were also detected from ISA vaccinated fish, particularly at the pre-smolt stage 14 wpv, whereby only 2 ISA vaccinated groups had produced antibody values significantly higher than controls and 10/48 fish were positive for ISA antibodies, although 6 of these fish were also positive for SHK-1 cell antigen. Antibody

responses to the ISA vaccine were not adversely affected by the inclusion of marker antigens in the vaccine formulation, in fact, there were always greater antibody values to ISAV in fish vaccinated with both ISA vaccine and marker antigens, which may be associated with effects of the additional adjuvant that those fish received from dual inoculation or even through an adjuvant effect provoked by the additional antigen as opposed to antigenic competition. This is a phenomenon that has been reported in mice after immunisation with 2 antigens at the same site (Monier, 1975), which was thought to be the result of T-cell cooperation with the presence of numerous macrophages. In mammals, administration of 2 antigens at the same time, has been shown not to provoke antigenic competition, as long as the antigens were not administered at the same lymphatic site (Brody and Siskind, 1972).

Kibenge *et al*. (2002) successfully screened wild infected and experimentally challenged Atlantic salmon for anti-ISAV antibodies, however, the serum titre found to be optimal in that study was also 1/20. Obtaining high specific anti-ISAV antibody titres was also difficult in the ELISA developed in the current study, although much of the signal was removed after optimisation of the ELISA by internal serum blocking to allow positive sera to be differentiated from controls as high backgrounds were observed in a preliminary trial, which made interpretation of ELISA results difficult (results not shown). Indeed a few control fish were positive for anti-ISAV antibodies at this concentrated serum dilution. This may have been due to natural antibody induction, e.g. by the marker as noted in fish immunised with KLH and the hapten trinitrophenol (TNP) in a previous study (Gonzalez *et al*., 1988). In the ELISA developed by Kibenge *et al*. (2002), non-specific antibody responses were also very high to SHK-1 cell antigen in some fish that were negative for anti-ISAV antibodies. Mikalsen *et al*. (2005) also remarked on high background staining during serological analysis of fish vaccinated with a DNA ISA vaccine and subsequently challenged, which emphasises difficulty for detecting specific anti-ISAV antibodies at a high serum

concentration. Furthermore, injection itself could elicit non-specific weak immune responses (Anderson, 1992) that may result in cross-reactive antibody responses to certain conserved epitopes. Non-specific antibody signals to SHK-1 cell antigen were also apparent in the current study and fish with stronger anti-ISAV antibody responses usually had high responses to SHK-1 cell antigen, thus cell culture derived antigens could possibly be utilised as markers of vaccination as well, if high titre, specific responses could be obtained, as these antigens would only be recognised from exposure to ISAV vaccine formulations and not infectious ISAV. Attempts to block such non-ISAV specific antibody attachment with low fat milk as serum diluent (Kim *et al*., 2007a) used for the ELISAs developed in the current study, is a common application for companion marker vaccine diagnostic tests for mammals and birds (James *et al*., 2008; Barros *et al*., 2009). Heat treatment of sera to 56°C for 30 min proved effective in the study by Kibenge *et al*. (2002) for reduction of background signals, however, numerous reports for mammalian ELISA tests have reported false positive results using this method (e.g. Hasselaar *et al*., 1990) and in fact heat treatment is used to eliminate the activity of IgM in mammalian and avian serology (Denzin and Staak, 2000) as previously applied for DIVA diagnostics for birds when detecting IgY (Tumpey *et al*., 2005). Therefore the internal blocking procedure was preferred in the current study. Although specific antibodies produced in Atlantic salmon to ISAV do constitute a protective effect (Falk and Dannevig, 1995; Lauscher *et al*., 2011), the majority of antibodies are thought to be directed to the nucleoprotein on ELISA (Falk pers. comm. cited in Wolf *et al*., 2013). The ISAV antigen used to coat ELISA plates in the current study had been characterised by TEM and RT-PCR and negatively stained purified ISAV antigen samples contained mostly unenveloped nucleocapsids. Due to disruption of virions during purification procedures a lack of envelope proteins on the ELISA plate, could result in the lack of antibodies detected in vaccinated fish. Salmon vaccinated with DNA and recombinant ISA vaccines expressing the surface HE

protein were negative by ELISA until they were challenged with infectious virus (Mikalsen *et al*., 2005; Wolf *et al*., 2013). This may also have affected antibody detection in the current study, as fish were vaccinated with a whole inactivated virion preparation, which likely consisted predominantly of enveloped particles with abundant protective HE protein.

ELISA methods for detecting antibody responses of Atlantic salmon, and other fishes, vary extensively in the literature. Where detection is expressed as absorbance value, especially in cases where only very low titres of specific antibody can be detected, as in the current study, the assay development method and wavelength at which the result is determined should be compared with caution. Anti-ISAV antibody responses reported by Kibenge *et al*. (2002) and Cipriano (2009) were determined using the alkaline phosphatase (AP) - *p*-nitrophenyl phosphate enzyme – chromogen system in which the optimal development time for assay was 24 h. This method enabled colour development proportional to the amount of suspected specific antibody in the fish serum with a final signal OD of > 4.0 at 405 nm for positive sera and only 0.5 for negative sera (Kibenge *et al*., 2002). Initially, however, the signal obtained was much lower after 10 min of development. This is in contrast to the final ODs obtained in the current trial where the highest responder had an OD of only 0.53 at 450 nm and the lowest negative sera only 0.04, using the HRP - TMB enzyme – chromogen system. The use of AP as a chromogen is less self-limiting than that of HRP, and thus enables the operator to increase sensitivity of the assay (Afolabi and Thottappilly, 2008) as the product of the AP – substrate reaction initiates a secondary cyclic enzyme reaction leading to an amplified signal (Fig. 3.13). Thus, the compromise for a rapid serological detection system for anti-ISAV antibodies, is perhaps an approach necessary for maximising signal output for detection of very low antibody responders similar to the method of Kibenge *et al*. (2002). Although the requirement of such a compromise to increase sensitivity may have negative implications with regards to developing a rapid serological assay, i.e. lateral

flow test, which is an important consideration for marker vaccines. Nonetheless, anti-ISAV antibody responses at a titre of 1/20 were detected, though only 28/109 (26%) of ISA vaccinated fish were sero-positive for ISAV and 13 of these also positive for SHK-1 cell antigen.

Previous ISA vaccination trials have proved successful for the protection of salmon following experimental challenge yet have failed to detect specific anti-ISAV antibodies (Brown *et al*., 2000) and only 1.2% of 1141 fish serum samples, screened by ELISA according to Kibenge *et al*. (2002), taken from wild salmon in the Penobscot, Merrimack and Connecticut River (U.S.A.) were found to be positive (Cipriano, 2009). While the latter study would appear promising, implying almost complete freedom of the virus in wild fish stocks in these rivers, the test sensitivity could also be questioned.

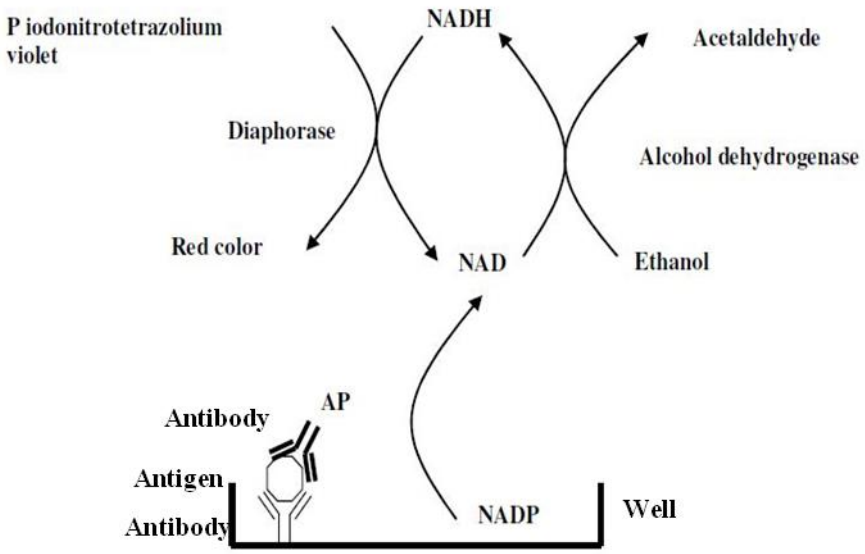


Fig. 3.13 Schematic diagram of sandwich enzyme linked immunosorbent assay (ELISA) showing chemical reactions involved in the alkaline phosphatase (AP) amplification system resulting in amplified signal over time. The bound alkaline catalyses the breakdown of nicotinamide adenine dinucleotide phosphate (NADPH) whose products initiate a secondary cyclic enzyme reaction, which results in a coloured product. Amplification occurs as the process is repeated several times. *After Afolabi and Thottappilly* (2008)

A recent investigation highlighted the possibility that detection of specific anti-ISAV antibodies may be associated with the dose of antigenic challenge, implying that specific antibody detection may be difficult unless the salmon immune system encounters an intense viral dose. Lauscher *et al*. (2011) found that fish vaccinated with a very high dose of inactivated ISAV produced very strong antibody responses of titres >1/3200 after 6 wpv., however, it was noted that fish vaccinated with lower doses, 80% less than the high vaccine dose, produced very poor antibody responses or did not respond at all after the same time period. Reduced relative percent survival has also been observed in fish vaccinated with low concentrations of inactivated ISAV following experimental challenge (Jones *et al*., 1999b). The lower dose vaccinated fish reported by Lauscher *et al*. (2011) were still poorly sero-responsive after 6 days post challenge (dpc) and were all negative after 21 dpc. This was in contrast to the high dose group, which continued to sero-convert with high titres of anti-ISAV antibody after 6 and 21 dpc (Lauscher *et al*., 2011). The temperature was constant during this vaccine – challenge trial at 12°C, whereas in the current study the temperature was < 6°C, thus higher temperatures may have accounted for greater antibody responses to ISA vaccination in the trial by Lauscher *et al*. (2011). However, subsequent vaccination trials using a DNA-layered salmon alphavirus (SAV)-derived replicon vaccine, also undertaken at 12ºC in pre-smolt salmon, did not induce antibody responses and only 12/18 fish were seropositive using an inactivated ISA vaccine (Wolf *et al*., 2013). The low antibody responses observed in the current trial may be attributed to insufficient antigenic dose in the Intervet Schering Plough ISA vaccine, as indeed the high dose applied by Lauscher *et al.* (2011) was not typical. This would perhaps represent one of the major hurdles for ISA marker vaccination as it is not likely to be economically feasible to produce such high quantities of inactivated virus for vaccination on a commercial scale, but vaccines lacking all virion proteins do not appear to induce detectable antibodies prior to infection (Mikalsen *et*

al., 2005; Lauscher *et al*., 2011; Wolf *et al*., 2013). However, in the current study the sampling of salmon sera was also undertaken at much later time points of 14, 20 and 25 wpv, thus there is unlikely to have been a high level of proliferating clonal B cell production after such a long period following primary vaccination. Detection of low level antibodies throughout the production cycle is however vital if marker vaccination can be potentially implemented for Atlantic salmon to control this virus. Thus the influence of the adjuvant as a depot is also key for which Montanide ISA may have been most suitable. The use of Montanide ISA adjuvant is considered to induce antibody responses with equivalent efficacy to that of Freund's complete adjuvant with less damage caused to tissue and granuloma formation (Stils, 2005). Experimental challenge in sea water may have revealed any protection provided by the vaccine used in this trial and any associated memory antibody response, but unfortunately, too few fish were available post salt water transfer.

The weak antibody responses observed in general may have been associated with the poor CF of the experimental fish. Overall CF declined, relative to salmon smoltification status, as expected, over the course of the experiment, but was lower than would be expected, which may have been associated with very low water temperatures experienced between December and March. It is important to take into consideration the effects of temperature and stage of smoltification on the overall humoral response of Atlantic salmon to different antigens for determining the reliability of serological testing for responses to marker vaccination.

The effects of smoltification and temperature on the immune response in Atlantic salmon have been widely researched (Specker and Schreck, 1982; Maule *et al*., 1987; Zapata *et al*., 1992). Immunosuppression of smolting salmon has been correlated to increasing cortisol levels in plasma and reduced lymphocytes in the spleen (Specker and Schreck, 1982;

Maule *et al*., 1987) and a reduced ability to deal with pathogenic insult (Zapata *et al*., 1992), however, no definitive indication of this complex metamorphosis of Atlantic salmon has been investigated with regards to antibody detection following ISA vaccination. The general trend in antibody response over the course of the pre-smolt, smolt and post-smolt stages was of a decline in the response against KLH from pre-smolt to post smolt, but in contrast, an increase in mean antibody values were observed to ISAV. However, no fish immunised with FITC antigen produced a true positive response regardless of smoltification status, which is in contrast to Atlantic salmon immunised ip with FITC-KLH at higher temperatures (~11ºC) and booster vaccinated, which produce significantly elevated anti-FITC antibodies (Valdenegro-Vega *et al*., 2013).

Smoltification is the physical transformation and physiological transition of parr, in fresh water, to smolt, in sea water. The process of smoltification involves a number of complicated events including the ability for increased hypo-osmoregularity, increased growth rate and rapid metabolic changes (Hoar, 1976; Stefansson *et al*., 2008; Björnsson *et al*., 2011). These changes include the reduction of body CF, as well as an increase in sodium potassium ATPase, peaking towards the end of smoltification (Zaugg and McLain, 1970). Previous studies have investigated the impact that vaccination may have on smoltification as well as the affects that smoltification has on the fish immune response, which may have implications on vaccine efficacy (Melingen *et al*., 1995a; b; Eggset *et al*., 1999). However, pre-smolt fish were vaccinated at least 6 weeks prior to smoltification, which is a window post-immunisation that has previously been found not to cause adverse effects on fish vaccinated with *Aeromonas salmonicida* and *Vibrio salmonicida* prior to the induction of smoltification (Eggset *et al*., 1999). Eggset *et al*. (1999) hypothesised that immunisation with antigens in oil adjuvant close to the initiation of smoltification, is perhaps a stage when fish are more vulnerable. Smoltification has been associated with reduced total IgM (Melingen *et*

al., 1995b), which was subsequently found to rise up to 2-fold post sea water transfer in the same study, which interestingly corresponds to the rising antibody titre to ISAV in the current study. It is likely that a significant portion of these antibodies, however, represent natural antibodies as false positive detection of control fish to ISAV was also evident at the post-smolt stage. Fish vaccinated at various stages of smoltification always tended to produce a strong antibody response between 4–10 weeks post vaccination (wpv) (Melingen *et al*., 1995a). However, this may have been associated with the high temperature at which these fish were vaccinated. Although Lillehaug *et al*. (1993) claimed that temperature had not affected the antibody responses observed after vaccination against *Vibrio salmonicida* at low temperatures, i.e. also 6°C, the ELISA employed in that study was similar to the assay utilised in the current study with sera tested at a dilution of 1/20. Interestingly, the antibody curves reported in the Lillehaug *et al*. (1993) study resembled the curve noted for anti-ISAV antibodies in the current study, whereby there was a significantly lower antibody response at 16 wpv compared to 8 wpv and 28 wpv. The ISA marker vaccination trial conducted here resulted in significantly lower antibody responses to ISAV at 14 wpv than at 25 wpv following sea water transfer. There may therefore be a trend whereby greater specific antibody production occurs following smoltification. Unfortunately, the stage of smoltification was not taken into account in the study by Lillehaug *et al*. (1993), although the fish were vaccinated as parr at 15 g, thus it is reasonable to hypothesise that the fish may have been smolting during the experimental period from 10 – 20 wpv, where a vast reduction in specific antibody production was detected. On closer examination, the antibody responses obtained in that study actually appear weak for which low temperature and/or smoltification may have been a contributing factor. Nonetheless, the vaccine still provided very good protection when fish were subsequently challenged, suggesting important non-humoral immune responses may have been involved in protection provided by the vaccine, which

portrays a similar inverse correlation of antibody response to protection reported by Eggset *et al*. (1997a) after vaccination and challenge against cold water vibriosis. Timing of primary vaccination is suggested not to be as vital as the secondary 'booster', although vaccination at low temperatures has been reported to adversely affect T-cells and thus the TD antibody response but not the TI antibody response (Clem *et al*., 1991 cited in Bly and Clem, 1992). This perhaps helps to explain why good antibody responses are usually obtained subsequent to bacterial vaccination and challenge, such as with *Vibrio salmonicida* and *Aeromonas salmonicida*, as the lipopolysaccharide (LPS) (Melingen *et al*., 1995a; Eggset *et al*., 1997a) epitopes constitute major TI antigens. The diversification of antibody production by lower vertebrates may also be adversely affected by low temperature as fish may rely on the germ-line genes in which the cell cycle may be prolonged. Therefore cells are relatively big and low in abundance thus wasted energy on lymphocytes may be considered biologically 'expensive' (Du Pasquier, 1982), which may present further challenges to DIVA serology in terms of inducing specific antibody responses to various antigens.

It has been suggested by Kaattari *et al*., (1999), that variations in the antibody form of fish IgM, may have major implications with regards to serological diagnostics as well as vaccine efficacy, as the proposed redox forms of the fish tetrameric IgM, may result in variation in epitope binding specificity and thus possible blocking effects resulting in poorly titrated specific antibody. Furthermore, immunological studies in salmonids with foreign antigens that have been well classified as immunogenic molecules in mammals, often reveal inconsistent results (Alcorn and Pascho, 2002), which may be due to variation of the experimental environment, condition of fish, genetic background and as noted from the current study, perhaps smolt status. Marker vaccination using serology may therefore be more feasible for non-anadromous fish species.

3.4.2 Exogenous marker vaccination for koi herpesvirus in carp

Green fluorescent protein has been demonstrated to possess immunogenic properties in mammals (Stripecke *et al*., 1999; Walsh *et al*., 2000a; b; Fang *et al*., 2008) and anti-GFP antibodies have been successfully detected in inoculated carp (Companjen *et al*., 2006). It was therefore investigated as a marker antigen for an inactivated KHV vaccine. During optimisation there were higher antibody values to the GK group than the G group, suggesting a possible adjuvant effect from the dual inoculation, however the antibody response of the K vaccine group was also higher than the G group. The GFP vaccine formulation consisted only of ISA montanide adjuvant, a water in oil adjuvant that provokes both Th 1 and Th2 responses (Mata *et al*., 2007). The KHV vaccine, however, is formulated in aluminium hydroxide, which induces strong Th2 responses (Cox and Coulter, 1997; Kool *et al*., 2008) and thus may have resulted in greater antibody induction in KHV vaccinated fish. However, the antibody responses of the K group, not immunised with rGFP, were also relatively strong against BSA implying non-specific, possibly natural antibody responses, which are characteristic of cyprinid IgM (Kachamakova *et al*., 2006; Sinyakov *et al*., 2002; 2006; Sinyakov and Avtalion, 2009). Non-specifically bound antibodies were completely eliminated following intensive blocking. It was noted that the cut-off used for the sandwich GFP ELISA, utilised by Companjen *et al*. (2006), was only OD $\geq$ 0.05 at 450 nm, which would imply that the antibody values observed were also very low, despite the authors reporting a positive titre of ~ 1/400 for primary inoculated positive control fish. However, following a booster immunisation, a high antibody titre was detected at a cut-off OD $\geq$ 0.2, thus a booster immunisation may be required to induce detectable anti-GFP antibody responses for detection of marker vaccinated fish. No antibody response to GFP from a single immunisation was observed in the current study following serum blocking, which may also have been the result of poor presentation to the immune system and rapid removal by

stimulated macrophages. It may be necessary for continuous antigen expression from a replicating virus with a GFP insert in order to activate a Th2 response by the MHC 2 class pathway to induce a measurable anti-GFP antibody response. Green fluorescent protein has previously been inserted into the genome of attenuated KHV without affecting replication (Fichtner *et al*., 2007; Costes *et al*., 2008), which may make it a useful endogenous marker strategy for an attenuated replicating virus as results for mammalian vaccines have previously been encouraging (Walsh *et al*., 2000a; b; Fang *et al*, 2008). Negative factors regarding the use of GFP as a marker antigen is that it is unlikely that such a vaccine could be licensed for food fish and there have also been reports of GFP-induced cell toxicity in cells expressing the protein *in vitro* (Liu *et al*., 1999). The expression system used in such marker vaccines is also vital for sufficient presentation of the antigen to B cells. A secreted expression system allowed GFP antigen to be easily internalised, degraded, presented and subsequently recognised by specific antibody receptors of B lymphocytes in a previous study (Walsh *et al*., 2000a). Anchorage of the same marker antigen to the membrane proved to be a more effective approach, which was thought to result in an efficient uptake from the cell surface and directed to the MHC II presentation pathway, thus allowing the marker antigens to activate B lymphocyte antibody production (Walsh *et al*., 2000a). Therefore, endogenous expression of GFP may be necessary for specific anti-GFP-antibody induction. It may not be feasible to induce specific antibodies to the marker by the simple exogenous marker vaccination approach employed in the current study. It may be necessary for recombinant GFP to be conjugated to a carrier (i.e. as it may constitute a TD antigen) in order to induce a good detectable antibody response, which could then be applied to an inactivated KHV vaccine. Weiss and Avtalion (1977) found it possible to induce an upregulation of anti-hapten antibody responses at low temperatures, as long as fish were pre-injected with modified carrier proteins at optimal temperatures to enable helper cell maturation. Cell co-operation

and antibody synthesis could then occur at low temperatures, which would be particularly beneficial for specific detection of antibodies to the marker antigen, as the effect of temperature has been found to influence antibody production in carp (Rijkers *et al*., 1980; Bly and Clem, 1992). Although natural antibody responses have previously been shown to bind strongly to some carrier proteins such as KLH (Kachamakova *et al*., 2006), which would have adverse effects on marker vaccine diagnostic test sensitivity.

Although there has been a lack of success with the application of heat inactivated KHV vaccines (Ilouze *et al*. 2011) the formalin-inactivated KHV vaccine in the current study has proved highly antigenic with strong anti-KHV titres produced and previous field trials have reported high levels of protection (Pardoe, Henderson Morley PLc., pers. comm., 2009; Clarke, 2009). Other studies have also demonstrated protective potential of formalin inactivated KHV administered orally (Yasumoto *et al*., 2006). Even following stringent blocking techniques similar to Kim *et al*. (2007a) with modifications to published assays from Adkison *et al*. (2005) and St-Hilaire *et al*. (2009), a sera dilution of 1/200 still resulted in false positive results in negative carp and only at a dilution of 1/800 was this eliminated.

The plate was coated with mostly fully enveloped KHV virions, which appeared to be similar to ultracentrifuge gradient purified KHV shown in previous studies (Hedrick *et al*., 2000; Michel *et al*., 2010b) as observed after negative staining and TEM analysis of the purified KHV sample. Although unenveloped capsid particles were still present, the virus coating antigen was otherwise relatively pure and the specific antibodies detected are likely to be directed to envelope glycoproteins. Depending on the carp strain, natural antibody levels can be highly abundant from a dilution of 1/80 (Kachamakova *et al*., 2006), which probably compromised the ELISA sensitivity in the current study. Although carp appear to produce strong, specific antibodies following formalin inactivated KHV vaccination

administered ip, improved sensitivity of the accompanying diagnostic test is required in order for marker vaccination to be feasible.

3.4.3 Concluding remarks

Suitably immunogenic foreign antigens reported for mammals may not be applicable for fish, as identified with TT, and the life cycle of the fish species should be considered during marker vaccine development. Due to the expense in adding antigens such as TT, alternative more cost-effective foreign antigens should be applied as exogenous vaccine markers, which can be detected reliably, i.e. throughout the production cycle, but this can be complicated for anadromous species like Atlantic salmon. Timing of vaccination with regards to temperature may be vital for enabling detection of antibody responses to marker antigens, but accompanying diagnostic test sensitivity must be optimal by using low serum dilutions, although high concentrations of serum, e.g. a 1/20 dilution for Atlantic salmon and 1/200 for carp, can result in false positive antibody detection possibly due to natural antibodies. In contrast to Atlantic salmon antibody responses to ISA vaccination, strong antibody responses were obtained to KHV vaccination and quantified using a fully optimised and working ELISA. Previous immunisation and challenge studies have highlighted the importance of antibody responses of carp to KHV, which, although only measured after 6 wpv in the current study, can reach high titres for long periods (Ronen *et al*., 2003; Adkison *et al*., 2005; St-Hilaire *et al*., 2009). Koi herpesvirus disease therefore represents a suitable model for further investigations on applying DIVA vaccination for fish species, i.e. carp, for which such a strategy may not only become highly beneficial, but necessary if safe and effective vaccination programmes can be introduced to control this highly fatal disease and prevent further spread.

On applying a suitable foreign antigen, e.g. perhaps utilising synthetic biomarkers as described by Root-Bernstein (2005), applications of exogenous marker vaccination may still prove useful for DIVA strategies. Vaccines carrying genetic deletions, or 'negative endogenous' markers, allow uninfected and immunised animals to be distinguished from infected animals based on sero-negative responses to the epitope absent in the vaccine, but the vaccine status of the host is subsequently masked. The inclusion of a foreign antigen administered with 'negative marked' vaccines enables detection of antibodies to alternate epitopes, i.e. a vaccinated and uninfected animal. Furthermore, live attenuated vaccines commercialised for protection against KHVD (Cavoy®, Novartis; KV3, KoVax) could benefit from the inclusion of exogenous markers in the vaccine as only naïve carp are subsequently at high risk of disease. These high risk fish could be screened and identified prior to transportation (i.e. sera from these fish will lack antibodies to the marker) and cohabitation with vaccinated, but potential carrier and reservoir fish. However, serology is not, and is unlikely to be, an accepted isolated method for diagnostics, especially with regards to notifiable diseases (OIE, 2012). Therefore accompanying molecular testing is paramount for successful disease control strategies.

Chapter 4

Early stage pathogenesis of KHV: Implications for diagnostic detection methods

4.1 Introduction

4.1.1 Acute aquatic viral pathogenesis and problems associated with detection methods

Detection of anti-KHV antibodies is often not feasible until later stages of KHV infection (Ronen *et al*., 2003; Perelberg *et al*., 2008; St-Hilaire *et al*., 2009; Matras *et al*., 2012), which may compromise the application of DIVA vaccination strategies as they require detection of anti-KHV antibodies. Additionally, anti-viral antibodies only indicate exposure to the virus, but do not confirm infection. Therefore accompanying methods for direct detection of the virus are required for its control, especially during the earliest stages of infection, so that appropriate measures can put in place.

Various methods have been used for detecting aquatic viruses within the host following infection, which have provided information not only on viral pathogenesis, but importantly, have also revealed the difficulties associated with the application of particular diagnostic methods with respect to specific stages of infection (Sano *et al*., 1991; 1992; 1994; Lopez-Jimena *et al*., 2011; 2012).

The various *in situ* detection methods have their advantages and disadvantages (Adams *et al*., 2008). Compared to immunohistochemistry (IHC) and immunofluorescent antibody tests (IFAT), the use of *In situ* hybridisation (ISH) is less prone to false negative results that can occur in the former two methods due cross-linking of antigens, and subsequent masking of epitopes, in tissues following formalin fixation. However, viral DNA detection by ISH does not indicate whether or not viral replication is taking place, whereas IHC and IFAT can indicate the presence of viral structural proteins (e.g. γ; late proteins), and hence replication. Unlike IHC and ISH, IFAT does not suffer from problems with non-

specific staining due to the presence of endogenous peroxidases, but pathological lesions and host responses cannot be determined by IFAT as the tissue is not counterstained (as in IHC and ISH). IFAT is often the most ideal approach for virus detection compared to IHC because of its superior sensitivity (Adams and Thompson, 2006; Adams *et al*., 2008). In terms of histopathology, IHC is preferred to ISH as the tissue integrity is usually well preserved in IHC (Adams and Thompson, 2008). Proteinase K digestion is required during ISH to enable hybridisation of probes to DNA and this often leads to structural denaturation.

There are many reports relating to detection and analysis of KHV for both diagnostics and research, where the authors utilised polyclonal rabbit anti-sera for antigen detection (Hedrick *et al*., 2000; Pikarsky *et al*., 2004; Rosenkranz *et al*., 2008; Kempter *et al*., 2009; Bergmann *et al*., 2010c), and only very few have used MAbs (Kempter *et al*., 2009; Bergmann *et al*., 2010c; Aoki *et al*., 2011) or ISH for diagnostics (Bergmann *et al*., 2006; 2007; 2009b; 2010c; Kempter *et al*., 2009; Lee *et al*., 2012). No studies have been undertaken with these methods do determine their sensitivity for KHV. Application of such methods could also provide vital information with regards to pathogenesis, i.e. the expression of known antigens in infected carp tissues, the portal of entry and target tissues for viral replication, especially with respect to asymptomatic carriers.

DNA probes have been previously used to detect other aquatic herpesviruses, such as Channel catfish virus (CCV) in asymptomatic fish using Southern blotting (Wise *et al*., 1985; Gou *et al*., 1991). Detection of viral nucleic acid, e.g. by expression ISH, does not always correlate with positive detection of viral protein by IHC, even within sections of the same tissue samples, as noted in turbot injected with a DNA vaccine against Nodavirus (Sommerset *et al*., 2005b). This can be associated with both assay sensitivity and time lag between transcription (detected by the ISH probe) and translation (detected by the IHC MAb) (Sommerset, *et al*., 2005b).

ISH has proved very sensitive for detecting viral DNA of eel herpesvirus (Herpesvirus anguillidae; HVA) at early stages (3 hpi) in infected cell cultures (Shih *et al*., 2003). Conserved genomic regions have been targeted in ISH for the detection of fish viruses (Alonso *et al*., 2004; Huang *et al*., 2004) such as the major capsid protein gene of Iridovirus (Huang *et al*., 2004). Alonso *et al*. (2004) detected the virus in cell culture after 8 hpi with ISH compared to 24 hpi with IHC. However, specificity is key for KHV bearing in mind that it is closely related to carp herpesviruses, CyHV-1 (carp pox virus) and CyHV-2 (Goldfish herpesviral haematopoietic necrosis virus) (Waltzek *et al*., 2005).

Using IFAT on experimentally infected carp enabled the progressive systemic infection of another aquatic herpesvirus, carp pox, to be investigated in detail where antigens were detected in the gills and gastrointestinal tract after only 2-3 dpi then later in the skin (Sano *et al*., 1991). However, due to various sensitivities of detection methods, there has been much debate regarding the pathogenesis and portals of entry in fish. For example with IHNV the use of bioluminescence, IHC, TEM and virus infectivity titration resulted in conflicting results (Yamamoto and Clermont, 1990; Yamamoto *et al*., 1990; Harmache *et al*., 2006). In a study on the pathogenesis of Red Spotted Grouper Nervous Necrosis Virus (RGNNV) in European sea bass, ISH was unable to detect viral genome in some tissues that were positive by RT-qPCR during a time course of experimental infection (Lopez-Jimena *et al*., 2011). Furthermore, IHC detected antigens in tissues where ISH was negative for genomic viral RNA in the same challenge (Lopez-Jimena *et al*., 2012). This, like other studies indicates the necessity to determine the most ideal target antigens of antibody-based methods as well as the target genes or sequences for ISH methods for in situ diagnostics.

Latency is a silent persistence of virus in the host (Pastoret *et al*., 1982), which results in less viral antigen within host tissues for detection by antibody based methods such as IHC and IFAT (Thiry *et al*., 1986). However, nucleic acid based detection methods such as ISH

have previously been successfully used to define mechanisms of herpesviruses during latent infections in mammals and fish (Teo and Griffin, 1990; Sano *et al*., 1994; Cardoso *et al*., 2012). Whereas viral antigens could not be detected during the period between acute infection and tumor formation in carp experimentally infected with carp pox, DNA could be detected by ISH (Sano *et al*., 1991; 1992; 1994).

It would useful to investigate the application of in situ diagnostic methods for detecting KHV infected carp during the early stages of infection, but it is vital to compare these methods with the most commonly, and reliably, used molecular detection methods from extracted DNA.

4.1.2 Importance and limitations of molecular detection of KHV

Like other herpesviruses, despite inhibition of viral replication at non-permissive temperatures, i.e. above 30°C, KHV retains infectivity and a persistent infection ensues (Dishon *et al*. 2007; Ilouze *et al*., 2012a). Latency as defined as 'the delivery of viral genome to the nucleus without the initiation of a productive infection' (Penkert and Kalejta, 2011) where the genome is maintained as a non-integrated episome and expression occurs in only a limited number of viral genes and microRNAs (Michel *et al*., 2010a). This occurs in specific cell types during other herpesvirus infections, but has not yet been proven for KHV, however many studies have demonstrated latency-like characteristics of the virus (Dishon *et al*., 2007; St-Hilaire *et al*., 2009; Eide *et al*., 2011a; Ilouze *et al*., 2012a). It is possible that latency may be a contributing factor to the problems encountered with detection of KHV-infected fish surviving an outbreak as low viral copy numbers are difficult to detect by PCR. This has important implications with regards to controlling and eradicating the disease, as the host appears healthy, but may subsequently transmit the virus to naïve carp, particularly during periods of stress and temperature fluctuation; indeed both seasonal changes and

transportation stress have been shown to reactivate KHV from persistent, potentially latent, infections (Bergmann and Kempter 2011; Eide *et al*., 2011a).

Koi herpesvirus disease is listed as notifiable by the OIE and EU, thus specific and sensitive detection of KHV in infected fish has been a principal area of research to assist in its control. The development of conventional polymerase chain reaction (PCR) vastly improved detection of the virus and viral DNA, especially from necrotic or frozen tissues of fish (Gilad *et al*. 2004), and in cases where isolation of KHV on cell lines proved impossible. Molecular-based techniques are now the most effective method for detecting the virus and a number of assays have been published to date, but it is important that the most reliable diagnostic methods are employed. Highly specific and sensitive conventional PCR assays (Gilad *et al*., 2002; Gray *et al*., 2002; Bercovier *et al*., 2005; Yuasa *et al.,* 2005), nested PCRs (Bergmann *et al.*, 2006; El-Matbouli *et al.*, 2007) semi-nested PCR (Bergmann *et al.*, 2010a), real-time PCR (Gilad *et al.*, 2004) and loop mediated isothermal amplification assays (Gunimaladevi *et al.*, 2004; Soliman and El-Matbouli 2005; 2010) have been developed for the detection of viral DNA in fish tissues with sensitivity limits as low as 1-5 genomic copies (Bergmann *et al*., 2010a) obtained by some of these (Gilad *et al*., 2004; Bergmann *et al*., 2006; 2010a).

While generally accepted that acutely infected fish undergoing clinical disease can be successfully diagnosed with KHV using the majority of the molecular methods developed, which normally requires lethal sampling procedures for screening DNA from gill and kidney biopsies, as these support the production of high virus loads (Gilad *et al*., 2004; Pikarsky *et al*, 2004; Eide *et al*., 2011b) false negatives are often reported and only rarely are 100 % of fish sampled from experimental challenges positive for KHV (Gilad *et al.*, 2003; Bergmann *et al.*, 2010a; b), which reflects the difficulties in diagnosing fish from populations that have experienced disease outbreaks. Although such results are often likely to be associated with

low viral copy numbers during a latent or persistent infection, they may also be attributed to an acute phase of infection, i.e. initial viraemia, where fewer genomic copies of virus DNA are present for amplification by PCR. The onset of KHVD can be rapid, with sub-clinically infected fish suddenly developing the disease and dying one or two days later (Bretzinger *et al.*, 1999; Antychowicz *et al*., 2005). Diagnosing sub-clinical KHV presence is therefore important, however, the efficiency of the various PCR methods cited for KHV detection has not yet been determined with regards to detecting KHV immediately after exposure. As clinical signs of KHVD are never evident in these fish, reliable diagnostics for early detection of the virus is vital to prevent false negative results. An inactivated KHV vaccine was found to induce high specific anti-KHV antibody titres in Chapter 3. However, as serological diagnostics will not be accepted in isolation (OIE, 2012), and antibodies are not detectable till later stages of infection, it is essential to accompany serology with the most reliable molecular diagnostic tests.

4.1.3 Aims

The goal of this study was to determine the most sensitive diagnostic method for detection of KHV during the early stages of infection. During the study the acute stages of KHV pathogenesis were investigated *in vivo,* and how this may influence the sensitivity of various detection methods targeting viral nucleic acid (e.g. ISH and PCR), or different viral antigens (e.g. using KHV-specific MAbs in IHC and IFAT) and virus particles (TEM). Serology was also used to try to measure early antibody responses to the virus. Samples were collected, either lethally (i.e. skin, gills, spleen, kidney, gut, liver and brain) or non-lethally (mucus and leukocytes) over a 10 day period from fish experimentally infected with the virus by immersion. The sensitivities of seven PCR-based methods, including single round PCR (Gilad *et al.*, 2002; Bercovier *et al.*, 2005; Bergmann *et al.*, 2010b), nested PCR (Bergmann *et al.*, 2006; Centre for Environment, Fisheries and Aquaculture Science (CEFAS) 2007

unpublished); semi-nested PCR (Bergmann *et al.*, 2010a) and real-time qPCR (Gilad *et al.*, 2004) were compared using these samples.

4.2 Materials and Methods

4.2.1 Experimental infection with KHV

4.2.1.1 Virus propagation by cell culture

KHV isolate KHV-E D 182 (kindly provided by Dr. Keith Way, CEFAS, Weymouth, UK) obtained from clinical KHVD, was propagated in CCB cells similar to that described in Section 2.2.2.2 and Section 2.3.2, with modifications. Fresh sub-cultured CCB cells were infected with Koi herpesvirus for 1 h at 20°C, then 10 mL Hanks buffered salt solution (HBSS) containing 10 % foetal calf serum (FCS) without antibiotics was added. After virus absorption, fresh Earle's minimum essential medium (EMEM) containing 5% FCS was resupplemented to the cells, which were incubated for 7-10 days at 26°C. Once a 95-100 % CPE had been obtained, cells and virus were harvested by a freeze-thaw cycle at -80°C, followed by centrifugation at 1000 x *g* for 20 min at 4°C (Hettich Zentrifugen, Tuttlingen, Germany) in order to remove the cell debris. The supernatant was used as the viral inoculum for the challenge.

4.2.1.2 Fish, experimental design and sampling

One year old specific pathogen-free carp (*n*=60) weighing approximately 130 – 150 g, obtained from a commercial farm in Thuringia, Germany, were used in the study. The fish were held in a quarantine facility in 1 m^3 re-circulating tanks at FLI at a water temperature of 20°C and flow rate of 300 L h^{-1} for 6 days prior to the trial to allow them to acclimate to these conditions. They were fed daily with a commercial carp diet (Ssniff, Germany). These fish tested negative for antibodies to KHV and carp pox (CyHV-1) using a serum neutralisation

test (SNT) and ELISA (Bergmann pers. comm.), and negative for virus nucleic acid using a range of different PCRs (Gilad *et al*., 2004; Bergmann *et al*., 2006). Prior to performing the infection, two carp from a group of 40 fish were sampled after lightly anaesthetising them with benzocaine (2 mL [2% v/v in ethanol] in 10 L). Mucus swabs were taken from skin, fin base and gill (Fig. 4.1) with a sterile cotton wool bud, which was placed directly into 200 µL ATL lysis buffer (Qiagen, Germany) containing 20 µL proteinase K supplied in the kit (Qiagen, Germany) and 2 µL Internal control (IC2, Bergmann *et al*., 2010a). Fish were then bled with sterile heparinised and non-heparinised 2 mL syringes for leukocyte separation (Bergmann and Kempter, 2011) and serum collection, respectively. The same carp were then killed by overdosing them in benzocaine (8 mL [2% v/v in ethanol] in 5 L). Tissue (skin, gills, spleen, kidney, gut, liver and brain) were sampled using new gloves and dissection tools between biopsies to prevent cross-over of KHV DNA between samples. The tissues were frozen and stored at -70°C for molecular analysis. The samples for histological and antibody-based analysis, i.e. IHC and IFAT, were placed in Davidson's solution (v/v: 35% water, 35% ethanol, 9% formaldehyde, 12% glycerol, 9% glacial acetic acid) and samples for TEM analysis were placed in Karnovsky's fixative (Science Services GmbH, Munich, Germany). Leukocytes (their isolation is described later in Section 4.2.1.3) were also fixed for TEM in 1 mL of a 500 mL Karnovsky's fixative stock (Science Services GmbH). Carp (*n*=38) were challenged with KHV (at a viral dose of 10^3 $TCID_{50}$ mL^{-1}) by immersion for 1 h in 30 L of water at 20°C, and then transferred randomly (*n*=19) to two separate 400 L tanks with a flow rate of 100 L h^{-1} at 20°C. Twenty additional carp, from the same acclimatised population, were immersed in non-infected culture medium instead of virus, as uninfected controls. The two experimental challenge tanks were each on a recirculation system with bio-filtration under identical conditions (Fig. 4.2).

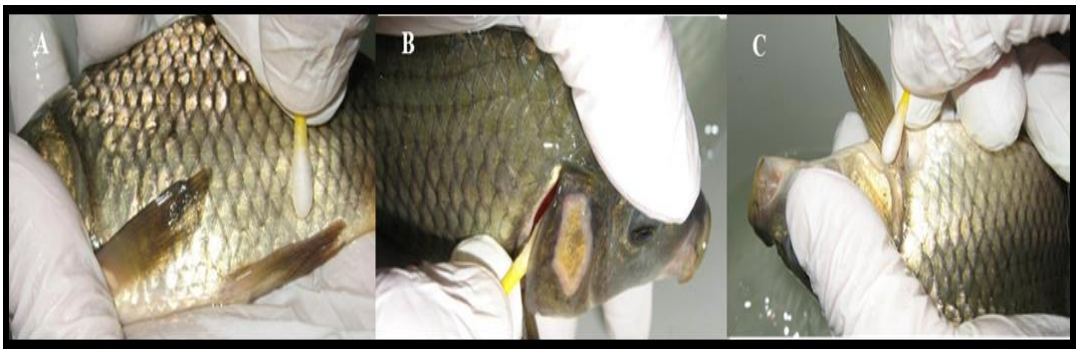


Figure 4.1. Non-lethal sampling of carp mucus for the detection of koi herpesvirus DNA. Mucus swabs were taken using sterile cotton wool buds, which were placed into lysis buffer (A) Skin swab, (B) Gill swab, (C) Fin base swab.

Figure 4.2. Experimental set up for koi herpesvirus (KHV) challenge. Fish were challenged with KHV by immersion in the same challenge tank (CT), then placed in separate tanks B15 and B16, attached to different recirculation systems, supplied with de-chlorinated water and a daily 50 % water change.

Two infected carp (one from each tank, B15 and B16) were sampled both lethally and non-lethally as described above at time points 1, 2, 4, 6 and 8 hours post-infection (hpi), then one fish sampled daily 1 – 10 dpi. A single control carp was also sampled daily. Due to the differential onset of disease between the two tanks (i.e. Tank B15 exhibited per-acute KHVD with all fish dying by 4 dpi and was designated the peracute disease tank, while a slower onset of mortalities were observed in B16 and was designated the acute disease tank), fish

could only be sampled from Tank 16 from 4 dpi. The condition of fish was monitored daily and morbidity and mortalities recorded. Maintenance of infected and control carp was identical throughout the experiment, which included a daily 50% water change, and monitoring ammonia, nitrite, pH and dissolved O_2 (dO_2) levels (Tetra Pond water test set, Tetra, Germany). Any mortalities were removed immediately, and the gills and kidney of these fish sampled individually as described above (tissues pooled) for viral DNA detection by PCR (Gilad *et al.*, 2002; Bergmann *et al.*, 2006). Each fish sample was analysed independently using a range of single round, nested and semi-nested PCR methods as well as real-time qPCR and all fish tissues were analysed by ISH. Blood serum samples were analysed by ELISA and SNT, and further analysis of fish harbouring a high concentration of viral DNA was undertaken on tissues and leukocytes using IHC, IFAT and TEM.

4.2.1.3 Leukocyte separation

Heparinised blood, sampled in Section 4.2.1.2., was diluted 1:5 with EMEM. Leukocytes were separated from this through a 1.075 % Percoll gradient by centrifuging at 800 x *g* for 40 min at 4°C. The buffy coat of leukocytes shown in Fig. 4.3 was collected and washed with phosphate buffered saline (PBS, 0.02M phosphate, 0.15M NaCl, pH 7.2) by centrifuging at 800 x *g* for 10 min. The cell concentration of the washed leukocytes was adjusted to at least 10^7 cells mL^{-1} (Bergmann *et al*., 2010c), which was placed directly into 200 µL lysis buffer containing 20 µL proteinase K and 2 µL IC2 for DNA extraction.

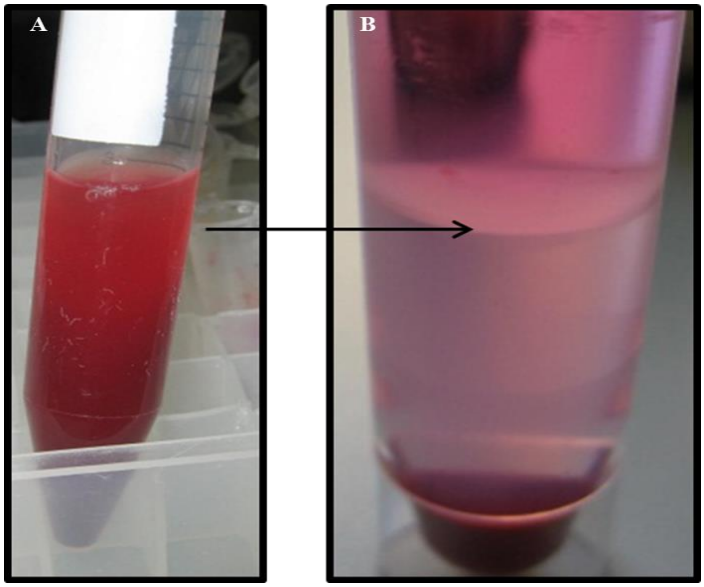


Figure 4.3. Isolation of carp leukocytes on a 1.075 % Percoll gradient. (A) Carp blood diluted 1:5 with EMEM media; (B) buffy coat of leukocytes (arrow) collected after centrifugation .

4.2.2 Molecular analyses

4.2.2.1 DNA extraction

DNA was extracted directly from the mucus samples placed into lysis buffer as described above (Section 4.2.1.2). For tissues, organs were dissected and 25-30 mg of tissue was placed in eppendorf tubes containing a metal bead and 80 µL PBS before mechanically lysing using a tissue lyser (Qiagen, Germany) for 2 min at 30 shakes sec^{-1}. Two hundred microliters of lysis buffer, 20 µL proteinase K and 2 µL IC2 were then added to the lysed tissue. This was heated at 56°C on a thermo shaker at 900 rotations min^{-1} (rpm) for 1 h. The lysed samples were then heated for 10 min at 70°C on the thermo shaker to deactivate the proteinase K. DNA extractions were then carried out using a QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions and excess tissues and leukocytes stored at -70°C for repeated DNA extractions and PCR testing.

4.2.2.2 Controls and plasmids

Positive internal controls for KHV DNA in qPCR were prepared as previously described by Bergmann *et al*. (2010a) using a plasmid preparation kit (vector pGEM®-T Easy: Promega, Mannheim, Germany) with a KHV fragment of 484 bp from open reading frames (ORFs) 89-90, accession no. AF411803 (Gilad *et al*., 2002) for which the plasmid concentrations and fragment copy numbers had been previously determined by Bergmann *et al*. (2010a). A standard curve from 1 – 10^6 gene copies was prepared and used for quantification of viral load from each sample. The internal control system (IC2) was used according to Hoffman *et al*. (2006) and a duplex real-time PCR was used, modified from Gilad *et al*. (2004) by Bergmann *et al*. (2010a).

4.2.2.3 PCR, nested PCR and semi-nested PCR

The PCR methods used in the study are highlighted in Table 4.1, together with relevant primers sequences, product sizes, cycle conditions and estimated sensitivity limit of the reactions according to a previous study (Bergmann *et al.*, 2010a). All PCRs were undertaken in duplicate for each extracted DNA sample. A random selection of samples was reanalysed to examine the reproducibility of the PCR results. A Go Taq Flexi DNA Polymerase Kit (Promega, Mannheim, Germany) was used in each assay except in the qPCR. Products were visualised on a 1.5 % agarose gel (in TAE buffer, 40mM Tris-acetate, 1mM EDTA, pH 8) containing ethidium bromide (0.5 μg ml^{-1}) under UV light after electrophoresis at 60 V for 60 min. All PCR reactions included no-template negative controls (DNAse-free water) and a second no-template control was included in the second round reactions of the nested PCR. DNA extractions, PCR reaction preparations and gel electrophoresis were undertaken in different rooms to prevent contamination. Positive controls (KHV, HP783, Israeli attenuated vaccine DNA) were always prepared and added in a different room to prevent cross-contamination of KHV DNA.

Conventional PCR methods were undertaken with the following Master Mix: 100 µM each of dNTPs, 2.5 mM $MgCl_2$, 0.5 µM each of forward and reverse primers, 0.6 Units Go *Taq*-polymerase enzyme, 1 x colourless Go Taq Flexi Buffer and 5 µl of extracted, purified DNA added as template. The reaction mixture used for the second round of the nested PCR was the same as the first round, using 2 µl of the first round reaction as DNA template. The reaction mixtures for both rounds were made up to 25 µl with molecular grade water. The one tube semi-nested glycoprotein gene PCR, according to Bergmann *et al.*, (2010a), was carried out using one forward primer and three reverse primers with annealing temperatures of 68°C for 5 cycles, then 65°C and 60°C for 20 cycles each. The master mix for this PCR consisted of 100 µM dNTPs, 5 mM $MgCl_2$, 1 µM forward primer Sn 1F improved, 0.2 µM reverse primer Sn 1R improved, 0.3 µM reverse primer Sn 2R improved, 0.6 µM reverse primer Sn 3R improved, 0.6 units Go *Taq*-polymerase enzyme, 1 x colourless GoTaq Flexi buffer, 5 µl template DNA and the final volume made up to 25 µl with molecular grade water. The reaction was performed in a thermo cycler (Master Gradient, Hamburg, Eppendorf, Germany) using the temperature regime shown in Table 4.1, with a final cycle of 5 min at 72°C to complete the reaction.

4.2.2.4 Real-time quantitative PCR

Real-time qPCR was carried out according to Gilad *et al*. (2004) with modifications made by Bergmann *et al*. (2010a) to amplify a fragment of the KHV genome (Accession No. AF411803). An internal control mix consisted of Enhanced green fluorescent protein (EGFP) primers 1F and 2R at 10 pmol μL^{-1} each and 1.5 pmol μL^{-1} EGFP-HEX (Hex phosphoramidite) IC2 probe (accession No. U55761). The KHV mix consisted of KHV primers 86F and 163R at 10 pmol μL^{-1} each and KHV probe KHV 109P at 1.25 pmol μL^{-1}. The 2 x reaction mix from Quanti Tect, multiplex PCR, No ROX kit (Qiagen, Germany) was used according to the manufacturer's instructions. Briefly, master mix was prepared with the

components described above with 2 µL IC mix (3 pmol EGFP-HEX IC2 probe), 2 µL KHV mix (20 pmol EGFP primers 1F and 2R), 12.5 µL 2 x reaction mix and 3.5 µL DNAse-free water plus 5 µL of template DNA or 'no template' control for each reaction. Thermal cycle conditions, shown in Table 4.1, were run in a MX3000P qPCR machine (Stratagene). Significant differences of medians of KHV viral load between per-acute, early acute and acute stages of infection and between different tissue, mucus and leukocyte samples were assessed by Kruskal-Wallis test and pair wise comparisons were assessed by Mann-Whitney U test using Minitab 16 statistical software.

4.2.2.5 In situ hybridisation (ISH)

(a) Preparation of KHV DNA for developing probes

The CCB cells were cultured and infected with KHV as described previously (Section 4.2.1.1) and DNA was extracted by the DNAzol method (Invitrogen, Kalsruhe, Germany) (Bergmann *et al*., 2010a). KHV DNA was amplified by PCR to prepare two different sized probes, one of 414 bp amplified with primers according to Bergmann *et al*. (2006) [for this a single round PCR was performed using the Gilad nested primers] and the other of 517 bp amplified with primers according to Hutoran *et al*. (2005) [using the cycling conditions of Gilad *et al*. (2002) without a nested step] (Table 4.2). These primer sets have been shown to not react with closely related heterologous viruses, including Channel catfish herpesvirus (CCV), Carp Pox virus (CyHV-1), Goldfish hematopoietic necrosis herpesvirus (CyHV-2) and herpesvirus anguillae (HVA) (Kempter *et al*., 2009). A dig-labelled Viral haemorrhagic septicaemia virus (VHSV) probe was used as an alternative virus negative control.

(b) Labelling of KHV DNA with Digoxigenine (DIG)

Koi herpesvirus DNA was labelled with Digoxigenin-11-2'-deoxy-uridine-5'- triphosphate (DIG-dUTP, 30%) using a DIG Probe Synthesis Kit (Roche) according to the manufacturer's

instructions, in which 2'-deoxythymidine 5'-triphosphate (dTTP, 70 %) was substituted with DIG-dUTP by PCR. The PCR used was similar to the single round PCR performed by Gilad *et al*. (2002) (Section 4.2.2.4; Table 4.1) except the annealing temperature was reduced to 60°C because of the large size of the probe. The buffers, enzyme and dNTPs supplied in the DIG Probe Synthesis Kit were used for the DIG-labelling PCR. The DIG Probe Synthesis Mix consisted of 20 µM each of dCTP, dGTP and dATP, but with 13 µM dTTP and 7 µM DIG-11-dUTP, which results in the substitution of DIG-labelled dUTP (DIG-11-dUTP) for dTTP in a ratio of 30:70, respectively.

A total of four reactions were undertaken during validation of probe labelling: PCR with and without DIG-dUTP for both the Gilad nested primers (Bergmann *et al*., 2006) and the Hutoran *et al*. (2005) primers. Gel electrophoresis was used, as described in Section 4.2.2.3, to show that the probes had been successfully incorporated into the amplified oligonucleotides.

Table 4.1. PCR conditions for the amplification of viral DNA and estimated sensitivity threshold

PCR	Primer sequence 5'-3'	Product size (bp)	Denaturation	Annealing	Extension	Copy no.*
Gilad 1 round (Gilad *et al.*, 2002)	F = KHVF: GACGACGCCGGAGACCTTGTG R = KHVR: CACAAGTTCAGTCTGTTCCTCAAC	484	**95°C – 5 min (x1)** 94°C – 1 min (x39)	68°C – 1 min (x39)	72°C – 30 s (x39) **72°C – 7 min (x1)**	10^{4-5}
Gilad nested (Bergmann *et al.*, 2006)	F = KHV-1Fn: CTCGCCGAGCAGAGGAAGGC R = KHV-1 Rn: TCATGCTCTCCGAGGCCAGCGG	414	**95°C – 5 min (x1)** 94°C – 1 min (x25)	68°C – 1 min (x25)	72°C – 30 s (x25) **72°C – 7 min (x1)**	1-5
TK one round (Bercovier *et al.*, 2005)	F = TKF: GGGTTACCTGTACGAG R = TKR: CACCCAGTAGATTATGC	409	**95°C – 5 min (x1)** 95°C – 30 s (x35)	55°C – 30 s (x35)	72°C – 1 min (x35) **72°C – 10 min (x1)**	10^{1-2}
TK nested (CEFAS, 2007 Unpublished)	F = TKFn: CGTCTGGAGGAATACGACG R = TKRn: ACCGTACAGCTCGTACTGG	348	**95°C – 5 min (x1)** 95°C – 30 s (x25)	52°C – 30 s (x25)	72°C – 1 min (x25) **72°C – 10 min (x1)**	10^{1-2}
Glycoprotein one round (KHV-U, ORF 56) (Bergmann *et al.*, 2010b)	F = SBM-gp-2F: ACGTCGGCGTGCGCCAC R = SBM-gp-2R: GGACGTGGTCTGCCACTAC	661	**95°C – 5 min (x1)** 95°C – 30 s (x35)	60°C – 30 s (x35)	72°C – 1 min (x35) **72°C – 10 min (x1)**	10^{2-3}
Semi-nested glycoprotein (ORF 56) (Bergmann *et al*. 2010a)	F = Sn 1F improved: GGTACTTGTTGGCGTACATGGC R1 = Sn 1 R: CGGTTGTCAGCAGCACCTCAA R2 = Sn 2- R Improved: GCGAGGAGCACATCGCGC R3 = Sn 3-R Improved: CGTGGTGGCCGTCGC	464 372 182	**93°C – 5 min (x1)** 93°C – 1 min (x5)	68°C – 1min (x5) 65°C – 1 min (x20) 60°C – 1 min (x20)	72°C – 1 min (x5) 72°C – 1 min (x20) 72°C – 1 min (x20) **72°C – 1 min (x5)**	1-5

TaqMan real-time (Gilad *et al*., 2004) with modifications acc. Bergmann *et al*. (2010a)	F = KHV-86 F: GACGCCGGAGACCTTGTG R = KHV-163 R: CGGGTTCTTATTTTTGTCCTTGTT KHV probe = KHV-109P (FAM) CTTCCTCTGCTCGGCGAGCACG-(BHQ1) IC2 probe = EGFP1-HEX (HEX) AGCACCCAGTCCGCCCTGAGCA-(BHQ1) IC2 F = EGFP1-F GACCACTACCAGCAGAACAC ‑ IC2 R =EGFP2-R GAACTCCAGCAGGACCATG	78	**95°C – 15 min (x1)** 95°C – 1 min (x42)	60°C – 30 s (x42)	72°C – 30 s (x42)	1-5

***Copy no. threshold necessary for KHV detection (Bergmann *et al*., 2010a), determined from known plasmid concentrations and fragment copy numbers of a KHV insert (ORF 89-90, Gilad *et al*., 2002).**

Cycling conditions in bold type indicate initial denaturation conditions and final extension conditions for the respective PCR. F = Forwards primer; R= reverse primer; sequences of probes and internal control (IC2) are shown below primers for real-time PCR

Table 4.2. Primer sets used to develop ISH probes

In situ hybridisation protocols (references)	Primer sequence 5'-3'	Product size (bp)
Bergmann *et al*. (2006) from Gilad single round PCR sequence (Gilad *et al*. (2002)	KHV-1Fn: CTCGCCGAGCAGAGGAAGCGC KHV-1Rn: TCATGCTCTCCGAGGCCAGCGG	414
Hutoran *et al*. (2005)	NH-1: GGATCCAGACGGTGACGGTCACCC NH-2: GCCCAGAGTCACTTCCAGCTTCG	517

(c) ISH on fixed tissue sections

Tissues fixed in Davidson's were dehydrated, impregnated and embedded with paraffin wax using an automated Tissue-TEK® VIP system (Miles Scientific) according to standard protocols. The wax-embedded tissues were sectioned (5 µm) using a Jung RM 2055 power microtome (Leica) and placed on Superfrost ® microscope slides (Microm International) for 18 h at 62°C. The tissues were dewaxed by 2 x 10 min incubations in Rotihistol (Roth), followed by 2 x 10 min incubations in 100 % ethanol before air-drying. Sections were framed with a wax Pap Pen (Merck, Darmstadt, Germany) and tissues treated with 100 µg mL^{-1} proteinase K (Appligen, Ilkirch, France) in TE buffer (50mM Tris, 10 mM EDTA, 10mM NaCl, pH 7.4) for 20 min at 37°C to permeabilise cell membranes. The sections were then further fixed by incubating for 1 min in 95 % ethanol followed by 1 min in 100 % ethanol. After air-drying, the sections were again framed with the Pap Pen and equilibrated with pre-hybridisation buffer by covering sections with approximately 200 µL hybridisation mixture [ISH-M: 4 x standard saline citrate (SSC, 0.6M NaCl, 0.06M Na-citrate, pH 7), 50% formamide, 1 x Denhardt's reagent, 250 µg yeast tRNA mL^{-1} and 10 % dextran sulphate] and incubated for 1 h at 42°C in a humid chamber. DIG-labelled probes (5 µL in 200 µL ISH-M) were added to the sections, which were then covered with a cover slip. The slides were placed on the *in situ* plate of a thermal cycler (Eppendorf Mastergradient) and heated to 95°C for 5 min to denature the DNA within the tissues. The slides were then cooled immediately on ice for 2 min before incubating overnight at 42°C in a humid chamber to allow the probes to hybridize to complimentary DNA. A solution of 0.4 x SSC was also incubated at 42°C overnight and the following day, the cover slips were removed by washing sections in 2 x SSC twice for 10 min at RT. To remove non-specifically bound probes, slides were incubated in pre-warmed 0.4 x SSC at 42°C for 10 min. The sections were subsequently submerged in a

bath of DIG 1 buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 1-2 min, then blocked with approximately 200 µL (or sufficient to cover the tissue) DIG 2 buffer (10 % Roti-block in DIG 1 buffer) for 30 min at RT. The slides were washed again for 1-2 min in DIG 1 buffer, and then covered with approximately 200 µL anti-DIG alkaline phosphatase-conjugated MAb diluted 1: 500 in DIG 2 buffer and incubated for 1 h at RT, covered with foil to prevent evaporation. The sections were washed 2 x 1 min in DIG 1 buffer, before equilibrating the tissues for 10 min with DIG 3 buffer (0.1M Tris, 0.1M NaCl, 0.05M $MgCl_2$, pH 9.5). The sections were finally incubated with approximately 200 µL nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) freshly diluted at a ratio of 1:50 in DIG 3 buffer at RT and the reaction stopped with DIG 4 buffer (100 mM Tris-HCl, 0.001M EDTA, pH 8). The sections were counter-stained with Bismarck-Brown Y (0.5 % w/v in 30 % ethanol) (Sigma-Aldrich, Steinheim, Germany) by washing the sections in dH_2O then incubation in Bismarck Brown Y for 1-2 min at RT. The sections were immersed 2 x in 95 % ethanol for 1 min then 2 x in 100 % xylene for 1 min. The slides were then mounted with Histokitt (Roth, Germany), cover slipped and visualised using a BX51 phase contrast microscope (Olympus, Japan) and images were recorded with an Infinity X U-CMAD3 camera (Olympus, Japan) with software. Positive staining was evident as violet-black foci in infected cells.

4.2.3. Histology

Tissues were fixed in 10 % buffered formalin for a minimum of 24 h. Post-fixed tissues were processed through an ethanol series over 24 h in a tissue processor (Shandon Citadel 2000, Thermo) and embedded in paraffin wax blocks. For hard tissues (e.g. cartilagionous gills) hardened blocks were sometimes placed in decalcifier for 10 min to 1 h. Sections were then

cut using a Shandon Finesse microtome (Fisher Scientific, UK) at 5 µm and routinely stained with haematoxylin and eosin (H&E).

4.2.4 Antibody-based detection methods

4.2.4.1 Immunohistochemistry (IHC)

A panel of anti-KHV MAbs were used for IHC, of which only a few had been partially characterised. The MAbs are described in more detail in Chapter 5 and three of these that produced promising signals in preliminary experiments on KHV-infected tissues were used in the analysis. A final protocol for the IHC was established after obtaining positive signals in infected tissues, while negative tissues exhibited no signal. Five µm sections were cut using a Shandon Finesse microtome (Fisher Scientific, UK) from carp tissues fixed in Davidson's solution and wax embedded, as described for ISH (Section 4.2.2.5c). Sections from KHV disease-free (control carp) and KHV positive carp from experimentally infected fish (conducted by Dr. Sven Bergmann), were used as negative and positive controls, respectively. The sections were deparaffinised and rehydrated 2 x 5 min in xylene baths followed by 100 % ethanol for 5 min and 70 % ethanol for 3 min and finally H_2O for 3 min. The tissues were outlined using a Pap pen (ImmEdge, Vector Laboratories Ltd, UK) and approximately 200 µL endogenous peroxidase blocking solution (glucose, glucose oxidase, sodium azide) was added to tissues and incubated for 1 h at 37°C on a Hybaid Omni (UK) plate to quench endogenous peroxidase activity (Andrew and Jasani, 1987). Slides were then washed 2 x 5 min in PBS and blocked with 10 % goat serum (Sigma-Aldrich, UK) in PBS for 45 min at RT. Monoclonal antibodies (hybridoma cells and ascites) produced against KHV (kindly provided by Dr. Malte Dauber, FLI, Germany) and against recombinant proteins of KHV ORF 62 and 68 (Aoki *et al*., 2011) (kindly provided by Professor Takashi Aoki,

University of Marine Science and Technology, Tokyo, Japan and Dr. Taesung Jung, Aquatic Biotechnology Center, College of Veterinary Medicine, Gyeongsang National University, Korea) were used to detect different antigens of KHV *in situ*. All MAbs were produced from hybridoma cell lines as described in Chapter 2, Section 2.1.1. Purified MAbs were used at a final concentration of 60 µg mL^{-1}, while MAb 10D10 was used at a 1/4 and MAb 7C6 at a 1/3 dilution. A commercially available anti-KHV MAb (Aquatic Diagnostics Ltd, UK) was also used at a 1/15 dilution. Ascites fluid from mice injected with either MAb 10D10 or MAb 7C6 hybridoma cells (Mabs against ORF 62 and ORF 68, respectively) were diluted 1/300. PBS was used as a diluent for MAbs and PBS containing 10 % goat serum was used as diluent for ascites dilutions.

The tissue sections were incubated with the MAbs for 1 h at RT, followed by 2 x 3 min washes with PBS. Anti-mouse IgG MAb conjugated to biotin (Sigma-Aldrich, UK) were added to tissue sections at a dilution of 1/250 in PBS for 30 min, which was followed by another wash step of 2 x 3 min with PBS. Streptavidin conjugated with horseradish peroxidase (HRP) (Vector Laboratories Ltd, UK), diluted 1/250 in PBS, was added to sections for 30 min RT and the sections were washed again 2 x 3 min with PBS. The sections were developed with a Vector VIP kit (Vector Laboratories Ltd, UK) according to the manufacturer's instructions. After 10 min the reaction was stopped by washing the sections for 1 min in PBS and then counterstaining them with methyl green (Vector Laboratories Ltd, UK) for 5 min at 60°C on the Hybaid Omni plate. The sections were washed 2 x 3 min in distilled H_2O, dehydrated through an alcohol series of 95 % ethanol for 3 min, 100 % ethanol for 3 min followed by 2 x xylene baths for 5 min each. The sections were mounted with Pertex and left to air-dry overnight.

MAbs recognising ISAV, previously used as a positive control for ISAV ELISA in Chapter 3, were used as negative control for the primary antibody as it had the same isotype as the anti-KHV MAbs. Ascites fluid produced against Keyhole Limpet Hemocyanin (AKLH: Sigma-Aldrich, US), used as a positive control for KLH ELISA in Chapter 3, was used here as a negative control for the IHC using ascites fluid. PBS or 10 % goat serum in PBS were negative controls for the secondary antibody.

Antigen retrieval was also attempted after de-waxing the sections by heat induction (heat induced epitope retrieval (HIER)). Briefly, slides were heated at 700 W in a microwave for 6 min in 300 mL of 0.1 M citrate buffer, pH 6, resting for 5 min before repeating the microwaving as before. After cooling for 15 min, slides were rinsed in PBS and outlined with a PAP pen for performing subsequent steps as described for IHC without HIER. Slides coated with 3-aminopropyltriethoxysilane (APES: Sigma Aldrich, UK) following the manufacturer's instructions, were used for sections in HIER to minimise tissue loss during the process.

4.2.4.2 Immunofluorescence Antibody Test (IFAT) on wax embedded tissue sections

Due to endogenous peroxidase activity observed in the kidney tubules of both infected and non-infected fish with the IHC procedure, IFAT was undertaken to confirm the staining specificity.

Five µm paraffin wax embedded tissue sections deparaffinised and rehydrated as described above were outlined with a PAP Pen (Vector Laboratories Ltd, UK), washed in PBS and then blocked with 10 % goat serum (Sigma-Aldrich, UK) in PBS for 1 h. Screening was undertaken with ASc10D10 diluted to 1:600 or anti-KHV MAb 20F10 (60 µg mL^{-1}), which were then added to sections for 1 h at RT. All reagents were diluted with 10 % goat serum in PBS. The slides were washed with PBS, and then incubated for 1 h in the dark with goat anti-mouse IgG conjugated to fluorescein isothiocynate (FITC: Sigma-Aldrich, UK)

diluted 1:50 in 10 % goat serum in PBS. Slides were washed with PBS, and 20 µg mL^{-1} propidium iodide (Vector Laboratories) was used as a counter stain and mounting medium for cover slipping. The sections were then viewed using a Leica TCS SP2 AOBS confocal laser scanning microscope (CLSM) (Leica Microsystems, Milton Keynes, UK) coupled to a DM TRE2 inverted microscope (Leica Microsystems, Milton Keynes, UK) and employing a X 63 oil/glycerol immersion objective, in conjunction with Leica confocal software (v. 621). A primary antibody isotype negative control, (anti-ISAV MAb at a 1:2 dilution) and a negative control with AKLH diluted 1:600 were also included. Imaging by confocal microscopy is described in detail in Chapter 5, Section 5.2.3.6.

4.2.5 Immunological analysis

4.2.5.1 Enzyme-linked immunosorbant assay (ELISA) to determine the anti-KHV antibody titre in the serum of infected fish

The indirect ELISA was used to screen serum from infected fish for antibodies against KHV, similar to that described in Section 3.2.5.3(c), but this assay was developed at the FLI, Germany as part of the EU project 'Epizone WP 6.1. (Denmark)'(Sven Bergmann, pers. comm.).

4.2.5.2 Serum neutralisation test (SNT) to determine the neutralising antibody titre against KHV in the serum of infected fish

The SNT used for determining the specific neutralising antibody titre against KHV in the serum of infected fish, was developed at the FLI, Germany, as part of the EU project 'Epizone WP 6.1. (Denmark)' (Sven Bergmann, pers. comm.).

4.2.6. TEM analysis of gluteraldehyde fixed tissues and leukocyte pellets

Tissues and leukocyte pellets were fixed with a 2.5 % gluteraldehyde fixative (2.5% v/v gluteraldehyde in 100 mM sodium cacodylate, pH 7.2) for at least 24 h at 4°C and then processed as described by Hayat (1989). Briefly, sections were rinsed in 0.1 M sodium cacodylate containing sucrose, pH 7.2, overnight at 4°C in order to remove excess fixative. The tissues and cell pellets were then post-fixed in 1 % buffered osmium tetroxide (1 % (v/v) osmium in cacodylate buffer, pH 7.2) and rinsed in distilled water (3 x 10 min). The tissues and leukocytes were then en-bloc stained in 2 % (w/v) uranyl acetate in 30 % (v/v) acetone for 1 h in the dark and dehydrated in an acetone series of 60 % for 30 min, 90 % for 30 min and 2 x 100 % acetone for 30 min and 1 h, respectively, prior to embedding in agar low viscosity resin (ALVR) (Agar Scientific, Essex, UK) mixed 1:1 with acetone. The tissues in ALVR were allowed to polymerise at 60°C for 24 h, before 100 µm ultra-thin sections were prepared from the resin blocks using a microtome (Reichert Ultracut E, Leica, UK) with a diamond knife (Diatome, US) and placed on 200 µm mesh Formvar-coated copper grids. These were first stained with 4 % uranyl acetate in 50 % ethanol for 4 min followed by Reynold's lead citrate for 7 min. The sections were finally observed under an FEI Tecnai Spirit G2 Bio Twin Transmission Electron Microscope (TEM).

4.3 Results

4.3.1 Virulence of KHV isolate in experimental infection

4.3.1.1 Mortality and morbidity

A differential onset of acute KHVD occurred between the two challenge tanks (B15 and B16) in which disease signs started as early as 2 dpi in the first tank (B15) and 100 % morbidity

was evident in both tanks after just 4 dpi (Fig 4.4). Despite all carp receiving the same virus dose, a peracute infection occurred in one tank (B15), whereby mortality started as early as 3 dpi, reaching 100 % after only 4 dpi, whereas the second tank (B16) exhibited a more typical acute onset of disease with mortalities starting after 7 dpi and all but 1 of the non-sampled fish dying after 11 dpi (Fig. 4.4). Tank B15 was therefore designated 'peracute KHVD tank' and tank B16 'acute KHVD tank'. Only live fish were randomly selected for sampling and DNA extractions. Gill and kidney pools taken individually from all dead fish were positive by PCR (Gilad *et al.*, 2002; Bergmann *et al.*, 2006) confirming the presence of KHV in dead fish (results not shown). It should be noted that all water parameters were within a normal, non-toxic range throughout the trial. There were no mortalities or morbidity observed in carp from the control tanks.

4.3.1.2 Clinical disease

Enophthalmos occurred as early as 3 dpi in the peracute KHVD tank followed by increasing mucus production and skin lesions, which became more pronounced in all fish after 6 dpi (Fig. 4.5 A, B). Infected carp sampled within the first 2 dpi appeared clinically healthy, indistinguishable from control carp (Fig. 4.5 D). Many fish lacked signs of disease until later stages of KHVD, and behaved normally until 4 dpi in the acute KHVD tank, at which point fish became lethargic. However, no obvious clinical KHVD was observed in the gills or in internal organs throughout the challenge. Blood taken from infected fish sampled after 4 dpi appeared much paler than uninfected controls (Fig. 4.5 C).

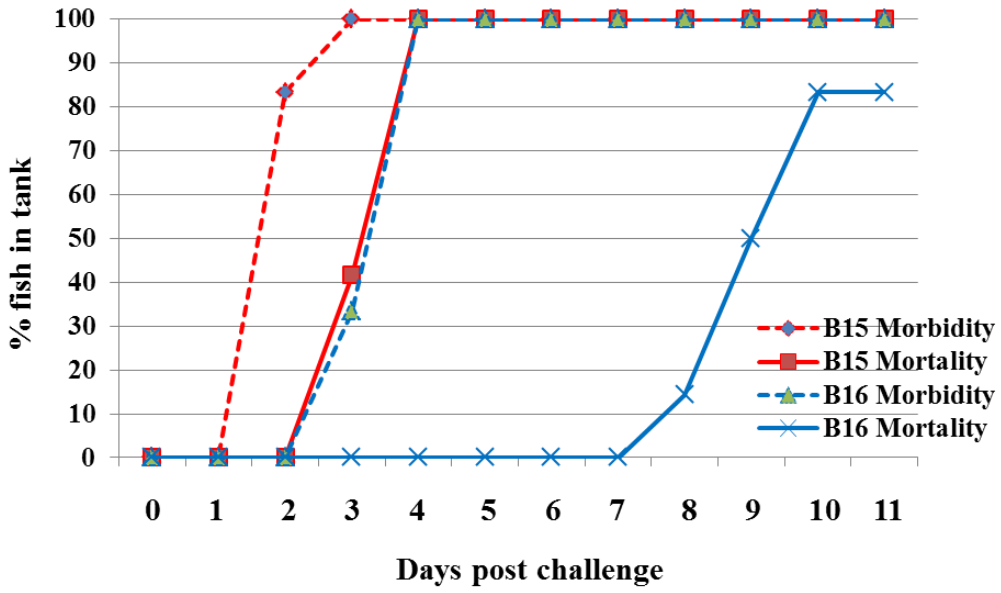


Figure 4.4. Morbidity and Mortality curves for carp experimentally infected with koi herpesvirus.

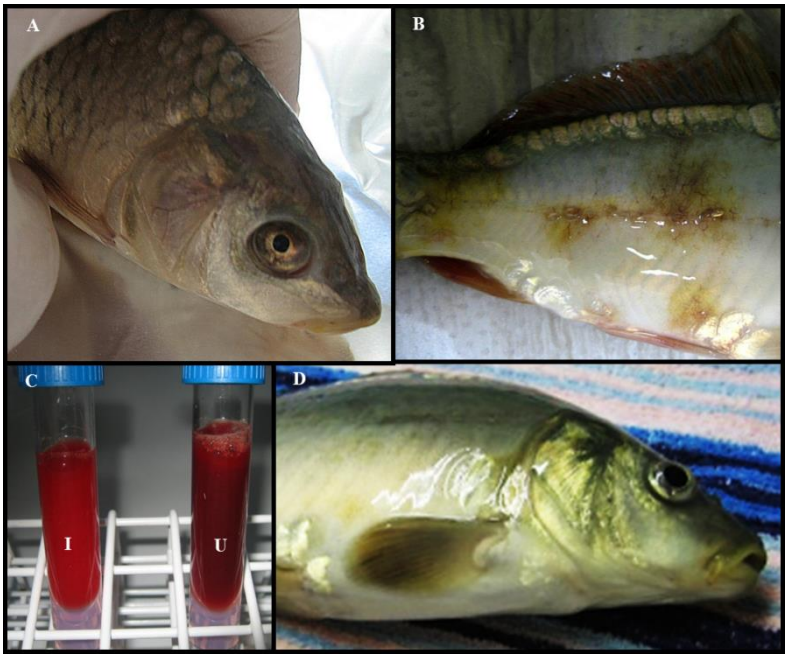


Figure 4.5. External signs in carp experimentally infected with koi herpesvirus disease (KHVD). (A) KHV-infected carp with KHVD-induced enophthalmia at 3 dpi; (B) KHV infected carp with KHVD-associated skin lesions at 6 dpi; (C) blood samples of (I) KHV infected fish at 4 dpi and (U) uninfected fish at 0 hpi diluted 1/5 with MEM); (D) uninfected carp sampled at 0 hpi

4.3.2 Early pathogenesis of KHV

4.3.2.1 Real time qPCR quantification of KHV DNA in carp tissues

(a) 1-8 hpi: Peracute and acute KHVD

Different concentrations of KHV DNA were observed in the mucus of fish from the two tanks during the first day of infection. Very high concentrations of virus, up to 80, 000 genomic equivalents (gen eq.), could be detected in swabs of skin and gill after only 1 hpi in the peracute tank (Tank B15) (Fig. 4.6 A). This level declined dramatically after only 4 hpi to ≤5,000 gen. eq. in skin mucus (from skin swabs and fin base swabs) (Fig. 4.6 A). In contrast, the level of KHV DNA in the equivalent mucus samples from the acute KHVD tank (Tank B16) increased dramatically from ~10,000 gen. eq. to 50,000 during the first 8 h of infection (Fig 4.6 B).

Mucus from gill swabs contained 1000 gen. eq. after 8 hpi in the peracute KHVD tank, despite a substantial initial attachment of virus (80,000 gen. eq). Gill swabs from the acute KHVD tank showed an even greater reduction of KHV DNA over the first 8 h of infection with 10,000 gen. eq. initially detected after 1 h, and 10 gen. eq. detected at 8 hpi. Notably higher levels of KHV DNA were noted in gill mucus of the peracute KHVD tank than the acute KHVD tank during the first 8 hpi (Fig. 4.6). There were also high levels of KHV DNA measured in gill biopsies, with between 1000-50,000 gen. eq. detected up to 2 hpi in the peracute tank (Fig. 4.7 A), however, <10 gen. eq. were found on gills of fish from the acute KHVD tank during the same time period (Fig. 4.7 B). Around 1000 gen. eq. were detected in skin biopsies of fish after 1 hpi in the peracute tank, however this decreased to undetectable levels in the majority of fish sampled during the first dpi (Fig. 4.7 A).

Despite fish sampled in the acute KHVD tank having a viral load of 5000 gen. eq. in skin samples at 4 hpi, there was no detectable viral DNA in other fish sampled during the first day of infection (Fig. 4.7 B).

Internally, in both the peracute and the acute KHVD tanks, very low levels of KHV DNA (< 10 gen. eq.) were detected within gut tissue and leukocytes (< 10 gen. eq.) from only 4 hpi and 6 hpi, respectively, but all other organs were negative during 1-8 hpi (Fig. 4.7 C, D).

(b) 1-4 dpi: Peracute and acute KHVD

Despite the decline in KHV DNA in the mucus of infected fish during the first few hours post- infection, >1000 gen. eq. could always be detected in skin and fin-base swabs in fish from both tanks from 1 - 4 dpi (Fig. 4.6 A-B). Levels of KHV DNA detected in the mucus from gills however, were more variable with <10 gen. eq. detected in the peracute KHVD tank, but >1000 in the acute KHVD tank (Fig. 4.6 A-B).

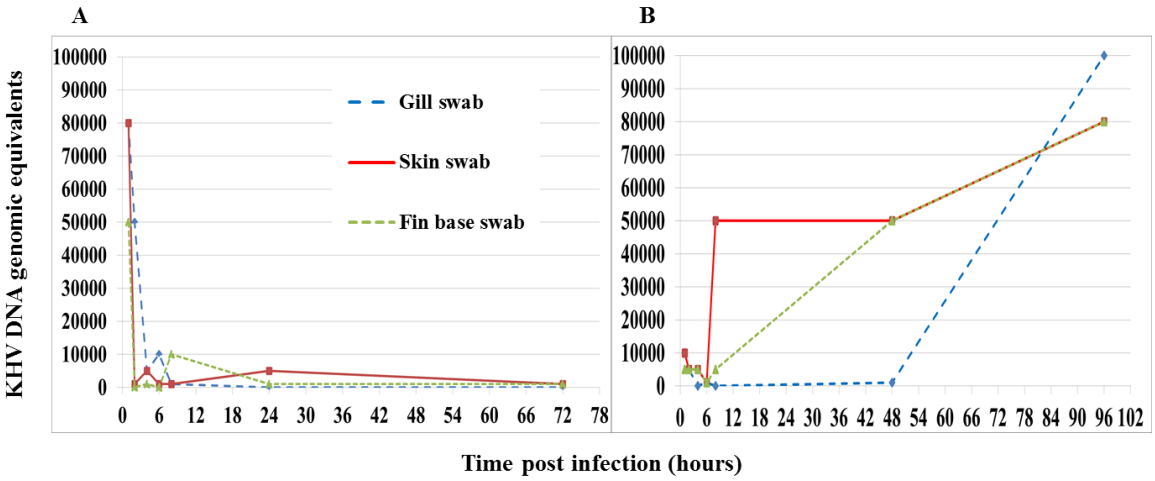


Figure 4.6. Koi herpesvirus DNA genomic equivalents measured in the mucus of infected fish by real-time qPCR during the first 4 days post infection (dpi). (A) fish from the peracute KHVD tank (B15); (B) fish from the acute KHVD tank (B16).

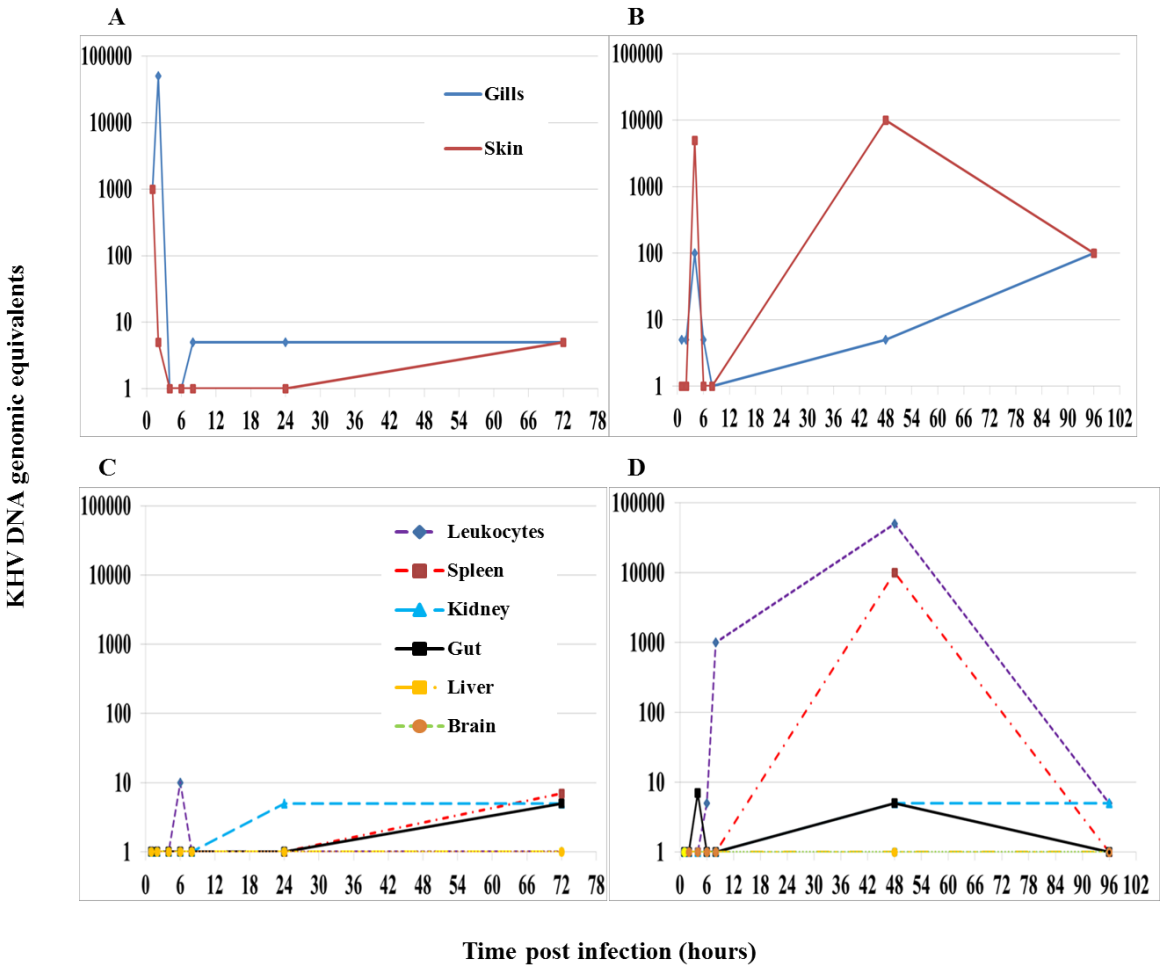


Figure 4.7. Koi herpesvirus DNA genomic equivalents measured in external and internal tissues and blood of infected fish by real-time qPCR during the first 4 dpi. Results are shown for (A, C) fish from the peracute KHVD tank (B15) and (B, D) acute KHVD tank (B16). (A) KHV DNA in external tissues of fish from the peracute KHVD tank; (B) KHV DNA in external tissues of fish from the acute KHVD tank; (C) KHV DNA in blood and internal tissues of fish from the peracute KHVD tank; (D) KHV DNA in blood and internal tissues of fish from the acute KHVD tank. Data are presented on logarithmic scales.

In the peracute tank, KHV DNA levels were very low in external tissues (gills and skin) from 1 - 3 dpi with <10 KHV DNA gen. eq. However, in the acute KHVD tank, much

higher concentrations could be detected from 2 - 4 dpi with up to10,000 gen. eq. measured, although there was substantial fish to fish variation (Fig. 4.7 A, B).

The levels of KHV DNA in the peripheral blood leukocytes increased steadily up to 50,000 gen. eq. by 2 dpi in the acute KHVD tank (Fig. 4.7 D). After 24 h, the dissemination of KHV within internal tissues was similar in both tanks with viral DNA detected in the kidney, spleen and gut up to 4 dpi, although concentrations were generally low, with the exception of a dramatic increase observed in the spleen (10,000 gen. eq.) after 2 dpi in the acute KHVD tank (Fig. 4.7 C, D).

(c) 5-10 dpi: Acute KHVD

During the course of the challenge, a considerable increase in KHV DNA gen. eq. was evident during the later stages of the infection from 5 – 10 dpi compared to the first 4 dpi. Between 4 x 10^5 and 1 x 10^7 gen. eq. were detected in mucus swabs from skin, fin bases and gills during this period with exception to one fish sampled at 9 dpi (Fig. 4.8 A). High levels of KHV DNA were also detected in skin and gills, with levels between 5,000 and 8 x 10^5 gen. eq. detected between 5 – 10 dpi, again with the exception of the fish sampled at 9 dpi (Fig. 4.8 B). All internal tissues and blood leukocytes harboured much higher concentrations of KHV DNA during the last 5 days of the challenge with the exception of the fish sampled at 9 dpi (Fig. 4.8 C). Concentrations as high as 50,000 KHV DNA gen. eq. were observed in blood leukocytes, brain, liver and gut, while the kidney and spleen harboured the highest viral loads with concentrations between 5,000 – 50,000 in the spleen and 50,000 – 400,000 in the kidney (Fig. 4.8 C). This was again with the exception of the fish sampled after 9 dpi, which contained a much lower viral load in all tissues and mucus sampled with levels no higher than 100 KHV DNA gen. eq. (Fig. 4.8 A-C). No viral DNA was detected in any carp from the control tank (results not shown) and no positive reactions occurred in NTC wells

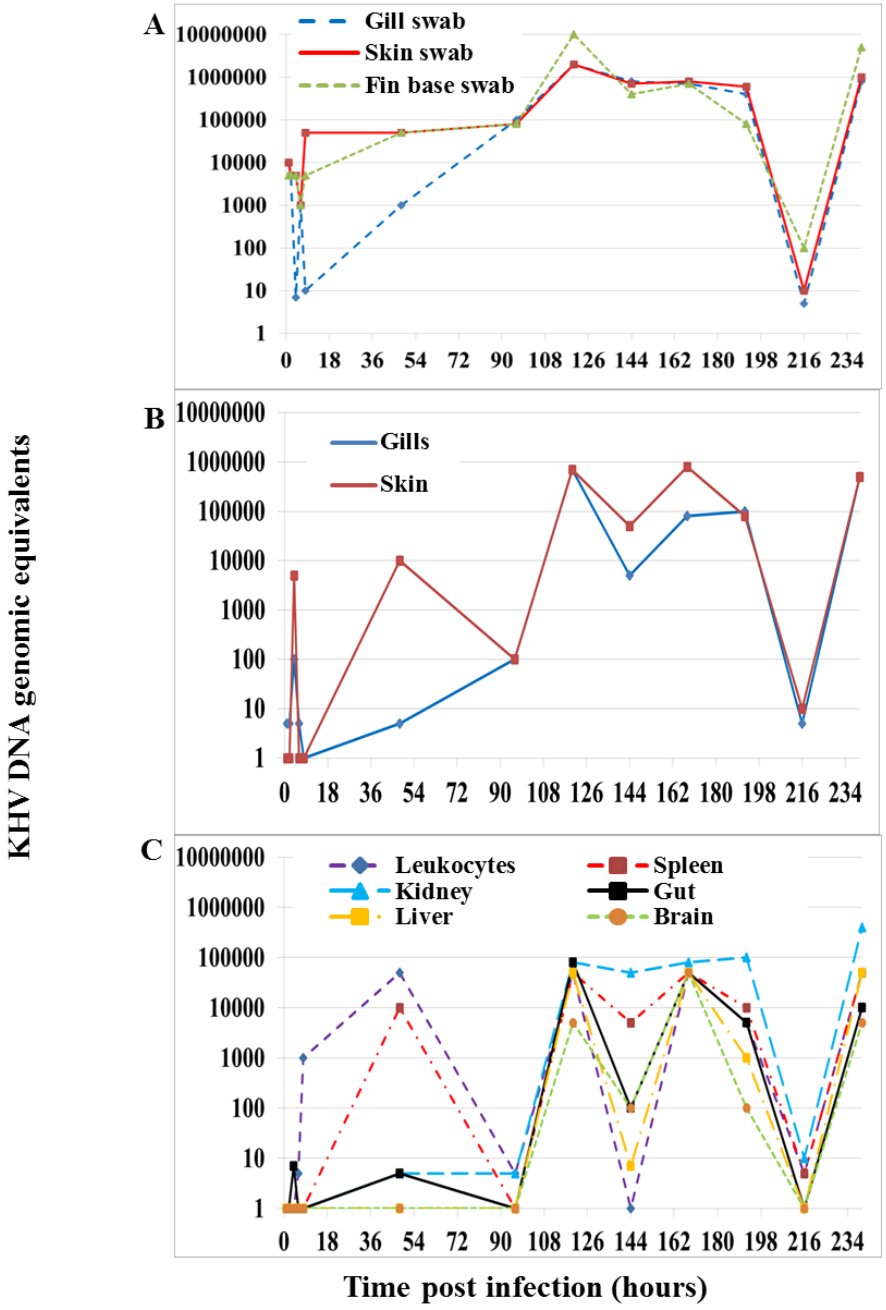


Figure 4.8. Koi herpesvirus DNA genomic equivalents in mucus, external and internal tissues and blood of infected fish from the acute KHVD tank measured by real-time qPCR over the 10 day course of the experimental challenge. . KHV DNA in (A) mucus; (B) external tissues; (C) blood and internal tissues. Data are presented on logarithmic scale.

(d) Comparison of mean viral load between peracute and acute (early and late) infection phases

Since only single fish were analysed per time point within each tank, where fish to fish variation can be expected, the mean viral loads were analysed between different infection periods, i.e. peracute KHVD and early stage acute KHVD (<5 dpi), and later stage acute KHVD (≥ 5 dpi), to gauge the trends in viral load within the various tissues at the different stages. A greater mean viral load was observed in gill biopsies of fish from the peracute KHVD tank compared to the early stage of acute KHVD, although not significantly different (p=0.31), whereas mean viral DNA copies present in skin were higher during the early stage of acute KHVD, within the first 4 dpi, but again were not significantly different from copies present in peracute fish skin (p=0.67) (Fig. 4.9 A). A fish with particularly high viral loads in the spleen (10,000 gen. eq.) of an acute KHVD fish increased the mean value in this tissue, but no other fish had high viral loads in the spleen during this period. Significantly higher copy numbers were observed in all fish tissues at later stages of infection (gill and gut: p<0.05; liver, brain, kidney, spleen and skin: p<0.01), however notably high levels of KHV DNA were observed in mucus in fish from both the peracute and acute KHVD tanks in the early stages of infection (Fig. 4.9 B). There were no significant differences between viral loads in the skin swabs between early acute and peracute stages compared to late stages of infection (p>0.05). There was however, significantly higher viral loads in leukocytes at later stages of the acute infection compared to peracute infected fish (p<0.015) as well as gill swab (p=0.03) and fin base swabs (p=0.03), but not between late stages of acute KHVD and early acute stages (leukocytes: p=0.16; gill swab: p=0.053; fin base swab: p=0.059). The mean viral load in the mucus from swabs increased markedly from early infection stages (1-4 dpi) to the later stages of the challenge (5-10 dpi) (Fig. 4.9 B).

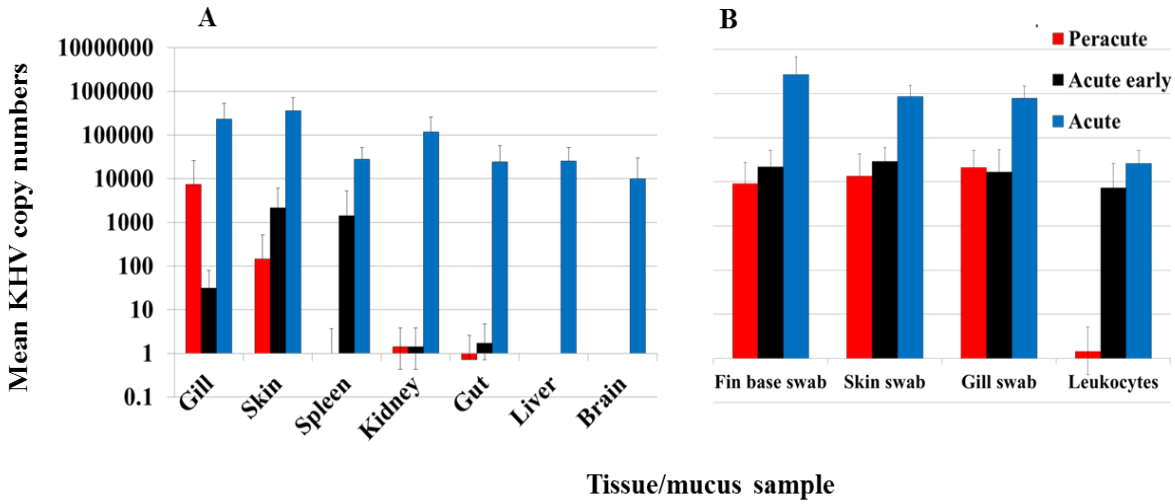


Figure 4.9 Koi herpesvirus DNA loads within tissues, mucus and white blood cells during experimentally induced peracute and acute KHVD as measured by TaqMan-qPCR. Data are shown as mean ± SD of *n*=7 for peracute KHVD tank fish sampled < 5 dpi, *n*=7 for acute (early) KHVD tank fish sampled < 5 dpi and *n*=6 for acute KHVD tank fish sampled ≥ 5 dpi.

4.3.2.2 Detection of viral DNA in tissues by ISH

(a) PCR of labelled and non-labelled probes

Probes were successfully labelled with DIG for both the 414 bp probe (Bergmann *et al*., 2006) and 517 bp probe (Hutoran *et al*., 2005), which was evident by comparing the molecular weight of unlabelled probes with labelled probes following electrophoresis (Fig. 4.10).

(b) Negative and positive control tissues

No KHV infected cells were found in any gill or kidney sections of negative control fish (Fig. 4.11 C, F). However, occasionally, there were signals observed externally on gill filaments, likely to be associated with non-specifically bound probes inside the mucus of gill samples. Positive controls expressed a large number of positive signals associated with respiratory epithelial cells of the gill lamellae, particularly at the base of the filaments (Fig. 4.11 B),

where inclusion bodies were also observed histologically in H & E staining (Fig. 4.11 A). A large number of melanomacrophages was seen in the kidneys of both positive and negative control carp, but no signals associated with viral DNA were observed in the latter (Fig. 4.11 D-F). The kidneys of positive controls also exhibited a high level of staining within the interstitium, but not associated with the tubules (Fig. 4.11 E), where inclusion bodies were observed in similar locations by H & E staining (Fig. 4.11 D). Probes detecting DNA to a heterogenous virus, VHS, were negative in all tissue sections (results not shown).

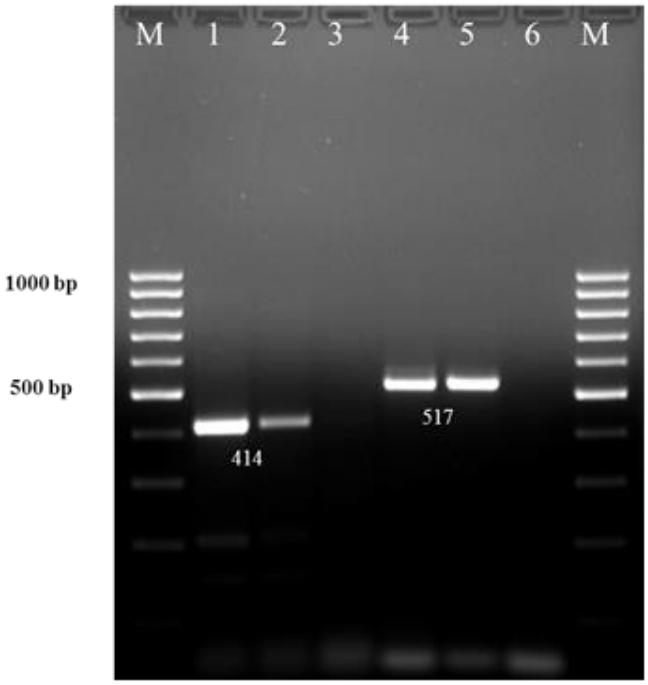


Figure 4.10. Agarose gel electrophoresis (1.5%) of labelled koi herpesvirus-specific ISH probes prepared by PCR. Lanes represent the results of labelled and non-labelled probes prepared using different primers sets. Lanes: (M) 100 bp molecular weight ladder; (1) PCR with primers KHV 1Fn-1Rn (unlabelled), (2) PCR with primers KHV 1Fn-1Rn (labelled), (3) No template control for primers KHV 1Fn-1Rn, (4) PCR with primers KHV NH1-NH2(unlabelled) (5) PCR with primers KHV NH1-NH2 (labelled) and (6) No template control for primers KHV NH1-NH2.

A scoring system was developed for differentiating the abundance of signals obtained by ISH based on comparisons of the signals recorded from sections of positive and negative control

carp tissues (n = 15). The scoring system used was similar to that applied in other studies using ISH for viral nucleic acid detection (Sano *et al*., 1994; Alonso *et al*., 2004; Huang *et al*., 2004; Lopez-Jimena *et al*., 2011).

(c) ISH analysis of early KHV pathogenesis

1-8 hpi: Peracute and acute KHVD

Both probes (Bergmann *et al*., 2006 and Hutoran *et al*., 2005) were used in ISH analysis of samples collected during the early stages of the KHV infection, but no observable differences were seen in the level of signal obtained between the probes.

The tissues from the two fish sampled at 0 hpi were negative by ISH, however non-specific staining was observed in mucus covering the skin. Melano-macrophages were observed in tissues such as the kidney, liver and the spleen in H&E stained sections, however no positive signals were evident in ISH (results not shown). During the first day of infection (1-8 hpi), there were differences noted between fish sampled from the tank exhibiting peracute KHVD (Tank B15) compared to fish from the tank exhibiting acute KHVD (Tank B16). The gills in fish from the peracute KHVD tank displayed stronger signals in ISH than those from the acute KHVD tank after 1 and 4 hpi (Table 4.3), where virus DNA was initially detected attached to gill epithelium or mucus from 1 hpi (Fig. 4.12 A), although many gill lamellae remained negative (Fig. 4.12 C).

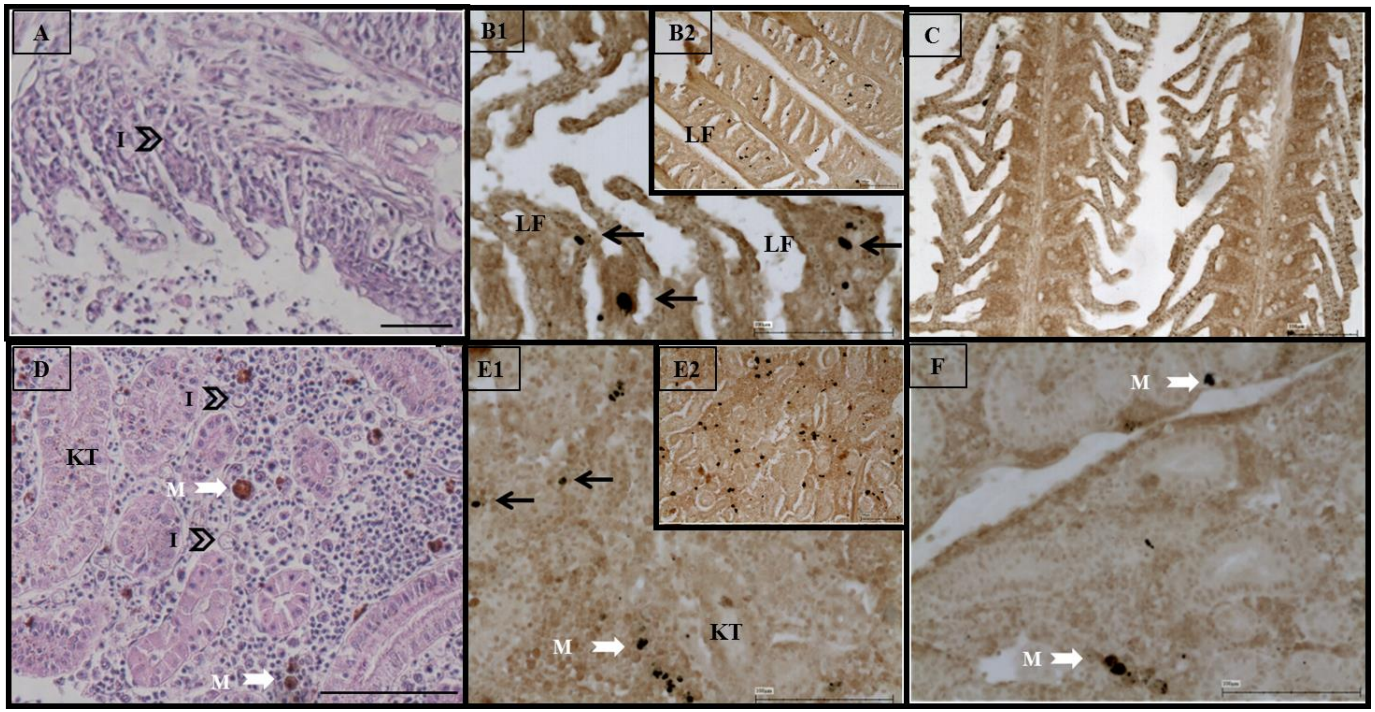


Figure 4.11. Detection of Koi herpesvirus in gill and kidney sampled from experimentally infected fish using ISH. (A) H&E stained gills from carp with clinical KHVD (Mag. x 50); (B) High mag. (B1) (x 50) and lower mag. (B2) (x 25) of gills from infected carp after ISH; (C) Gills from uninfected carp after ISH (Mag. x 25); (D) H&E stained kidney from carp with clinical KHVD. (Mag. x 25); (E) High mag. (E1) (x 50) and low mag. (E2) (x 25) of kidney from infected carp after ISH; (F) High mag. of kidney from uninfected carp after ISH, Mag. x 50. Scale bar = 100 µm. Black arrows indicate infected cells expressing signals for KHV DNA while white arrows indicate melanomacrophages. I = Inclusion body; LF = Secondary lamellar fusion; KT = Kidney tubules. Note that sections are not sequential.

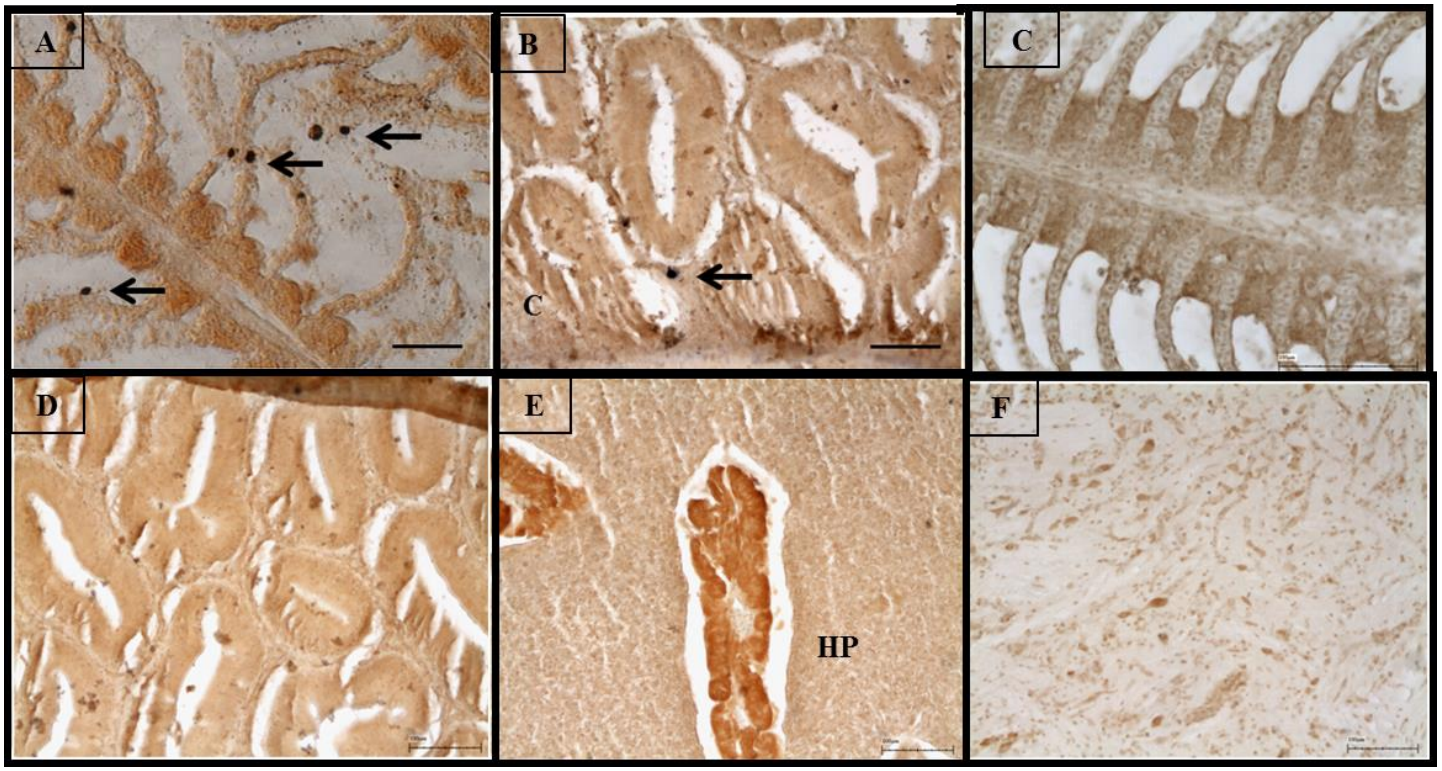


Figure 4.12. Koi herpesvirus infected carp tissues after ISH, 1 hpi. (A) Positive signals in gill mucus and filament epithelium (Mag. x 25); (B) Positive signals in gut, possibly associated with crypts (Mag. x 25); (C) Negative region of gill (Mag. x 50); (D) Negative area of gut (pyloric caecae) (Mag x 25); (E) Negative area of liver and hepatopancreatic tissue (Mag. x 25); (F) Negative brain tissue, (Mag. x 25). All tissues from acute KHVD tank. Scale bar = 100 µm. Black arrows indicate infected cells expressing signals for KHV DNA. C = Crypts; HP = Hepatopancreas.

However, virus DNA appeared to be located within gill epithelium as early as 2 hpi (Fig. 4.13 A) and by 6 hpi there were strong signals associated with the base of the gill filaments and in close proximity with the central venous sinus (Fig 4.13 D). Positive signals by ISH could only be detected in skin after 6 and 8 hpi, from both fish sampled from the tank exhibiting acute KHVD (Table 4.3), although it was not possible to analyse the skin of every fish up to this point. However, positive signals could be detected in the skin mucus from 1 hpi in every section analysed (results not shown).

The ISH signals obtained from internal organs of fish from the two challenge tanks varied during the first day of KHV infection. Viral KHV DNA could be detected in the blood vessels of the kidney, spleen and liver as early as 1 hpi from sampled fish of the acute KHVD tank only, although these signals were quite focal and the tissue and vessels of these organs remained predominantly negative (Fig. 4.12 E). Throughout the first day of the time course the positive signals found in the liver were consistently focal and associated with the vessels (Fig. 4.13 F; 4.14 D; Table 4.3), although not all hepatic vessels exhibited positive signals and some signals were in close association with the hepatopancreas (4.14 B). Similar staining of splenic vessels was also apparent until 4 hpi, after which some intracellular staining was also evident within the pulp (Fig. 4.14 C; Table 4.3). Apart from the first hpi, there were no noticeable differences between the abundance of signals of infected liver and spleen tissues in fish from either tank sampled at each time point during the first day of infection (Table 4.3). The positive KHV DNA signals observed in the vessels of the kidney after 1 hpi were followed by the observation of positive signals in the interstitial tissue after 4 hpi, which were not associated with the tubules, however, only one fish kidney from the peracute KHVD tank could be analysed at this time point (Table 4.3). After 6 and 8 hpi, again there were signals observed in the kidney tissues (Fig. 4.13 E), which were stronger in fish from the peracute

KHVD tank than the acute KHVD tank (Table 4.3). Regardless of disease propagation, signals in the kidney vessels were consistent during these early stages of infection (Fig.4.13 B; Table 4.3).

After 1 hpi, there were also a few positive signals noted in the gut, which seemed to be associated with the crypts (Fig. 4.12 B); however this was difficult to confirm due to the degraded architecture of the digested tissue. These signals were again very focal and the majority of gut and pyloric caecae remained negative (Fig. 4.12 D).

Table 4.3. Scoring of ISH signals in tissues of carp during the early stages of Koi herpesvirus infection in experimentally challenged carp

Fish	Time post infection (hpi)	Gills	Skin	Spleen	Kidney	Gut	Liver	Brain
1	0	-	-	-	-	-	-	-
2	0	-	-	-	-	-	-	-
3*	1	++	-	-	-	+	-	-
4	1	+	NA	+ *v*	+ *v*	+	+ *v*	-
5*	2	++	NA	+ *v*	+ *v*	+	+ *v*	-
6	2	++	NA	+ *v*	NA	+	+ *v*	-
7*	4	++	-	NA	+	NA	+ *v*	-
8	4	+	-	+	NA	+	+ *v*	-
9*	6	++	-	+	++	-	+ *v*	-
10	6	++	+	+	+	-	+ *v*	NA
11*	8	++	-	+	++	-	+ *v*	NA
12	8	++	+	+	+	+	+ *v*	-
13*	24	+++	NA	+	++	++	+	-
14	48	++	+	+	++	NA	+	-
15*	72	+++	NA	+	++	-	+	-
16	96	+++	+	NA	NA	+	+	-
17	120	+++	-	++	+++	+	++	+ *v*
18	144	+++	+	+	+++	NA	+	-
19	168	+++	+	++	+++	+	+	+ *v*
20	192	++	NA	+	++	+	+	+ *v*
21	216	+++	++	+	+++	+	+	-
22	240	+++	+	+	+++	+	+	+ *v*

NA = non-applicable (not possible to observe reaction as tissue over-digested); * = fish sampled from peracute KHV disease; *v* = signals only associated with vessels; + = few signals, ++ = moderate signals, +++ = many signals, - = negative/no signals

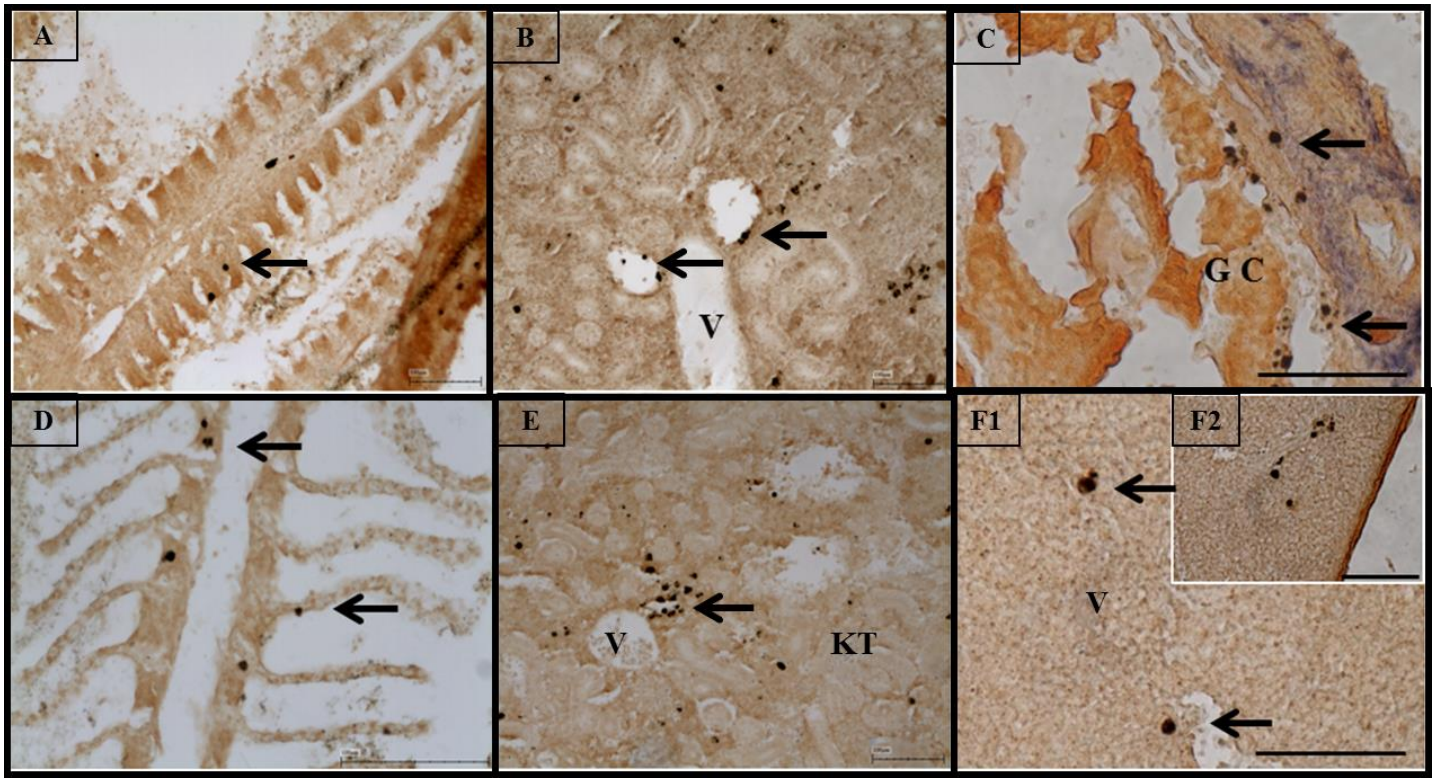


Figure 4.13. Koi herpesvirus infected carp tissues after ISH, 2 – 6 hpi. (A) Positive signals in gill filament epithelium (Mag. x 25), 2 hpi; (B) Positive signals in kidney associated with the vessels (Mag. x 25), 2 hpi; (C) Positive staining of gut contents (Mag. x 50), 2 hpi; (D) Positive signals in gill filament epithelial cells, Mag. x 25, 6 hpi; (E) Positive signals throughout interstitial renal tissue in kidney, but not tubules. Note close association with vessels, Mag. x 25, 6 hpi; (F) High mag. (F1) (x 50) and low mag. (F2) (x 25) showing positive signals in liver vessels, 6 hpi. All tissues are from peracute KHVD tank fish. Scale bar = 100 µm. Black arrows indicate infected cells expressing signals for KHV DNA. V = Vessel; KT = Kidney tubules; G C = Gut contents

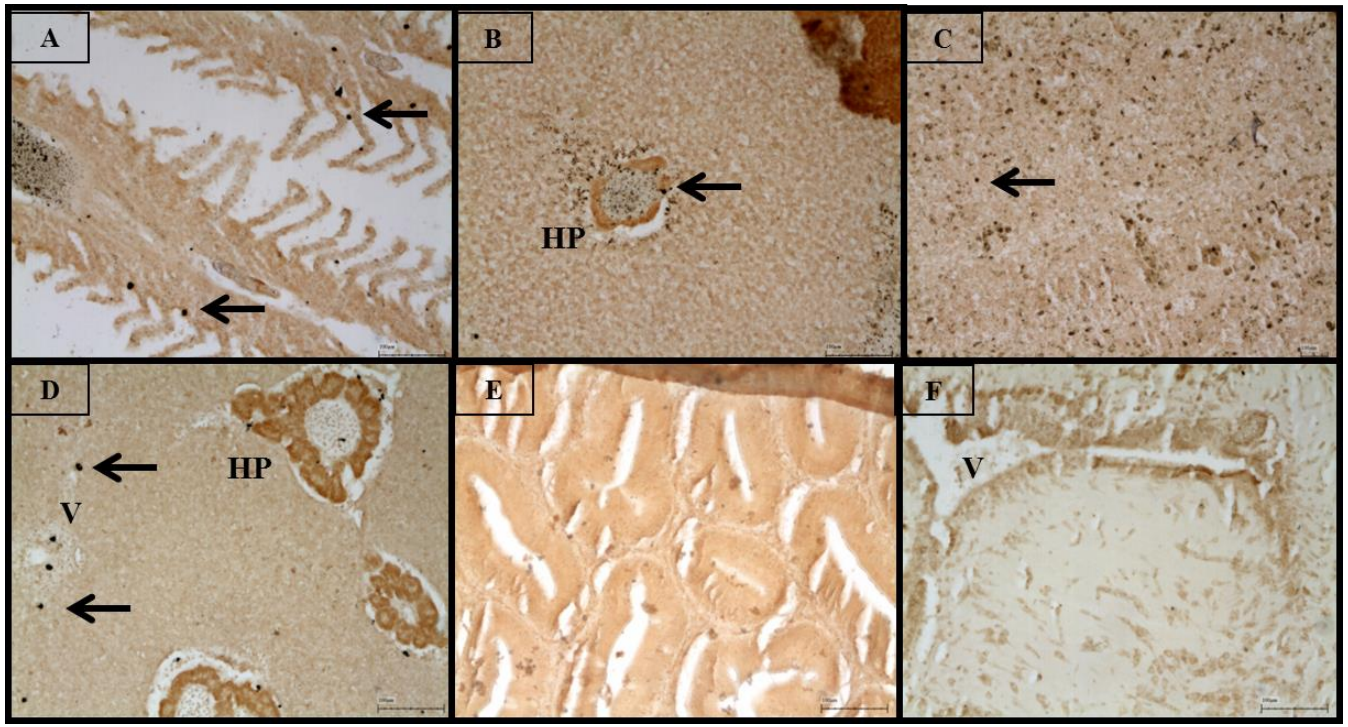


Figure 4.14. Koi herpesvirus infected carp tissues after ISH, 8 hpi. (A) Positive signals in gill epithelium. (Mag. x 25); (B) Positive signals in liver vessels and pancreatic tissue (Mag. x 25) (C) Positive signals in spleen pulp (Mag. x 25) (D) Positive signals in liver associated with vessels and hepatopancreatic tissue (Mag. x 25); (E) Negative region of gut (pyloric caecae) (Mag. x 25); (F) Negative brain (Mag. x 25). Tissues A-C are from peracute KHVD fish; tissues D-F are from acute KHVD fish. Scale bar = 100 µm. Black arrows indicate infected cells expressing signals for KHV DNA. HP = Hepatopancreas; V = Vessels.

There were no real differences in level of signal observed in the gut of fish from either the peracute KHVD tank or the acute KHVD tank throughout the first day of the infection (Table 4.3). After 2 hpi there were positive signals within the epithelium of the gut in close association with positive stained gut contents (Fig. 4.13 C).

After 4 hpi there were only a few cells exhibiting positive signals in the gut of fish from the acute KHVD tank. Interestingly, there were no signals observed in the gut and pyloric caecae of fish sampled at 6 hpi, but after 8 hpi signals were again observed from a fish sampled from the acute KHVD tank, although these were still focal and the majority of tissue remained negative (Table 4.3; Fig. 4.14 E). No signals were observed in the brain (Fig 4.12 F; 4.14 F) throughout the first 8 h of infection (Table 4.3).

1-4 dpi: Peracute and acute KHVD

After 1 dpi, only a single fish was sampled each day for analysis. The fish sampled at 1 dpi was from the tank exhibiting peracute KHVD. There were strong signals in the gills, again particularly around the base of the filaments, and numerous signals were associated with the blood vessels in the spleen, liver and interstitial tissue of the kidney. Particularly strong signals were found in the gut at this stage of infection (Table 4.3). Positive signals were also noted for the first time in liver hepatocytes. However, the brain was negative (Table 4.3). After 2 dpi, a fish sampled from the tank exhibiting acute KHVD expressed strong signals in the gills and kidney (Table 4.3) and there were signals both within the tissue and vessels of the spleen. Positive signals were found in the skin accompanied by strong signals in the mucus (not shown). After 3 dpi, the fish sampled from the peracute disease tank was found to be surprisingly negative in the gut, but the gill, kidney and spleen exhibited relatively strong signals. The liver was also positive, but the brain was still negative (Table 4.3). After 4 dpi, a

fish sampled from the acute KHVD tank had very strong signals along the gill filaments (Fig. 4.15 A-C), and there were also signals noted in the skin and gut. Virus could be detected within the gut lumen. Hepatocytes of the liver were also still positive and signals were observed throughout blood vessels (not shown), but it was not possible to analyse the spleen and kidney samples of this fish due to over-digestion of the tissue by proteinase K in the ISH procedure.

5-10 dpi: Acute KHVD

After 5 dpi, all fish analysed by ISH were from the tank exhibiting acute disease as all fish from the peracute infection had died. There were similar signals detected within the gills as those noted after 4 dpi, positive signals were observed in the vessels of the brain, and there were positive signals in the gut (Table 4.3). Although the abundance of signals fluctuated between fish sampled from 5-10 dpi, generally, all fish exhibited positive signals in most tissues analysed. Brain was the exception, with occasional positive signals observed only in the vessels and never within the tissue (Table. 4.3). The gill filaments consistently exhibited strong positive signals (Table 4.3), especially within the respiratory epithelium (Fig. 4.15 D). Regions of infection were still focal, whereby some areas of splenic pulp or hepatic tissue were positive (Fig. 4.15 E), while elsewhere remained negative for virus (Fig. 4.15 F). Even in heavily infected kidneys, areas of negative lymphatic tissue were evident. After 10 dpi huge aggregates of viral DNA were found on the skin (not shown). Histologically, it is interesting to note that no pathology was observed by H&E staining in any of the tissues during the 10 dpi (not shown). The scores for signals obtained from all the challenged carp tissues are shown in Table 4.3.

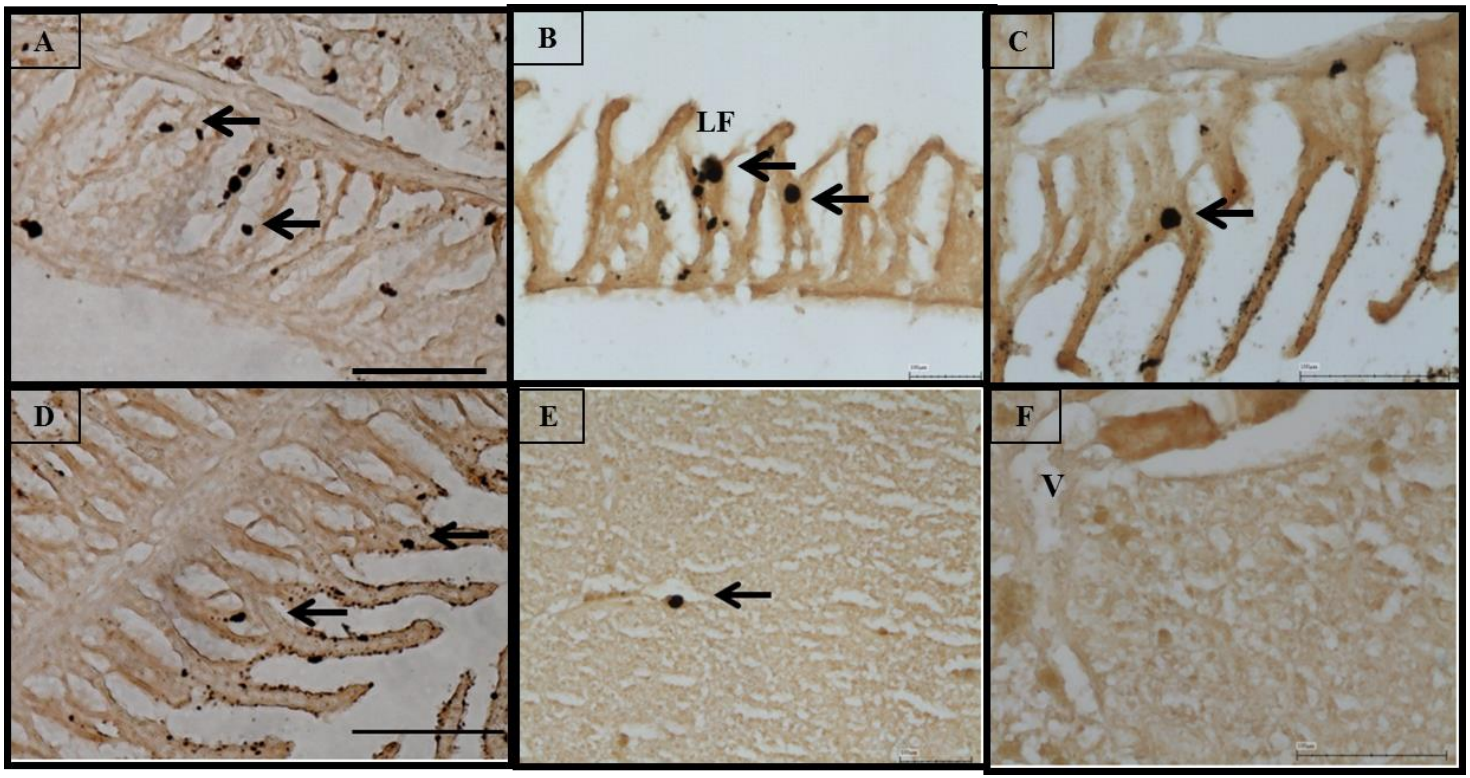


Figure 4.15. Koi herpesvirus infected carp tissues after ISH, 4-6 dpi. (A) Positive gills. Note great abundance of infected epithelial cells, 4 dpi (Mag. x 25); (B) positive gills with infected epithelial cells of fused filament, 4 dpi (Mag. x 50); (C) gill filaments, 4 dpi (Mag. x 50); (D) Gill lamella with high abundance of positive signals throughout the filaments, 6 dpi (Mag. x 25); (E) Liver with infected hepatic cells, 6 dpi (Mag. x 25); (F) Negative region of liver tissue and vessel, 6 dpi (Mag. x 50). All tissues are from acute KHVD tank fish. Scale bar = 100 µm. Black arrows indicate infected cells expressing signals for KHV DNA. LF = Secondary lamellar fusion

4.3.2.3 KHV antigen expression detected by MAbs using IHC and IFAT

Screening for KHV antigens *in situ* by IHC was not successful for the majority of MAbs. With initial screening using KHV-infected carp tissues, minor signals were observed in the kidney, liver and gills with MAb 20F10, detecting a capsid protein, at a concentration of 60µg mL^{-1} and with MAb 7C6 hybridoma supernatant diluted 1/3 or 7C6 ascites fluid (Asc 7C6) diluted 1/300, both detecting an antigen of KHV ORF68. However, these signals were inconsistent. The greatest signals were obtained using MAb 10D10, detecting a protein of ORF62, with both hybridoma supernatant, diluted 1/4, and ascites fluid diluted 1/600. The gill epithelium of both infected and non-infected (negative and positive control) fish had a large number of positively-stained cells when screened with ascites fluid containing MAb 10D10, (Fig. 4.16 A, D). No difference was seen in the level of staining between infected and non-infected gill samples with this MAb at 6 dpi (Fig. 4.17 A). There was strong staining in negative as well as positive kidneys, observed in both tubules and interstitial tissue, which must have been due to non-specific staining or endogenous peroxidise activity (Fig. 4.16 B, E). Similar staining was observed in the kidneys after 6 dpi when screened with hybridoma supernatant containing MAb 10D10 (Fig. 4.17 D). The positive signals in the liver tissue appeared more promising, in terms of being specific for the virus, whereby the negative controls with PBS had remained negative (Fig. 4.16 F), but focal staining around the vessels could be observed with MAb 10D10 (ascites fluid) in infected fish after 7 dpi (Fig. 4.16 C). The focal staining in the liver was also evident in fish sampled after 6 dpi with MAb 10D10, which appeared to be more associated with hepatocytes of the parenchyma (Fig. 4.17 B-C). Although focal signals were also noticed with alternative MAbs such as 7C6, the signals obtained differed between each MAb (Fig. 4.17 E), and were very patchy and inconsistent,

sometimes appearing negative in the same tissue section (Fig. 4.17 F). The commercial anti-KHV MAbs were always negative (not shown).

Due to non-specific staining observed in the kidney tubules, IFAT was undertaken with MAbs 7C6 and 10D10 to establish if this was associated with endogenous peroxidase activity present in the fish tissue. No staining was observed with MAb 7C6 in the liver, gills, gut or the interstitial tissue of the kidney in IFAT (results not shown).

The gills, liver, kidney and gut of carp undergoing clinically acute KHVD after 5 dpi exhibited positive fluorescent signals when screened with ASc. 10D10. Strong signals were also observed within endothelial cells of the kidney tubules as well as signals in the interstitium (Fig. 4.18 B, C), making it impossible to confirm that the positive signals observed were specific for KHV. A large number of positive epithelial cells were also observed in the gill filaments and pyloric caecae of infected fish, which appeared too abundant to be specific for the virus at this early stage of infection (Fig. 4.18 A, D). Signals observed in the liver appeared to be more focal and there was a variation in intensity, which could represent specific signals in the parenchyma (Fig. 4.18 E-F). Since the consistency of ascites fluid may have contributed to the non-specific staining, hybridoma supernatant containing MAb 10D10 was also used, but the detection of the virus was inconsistent over the course of infection trial and therefore not all tissues were analysed using IHC and IFAT. All tissues screened with PBS were, however, negative (Fig. 4.18 G) and positive tissues were negative when screened with other anti-KHV MAbs (Fig, 4.18 H).

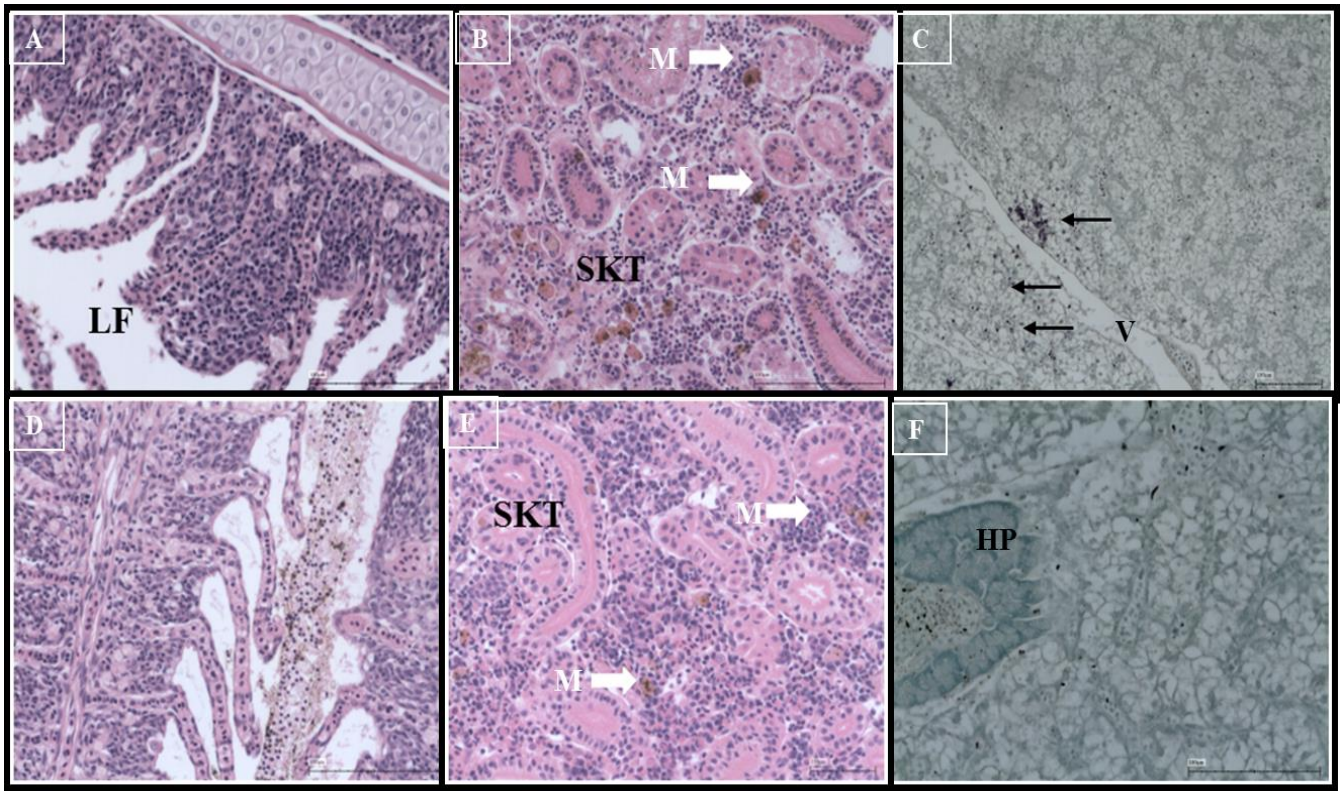


Figure 4. 16 IHC with MAb 10D10 (ascites fluid) on Koi herpesvirus infected tissues. (A) Positive control gills with strong staining (Mag. x 50); Note the fused secondary lamellae (LF); (B) Positive control kidney with strong staining surrounding melanomacrophages in the interstitial tissue. The kidney tubules also stained (Mag. x 50); (C) Positive staining associated with the vessels in the liver of experimentally infected carp, 7 dpi (Mag. x 25); (D) Negative control gills with non-specific positive signals (Mag. x 25); (E) Negative control carp kidney with stained tubules, but also strong sporadic signals throughout the interstitial tissue. (Mag. x 25) (F) Positive control carp liver incubated with PBS (Mag. x 25). Scale bar = 100 µm. LF = Lamellar fusion of secondary lamellae; SKT = stained kidney tubules; HP = Hepatopancreas; V = vessels. White arrows (M) = melanomacrophages; Black arrows = Focal staining of hepatocytes.

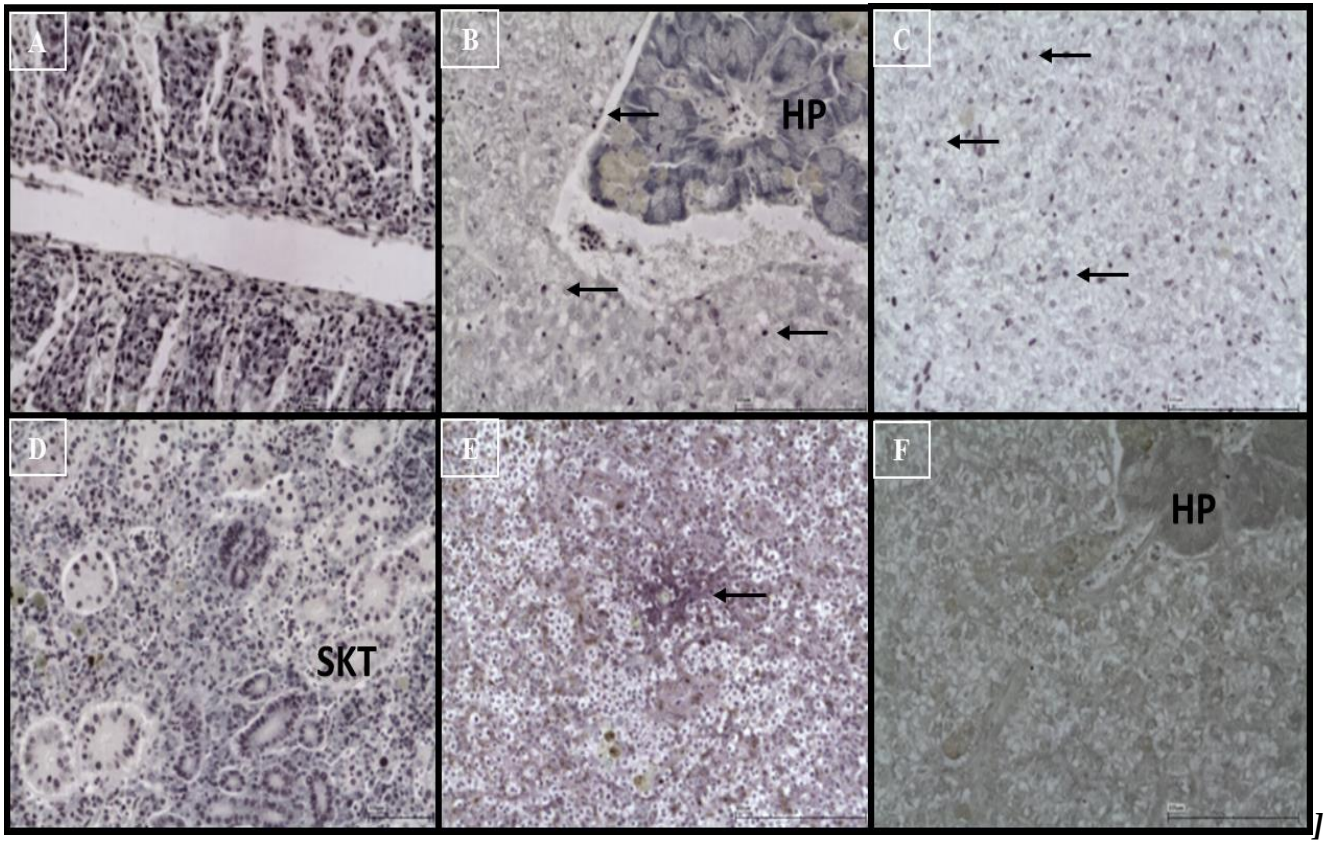


Figure 4. 17 IHC using MAb 10D10 or MAb 7C6 with Koi herpesvirus infected carp tissues, 6 dpi. (A) High abundance of signals in the gill filaments (MAb 10D10 diluted 1/4) (Mag. x 50); (B) Positive liver with staining near hepatopancreas (MAb 10D10 diluted 1/4) (Mag. x 50); (C) Positive signals in hepatocytes (MAb 10D10 diluted 1/4) (Mag. x 25); (D) High abundance of signals in the kidney. Note tubules are stained as well as interstitium (MAb 10D10 diluted 1/4) (Mag. x 25); (E) Focal staining in the liver parenchyma (MAb 7C6 diluted 1/3) (Mag. x 25); (F) Negative region of liver tissue (MAb 7C6 diluted 1/3), Mag. x 50. All tissues are from acute KHVD fish. Scale bar = 100 µm. Black arrows indicate focal positive signals. HP = Hepatopancreas; SKT = Stained kidney tubules.

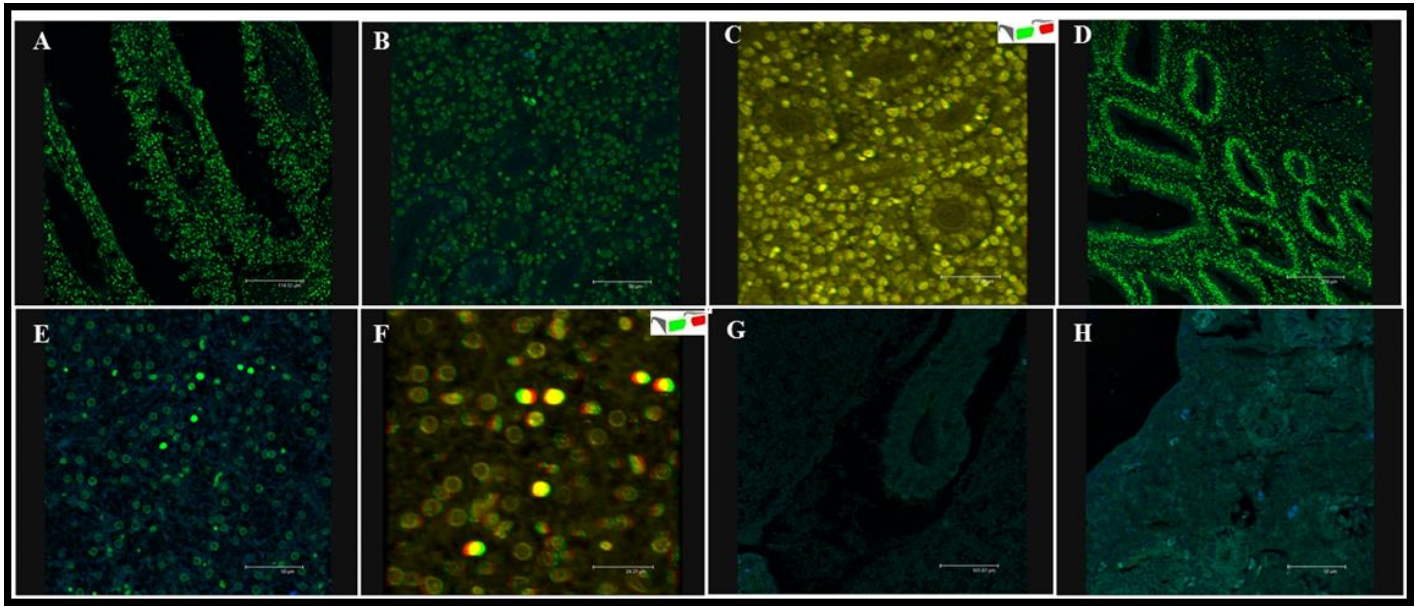


Figure 4.18 IFAT with Koi herpesvirus infected carp tissues after 5 dpi with MAb 20F10 or MAb 10D10 (As.). (A) Positive gills showing a high abundance of signals throughout the filaments (As 10D10 diluted 1/600) (Mag. x 25); (B) Positive kidney showing signals within the interstitial tissue, but also kidney tubules (As 10D10 diluted 1/600) (Mag. x 50); (C) 3D imaging of B showing positive signals expressed throughout the epithelium, but also endothelial cells of the tubules; (D) Positive pyloric caecae showing high abundance of signals observed throughout the epithelium (As. 10D10 diluted 1/600) (Mag. x 25); (E) Positive liver showing focal signals with varying degree of intensity within the parenchyma (As 10D10 diluted 1/600) (Mag. x 50); (F) 3D imaging of E showing the variation in signal intensity amongst possible infected hepatocytes (Mag. x 50); (G) Positive kidney used as secondary antibody control showing no signals (PBS) (Mag. x 25); (H) Negative control kidney showing no signals. (MAb 20F10 used at 60 µg mL^{-1}). All tissues are from acute KHVD fish. Scale bar = 100 µm. Visual interpretation of C and F require 3D spectacles as indicated by

4.3.2.4 Immunological analysis: Detection of anti-KHV antibodies by ELISA and SNT

All fish were negative for anti-KHV antibodies during the first 10 dpi at serum dilutions of 1/50 – 1/1600 as measured by indirect antibody ELISA and SNT according to Bergmann *et al*. (2012, pers. comm.). The surviving carp from the acute KHVD tank sampled after 70 dpi had a specific KHV antibody titre of 1/10,000 and a neutralising antibody titre of 1/45 (results not shown).

4.3.2.5 TEM analysis

Ultrastructure analysis of tissues and peripheral blood leukocytes did not reveal any KHV virus particles at any of the sampling points during acute clinical KHVD (from 5 dpi) when internal virus concentrations were > 400,000 gen. eq. (i.e. in the kidney) (Fig. 4. 19).

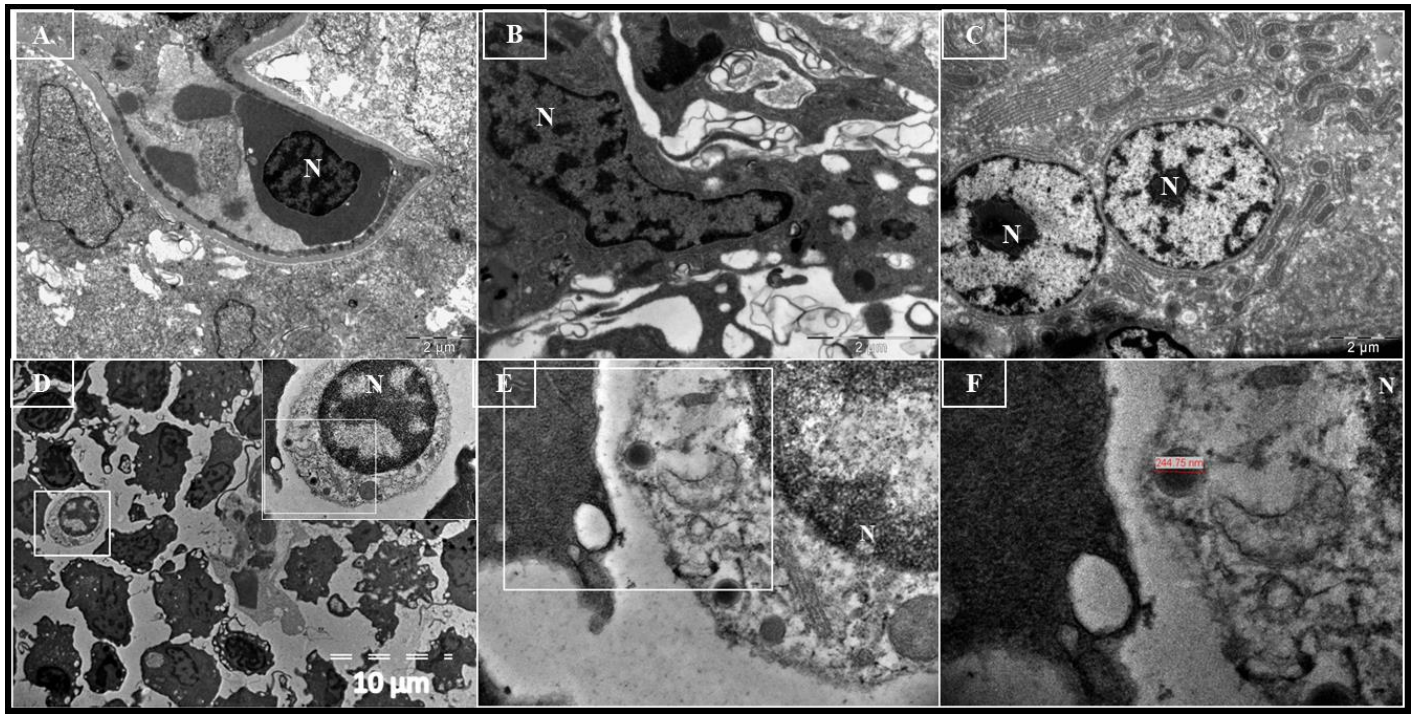


Figure 4.19 TEM micrographs of koi herpesvirus infected carp tissues and blood leukocytes, 10 dpi and 5 dpi, respectively. Organs shown contained the high concentrations of viral DNA (A) Gill tissue where no virus particles were observed, 10 dpi (B) Kidney tissue where no virus particles were detected (C) Cells of gut tissue with no virus particles; (D) Low mag. of concentrated, pelleted peripheral blood leukocytes. The square indicates a putative lymphocyte; (E) High mag. of electron dense spherical structures of interest in putative lymphocyte of D; (F) Measurement of electron dense spherical structure of interest. The large (245 µm) diameter of the structure and the lack of envelope revealed that these structures were not KHV virions. Scale bars are indicated in micrographs. *N* = Nucleus. The cells were not identified, but the gut cells in C appear to be undifferentiated due to the large chromatin dense nucleolus and achromatic nucleus. White box indicates the area magnified for the following micrograph in sequence.

4.3.3 Early molecular detection of KHV

4.3.3.1 Sensitivities of PCR for KHV DNA detection in organs by lethal sampling methods

There was a vast variation in the detection sensitivity of different PCR assays for amplifying KHV DNA in different lethally obtained biopsies during the earliest stages of infection (< 5 dpi) (Fig. 4.20; Table 4.4). The semi-nested PCR and real-time PCR amplified KHV DNA in more samples than any other assay within the first four dpi, although this was still < 50% (Table 4.4). This was in contrast to samples from later stages of infection (≥ 5 dpi) where 6/7 assays were positive for >80% of lethally obtained biopsies (Table 4.4) and all infected fish were confirmed as KHV positive with every PCR assay used. The Gilad PCR, however, gave <70% positive reactions even at these later stages of infection.

The large number of false negative results obtained during the early infection stages from biopsies of fish from both the peracute KHVD tank and acute KHVD tank, resulted in a number of fish falsely diagnosed as negative, where only a total of 3 fish sampled < 5 dpi were detected as positive using the Gilad PCR despite a total of 10 fish being positive by glycoprotein PCR and semi-nested PCR and 11 by real-time PCR (Table 4.4). A large number of false negative results were also obtained with the Gilad nested and TK nested PCRs. The OIE recommended TK PCR was positive for only 2 fish (from 7) from the peracute KHVD tank and 4 from the acute KHVD tank (Table 4.4). False negative screening of these fish at early stages post infection occurred despite 7 different organs being tested individually without pooling of samples.

Table 4.4 Positive KHV tissues using different PCRs

	Presentation of KHVD	Time (dpi)	Gilad	Gilad nest.	TK	TK nest.	Glyco.	Semi-nest	Real-time
[a]% PCR-pos. samples									
	Peracute (*n*=49)	<5 dpi	4.1	20.4	10.2	16.3	34.7	49	26.5
	Acute (*n*=49)	<5 dpi	4.1	10.2	14.3	18.4	16.3	32.7	26.5
	Acute (*n*=42)	≥5 dpi	66.7	90.5	83.3	85.7	88.1	88.1	90.5
	Survivor (*n*=7)	70 dpi	0	0	0	42.9	42.9	0	0
[b]No. pos. fish detected									
	Peracute (*n*=7)	<5 dpi	1	5	2	3	6	5	5
	Acute (*n*=7)	<5 dpi	2	2	4	4	4	5	6
	Acute (*n*=6)	≥5 dpi	6	6	6	6	6	6	6
	Survivor (*n*=1)	70 dpi	0	0	0	1	1	0	0

Abbreviated PCR assays used are: Gilad (Gilad *et al*. 2002); Gilad nest. (Bergmann *et al*. 2006); TK (Bercovier *et al*. 2005); TK nest. (CEFAS 2007 Unpublished); Glycol. (KHV-U, ORF 56) single round (Bergmann *et al*. 2010b); Semi-nest. (Bergmann *et al*. 2010a); real-time (Gilad *et al*. 2004; Bergmann *et al*. 2010a)

[a]**Samples screened by PCR include 7 tissues per fish (gill, skin, spleen, kidney, gut, liver, brain)**

[b]**Fish screened include all peracute KHVD fish (1 hpi – 3 dpi), early acute KHVD fish (1 hpi – 4 dpi) and later acute KHVD fish (5 dpi – 10) dpi)**

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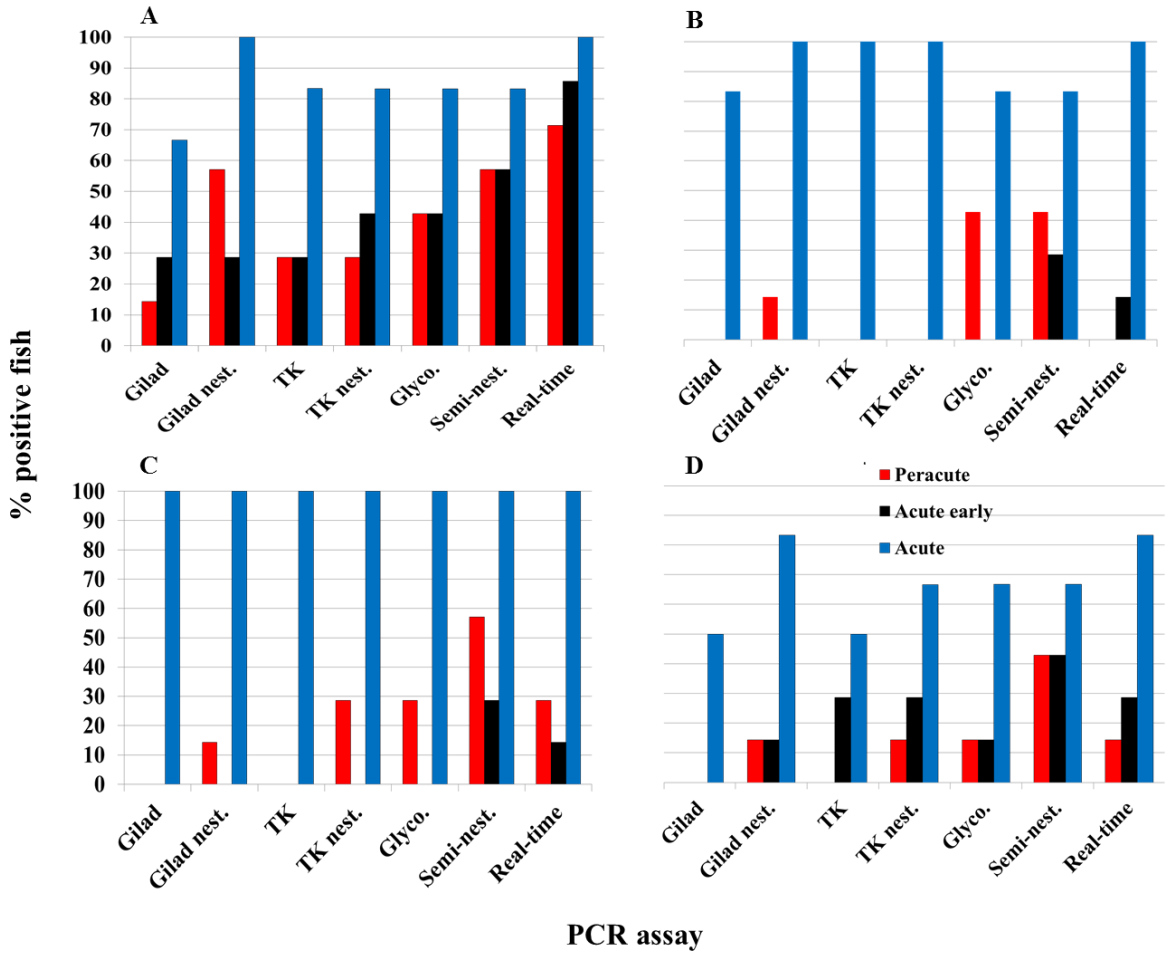


Figure 4.20 Detection of koi herpesvirus (KHV) DNA by various PCR assays in lethally acquired biopsies during peracute, early acute and acute KHVD. (A) gill; (B) spleen; (C) kidney; (D) gut. Bars represent the % of fish positive from early or later stage KHVD. Peracute = Tank B15 (sampled <5 dpi); Acute early = Tank B16 (sampled <5 dpi); Acute late = Tank B16 (sampled ≥ 5 dpi). Abbreviated PCR assays that were used for the same extracted DNA template are indicated on the *x*-axis: Gilad (Gilad *et al*. 2002); Gilad nest. (Bergmann *et al*. 2006); TK (Bercovier *et al*. 2005); TK nest (CEFAS 2007 Unpublished); Glycol. (KHV-U, ORF 56); (Bergmann *et al*. 2010b); Semi-nest. (Bergmann *et al*. 2010a); real-time (Gilad *et al*. 2004; Bergmann *et al*. 2010a)

Investigations undertaken on specific tissue biopsies, namely those recommended by the OIE manual for KHV diagnostics (OIE, 2012), revealed that gill samples provided a reasonably reliable sampling tissue for early KHV detection, but only if the most sensitive assays were employed (Fig. 4.20 A). The semi-nested PCR detected KHV DNA in 57% (4/7) of early (< 5 dpi) sampled fish gill biopsies from both the peracute and acute KHVD fish while the real-time PCR was able to detect 71% (5/7) in fish from the peracute KHVD tank and 85% (6/7) in fish from the acute KHVD tank during the early infection stages (< 5 dpi). All other PCR assays were positive for <50% (Fig. 4.20 A). In the other three organs recommended by the OIE, the spleen, kidney and gut, even the most sensitive PCR assays were unable to detect KHV DNA in the majority of fish (Fig. 4.20 B, C, D). The semi-nested PCR was able to detect KHV DNA following early infection, < 5 dpi, more often than any other of the assays in these tissues with 43% (3/7) and 29% (2/7) positive spleen tissues, 57% (4/7) and 29% (2/7) positive kidney tissues and 57% (4/7) and 29% (2/7) positive gut tissues of fish from the peracute KHVD tank and acute KHVD tank, respectively (Fig. 4.20 B, C, D). During the early stages of infection (< 5 dpi) in only 36% (5/14) of fish could KHV DNA be detected using gut biopsies and 43% (6/14) using spleen and kidney biopsies (not shown). Skin biopsies were also investigated to assess their effectiveness as a diagnostic sample for early KHV detection. The glycoprotein and semi-nested PCRs detected KHV DNA in 57% (4/7) of fish using these samples from the peracute KHVD tank. Interestingly, all other PCRs, including the real-time PCR, were only positive for <50% of fish with these same samples. In only 29% (2/7) of fish could KHV DNA be detected at early stages (< 5 dpi) in acute KHVD fish using any PCR with skin samples (Fig. 4.21).

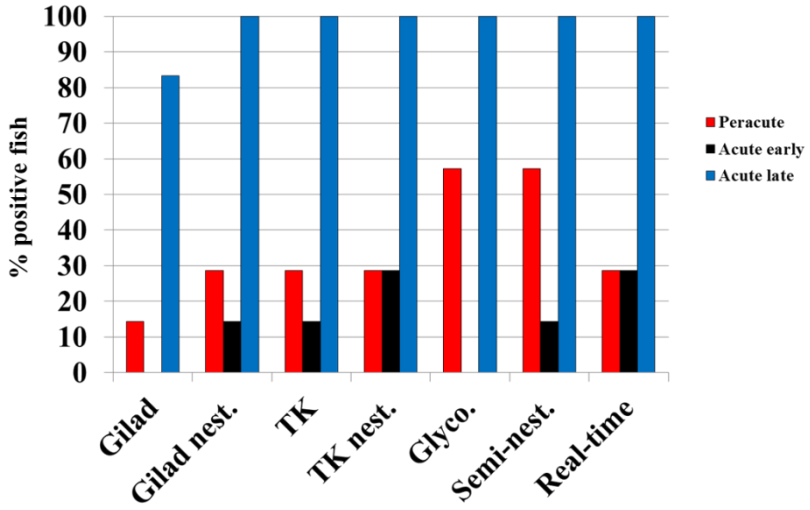


Figure 4.21 Detection of koi herpesvirus (KHV) DNA by various PCR assays in lethally acquired skin biopsies during peracute, early acute and acute KHVD. Bars represent the % of fish positive from the early or late stage KHVD. Peracute = Tank B15 (sampled <5 dpi); Acute early = Tank B16 (sampled <5 dpi); Acute late = Tank B16 (sampled ≥ 5 dpi). Abbreviated PCR assays that were used for the same extracted DNA template are indicated on the *x*-axis: Gilad (Gilad *et al*. 2002); Gilad nest. (Bergmann *et al*. 2006); TK (Bercovier *et al*. 2005); TK nest (CEFAS 2007 Unpublished); Glycol. (KHV-U, ORF 56); (Bergmann *et al*. 2010b); Semi-nest. (Bergmann *et al*. 2010a); real-time (Gilad *et al*. 2004; Bergmann *et al*. 2010a)

The thymidine kinase (TK) gene, was of particular interest as it is deemed one of the most sensitive and commonly used single round PCR assays and is included in the OIE manual for aquatic diseases (OIE, 2012), but could only detect KHV DNA at an early stage in 28% (2/7) of fish from both challenge tanks using gill biopsies and only from the acute KHVD tank using gut samples, whereas all fish were apparently negative using kidney and spleen samples (Fig. 4.20 A-D). Samples of liver and brain tissues did not provide a better alternative to OIE recommended tissues (gills, spleen, kidney and gut) for detecting KHV DNA during these early stages of infection (not shown). At later stages of the infection (5-10 dpi), most fish were detected positive using all PCR assays in these tissues where 86% of all PCR reactions were positive for fish tissues compared to just 33% during the earlier stages of

infection (not shown), although a relatively high number of false negatives were still observed with the single round Gilad PCR. After 5 dpi all kidney samples and >80% of spleen and gills were positive for KHV DNA by all PCRs, apart from the Gilad PCR, where <70% of fish were still detected positive in gill samples (Fig. 4.20 A-C). In spite of this, even at relatively late stages of infection, i.e. 9 dpi, discrepancies were still observed in OIE recommended samples, such as gut biopsies, using highly sensitive nested PCR assays (Fig. 4.22).

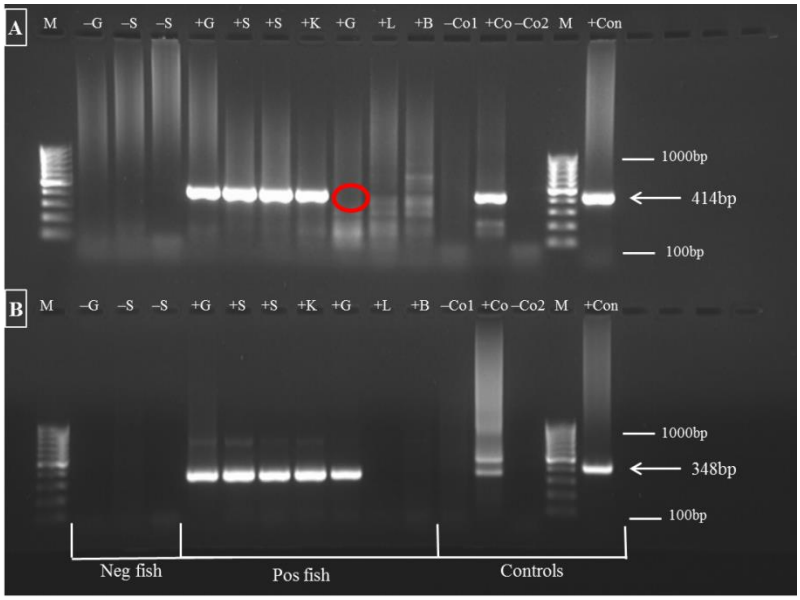


Figure 4.22 Agarose gel electrophoresis (1.5%) of PCR products showing discrepancies between PCR diagnostic assays in acute koi herpesvirus infected carp, 9 dpi. Even at late stages there were discrepancies in the detection of KHV DNA in the same fish by lethal sampling depending on the biopsy sample. (A) Gilad nested PCR detecting a 414 bp product, (B) TK nested PCR detecting a 348 bp product. Note the false negative result in the gut by the Gilad nested PCR compared to the TK nested PCR (red circle). Headers for lanes: M = marker ladder; -G = neg. gill; -S = neg. skin; -S = neg. spleen; +G = pos. gill; +S = pos. skin; +S = pos. spleen; +K = pos. kidney; +G = pos. gut; +L = pos. liver; +B = pos. brain; -con 1 = neg. control 1; +con = pos. control; -con 2 = neg. control 2; M = marker ladder; + con = pos. control

Real-time PCR was sometimes negative where other PCR assays, such as Gilad nested, TK nested and glycoprotein PCR, were positve in the spleen, kidney and gut (Fig. 4.20 B, C, D).

4.3.3.2 Sensitivities of PCR for KHV DNA detection in mucus and white blood cells by non-lethal sampling methods

Using non-lethal samples, > 75% were positive for KHV DNA at the early stages of infection (< 5 dpi) with 5/7 of the PCR assays, including the TK PCR (Table 4.5). The Gilad PCR and nested PCRs, however, still produced many false-negative results at this stage with the Gilad PCR detecting KHV DNA in only 18% and 29% of non-lethally acquired samples from the peracute and acute KHVD tanks, respectively. Furthermore, all fish, but 1 from the acute KHVD tank, could be detected as KHV-positive by all PCR assays during the first 4 dpi using these samples except for the Gilad and nested Gilad PCR assays (Table 4.5).

All fish tested positive with the real-time PCR using non-lethal mucus swabs, regardless of the time post-infection (Fig. 4.23 A, B, C). Glycoprotein gene PCR and semi-nested PCR, were the most effective for KHV detection in leukocytes, but were still only positive for 42% (3/7) and 57% (4/7) of fish from the peracute KHVD tank, respectively and 71% (5/7) and 43% (3/7) from the acute KHVD tank, respectively, during the early infection stages (Fig. 4.23 D). A noteworthy observation was the fewer positive leukocyte samples by real-time PCR during the early infection stages compared to conventional PCRs: TK PCR, TK nested PCR, glycoprotein PCR and semi-nested PCR (Fig. 4.23 D). At the later stages of infection KHV DNA could be detected in >70% of fish leukocytes by all assays except the Gilad PCR (Fig. 4.23 D). Detection efficiencies of TK PCR, TK nested PCR, glycoprotein PCR and semi-nested PCR were equivalent to real-time PCR for detecting KHV in fish

during early stages using mucus swabs of skin, i.e. fin base swab and skin swab and blood leukocytes (Fig. 4.23 B - D).

No inhibition was observed in any of the PCRs including real-time qPCR assays according to the internal control used and all negative controls (template controls and uninfected fish tissues) were negative suggesting no false-positive results had occurred throughout the analysis.

4.3.3.3 KHV DNA detection in surviving carp by lethal sampling methods using PCR

Only one fish survived the highly virulent experimental challenge, which appeared completely healthy with no signs of disease when sampled at 70 dpi.

The nested PCR detecting the thymidine kinase gene (CEFAS, unpublished 2007) and single round PCR detecting the glycoprotein gene were the only PCR assays able to detect KHV DNA in the tissues of this fish. The gill, spleen and kidney were positive by the nested TK gene PCR, while the spleen, kidney and gut were positive by the single round glycoprotein gene PCR (Table 4.4).

Table 4.5 Positive detection of KHV in white blood cells and mucus using different PCRs

	Presentation of KHVD	Time (dpi)	Gilad	Gilad nest.	TK	TK nest.	Glyco.	Semi-nest	Real-time
[a]% PCR-pos. samples									
	Peracute (*n*=28)	<5 dpi	17.9	60.7	78.6	78.6	85.7	89.3	78.6
	Acute (*n*=28)	<5 dpi	28.6	50	75	75	78.6	75	85.7
	Acute (*n*=24)	≥5 dpi	66.7	91.7	91.7	91.7	95.8	87.5	95.8
	Survivor (*n*=4)	70 dpi	0	0	0	100	100	0	75
[b]No. pos. fish detected									
	Peracute (*n*=7)	<5 dpi	2	6	7	7	7	7	7
	Acute (*n*=7)	<5 dpi	4	4	6	6	6	6	6
	Acute (*n*=6)	≥5 dpi	5	6	6	6	6	6	6
	Survivor (*n*=1)	70 dpi	0	0	0	1	1	0	1

Abbreviated PCR assays used are: Gilad (Gilad *et al*. 2002); Gilad nest. (Bergmann *et al*. 2006); TK (Bercovier *et al*. 2005); TK nest. (CEFAS 2007 Unpublished); Glycol. (KHV-U, ORF 56) single round (Bergmann *et al*. 2010b); Semi-nest. (Bergmann *et al*. 2010a); real-time (Gilad *et al*. 2004; Bergmann *et al*. 2010a)

[a]Samples screened by PCR include 3 mucus swabs per fish (gill swab, skin swab, fin base swab) and separated peripheral blood leukocytes

[b]Fish screened include all peracute KHVD fish (1 hpi – 3 dpi), early acute KHVD fish (1 hpi – 4 dpi) and later acute KHVD fish (5 dpi – 10) dpi)

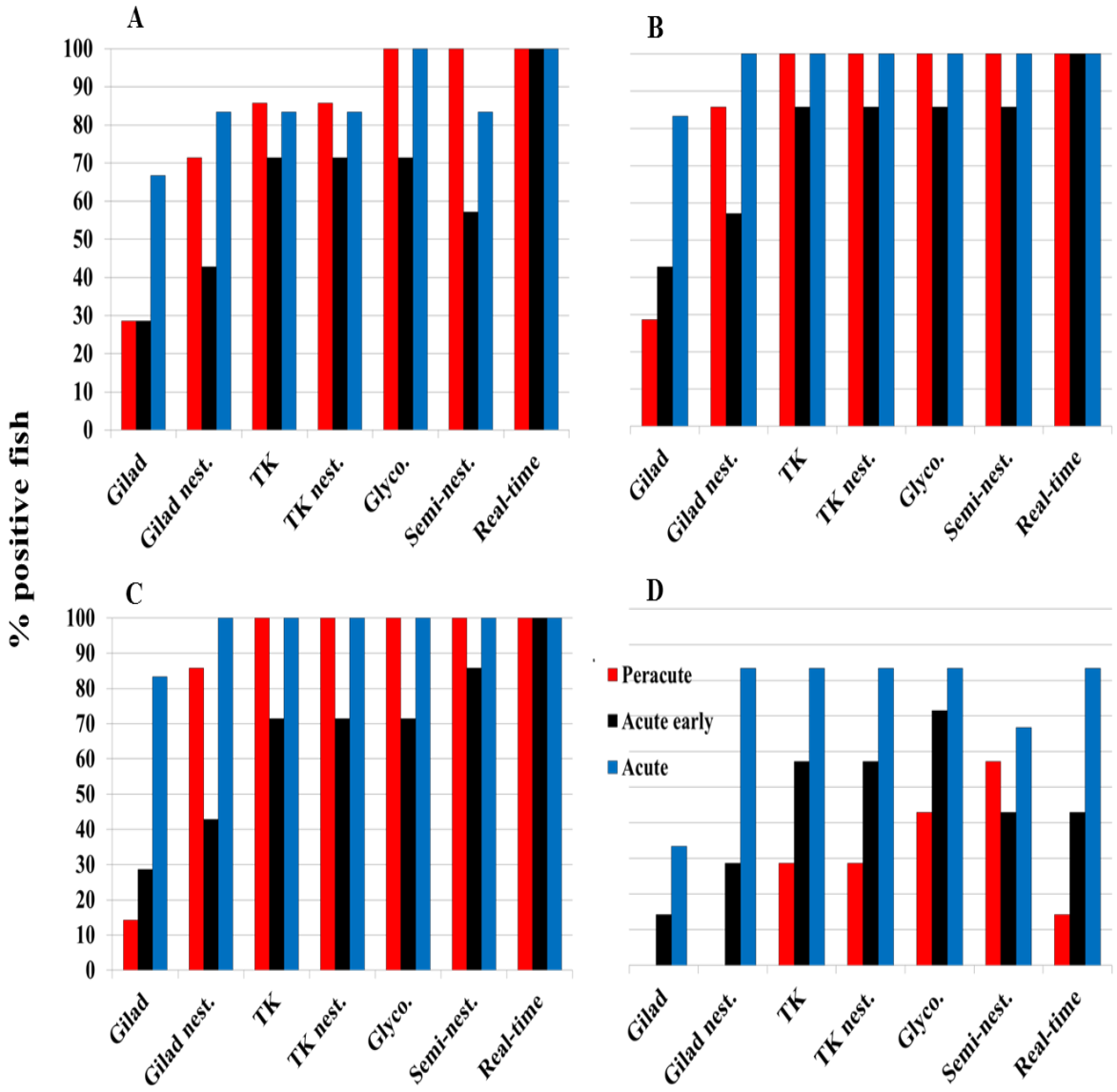


Figure 4.23 Detection of koi herpesvirus (KHV) DNA by various PCR assays in non-lethally acquired mucus and blood leukocytes during peracute, early acute and acute KHVD. (A) gill swab; (B) skin swab; (C) fin base swab; (D) blood leukocytes. Bars represent the % of fish positive from the early and late stages post infection. Peracute = Tank B15 (sampled <5 dpi); Acute early = Tank B16 (sampled <5 dpi); Acute late = Tank B16 (sampled ≥ 5 dpi). Abbreviated PCR assays that were used for the same extracted DNA as template are indicated on the *x*-axis: Gilad (Gilad *et al*. 2002); Gilad nest. (Bergmann *et al*. 2006); TK (Bercovier *et al*. 2005); TK nest. (CEFAS 2007 Unpublished); Glycol. (KHV-U, ORF 56); (Bergmann *et al*. 2010b); Semi-nest. (Bergmann *et al*. 2010a); real-time (Gilad *et al*. 2004; Bergmann *et al*. 2010a)

4.3.3.4 KHV DNA detection in surviving carp by non-lethal sampling methods using PCR

Only a few of the PCR assays provided positive signals from the non-lethally sampled surviving and unstressed carp. The nested TK gene PCR and the glycoprotein gene PCR were positive for all swab samples and leukocytes. Real-time PCR was only positive for skin swabs and fin base swabs while the Gilad nested PCR was positive for a single fin swab (Table 4.5). Faeces samples had also been collected, but were only positive by the single round glycoprotein gene PCR (data not shown).

4.4 Discussion

4.4.1 Pathogenesis of KHV during peracute and acute disease

A number of studies have previously been undertaken to investigate KHV pathogenesis using histological methods (Miyazaki *et al*., 2008; El-Din, 2011), molecular methods (Gilad *et al*., 2004; Pikarsky *et al*., 2004; Adamek *et al*., 2013), and bioluminescent imaging (Costes *et al*., 2009; Raj *et al*., 2011; Fournier *et al*., 2012), which highlighted KHV entry and dissemination through the tissues within the first few hours and days of infection. In the current study, analysis of KHV pathogenesis was undertaken within a much narrower window following virus exposure. The first few hours of infection, i.e. in 10 fish from 1-8 hpi, as well as the following 10 days were investigated by exposing carp to a highly virulent virus isolate by immersion to simulate a natural infection. Detection of viral DNA within the tissues was achieved using ISH and the concentration of viral DNA was determined using a TaqMan real-time qPCR (Gilad *et al*., 2004).

Despite analogous challenge conditions, differential disease progression was observed between the 2 tanks of carp infected with KHV, which is not uncommon (Shapira *et al*. 2005,

Fuchs *et al*. 2011). Considering this, together with the rapid mortality in fish exhibiting peracute (100 % mortality in 3-4 dpi, tank B15) and acute (83 % mortality in 7-11 dpi, tank B16) KHVD in the current study, which is similar to previous reports of experimental KHV infections of carp by cohabitation, ip injection and immersion (Perelberg *et al*., 2003; Pikarsky *et al*., 2004; Dishon *et al*., 2005; Antychowicz *et al*., 2005; Shapira *et al*. 2005, Bergmann *et al*. 2010b; Dong *et al*., 2013), emphasises the necessity for reliable diagnostics and highlights the need for early detection to control this virus and minimise its unpredictable disease transmission. Although clinical KHVD was not observed internally during the early stages of the infection, some disease signs were evident in infected fish as early as 3 dpi. These included excessive mucus secretion, which may explain the early development of disease signs such as enophthalmos and progressive body emaciation as a result of dehydration.

The lack of notable pathognomonic disease signs in infected fish, in spite of the hyper-virulent nature of the virus isolate, is similar to that described by Perleberg *et al*. (2003), where fish were asymptomatic during the first 5 dpi, but >90% mortality occurred within the first 11 days post exposure (dpe) in fish challenged by cohabitation, and mortality rates did not differ considerably in fish infected by immersion or ip injection. Clinical signs are also often lacking in natural outbreaks of the disease (Sano *et al*., 2004; Tu *et al*., 2004). Although the early stages of KHVD have been investigated previously, these have only focused on the pathogenesis predominantly post 24 hpi, although Costes *et al*. (2009) did report preliminary findings as early as 12 hpi, and most recently Adamek *et al*. (2013) reported findings focused specifically on infected carp skin, from 6 and 12 hpi. In the present study, samples were taken from 7 organs, blood leukocytes and mucus during the first 8 hpi, the results of which support previous suggestions for the portal of entry of KHV through the gills (Hedrick *et al*., 2000;

Pikarsky *et al*., 2004; Yasumoto *et al*., 2006; Miyazaki *et al*., 2008; El-Din, 2011) or gut (Perelberg *et al*., 2003; Dishon *et al*., 2005; Lee *et al*., 2012) questioning whether the skin is actually the only portal of entry after infection of the virus by immersion as previously suggested (Costes *et al*., 2009; Fournier *et al*., 2012).

Role of gills as portal of virus entry

High virus copy numbers were found attached to gill and skin mucus within 1 hpi, particularly with peracute KHVD fish (80,000 gen. eq.), but also with fish from the acute KHVD tank (10,000 gen eq.). Viral DNA in the skin mucus varied between fish and tanks during the first dpi, but was always >1,000 gen eq. In contrast, KHV DNA concentrations in gill mucus declined from 1 - 8 hpi in both tanks, which was possibly associated with virus uptake through the gills. As there was approximately >100-fold more KHV DNA in gill and skin tissues of peracute KHVD carp within a very narrow window post exposure, (i.e. 1-2 hpi), this may reflect the difference in disease progression between the two tanks. Higher copy numbers were observed in acute KHVD tank fish at the later time point of 4 hpi (100 gen. eq.), compared to the peracute KHVD tank after 6-8 hpi when <10 gen eq. were present in gill tissue of fish from both tanks. Although it cannot be certain that every fish was infected with KHV at 0 hpi, and despite only one fish being sampled at that time point, it can only be hypothesised that a much higher intake of virus occurred within the initial 2 hpi in the peracute KHVD-fish compared to a progressive accumulation of virus up to 4 hpi that resulted in more typical acute KHVD.

More intensive positive signals were seen in gill sections of peracute KHVD fish compared to acute during the first 4 hpi by ISH, which was in agreement with higher viral loads in gill mucus and tissue by qPCR. This supports the hypothesis that higher

concentrations of attached virus to gill epithelial cells resulted in the peracute infection if virus entry was via the gills. The increasing intensity of ISH signals in gill filament tips, within the respiratory epithelium and at filament bases in close proximity to the central venous sinus, during the first 8 hpi also suggests that gills may be involved in early KHV pathogenesis. Although there were signals in the mucus, this occasionally occurred in fish sampled at 0 hpi, which may have resulted from the probes sticking non-specifically to the mucus. Lee *et al*. (2012) and Bergmann *et al*. (2006) also reported strong ISH signals in gill epithelial cells and basal mucus cells in KHV-infected carp tissues, and Pikarsky *et al*. (2004) reported extensive gill pathology with a similar KHV experimental infection. They showed a loss of lamellae and a mixed inflammatory cell infiltrate after just 2 dpe. Miyazaki *et al*. (2008) showed, by ultrastructural analysis of KHV infected carp gills after a per-gill inoculation method, that respiratory epithelial cells contained intranuclear inclusion bodies and exhibited vacuolation and nuclear degeneration. They also observed macrophages containing intranuclear inclusion bodies within the lumen of the gill lamella capillaries (Miyazaki *et al*., 2008). This may explain viral loads detected internally in blood leukocytes in 3/4 fish between 6-8 hpi in the current study, which may be through a similar method used by other viruses to cross mucosal epithelial layers where infected macrophages migrate across the epithelium (Bergelson, 2003). The resulting viraemia may lead to the rapid dissemination of virus that can be detected in multiple internal organs within the first day of infection (Gilad *et al*., 2004; Pikarsky *et al*., 2004). This is followed by complete effacement of gill architecture and severe inflammation characterised by necrosis and sloughing of surface epithelium and mucus from 6–10 dpe (Pikarsky *et al*., 2004; Ilouze *et al*., 2006a). Although viral DNA in the gills of infected fish was not quantified in their study, they proposed that the virus enters through the gills where it replicates, resulting in this pathology,

which agrees with similar pathological findings of Hedrick *et al*. (2000). Furthermore, a previous challenge model (and subsequent experimental challenges (Yasumoto *et al*., 2006)) for KHV by per-gill inoculation resulted in intensive infection of gill respiratory epithelium containing large numbers of virus particles, as well as systemic infection in kidney, spleen, liver and blood macrophages observed by light microscopy and TEM (Miyazaki *et al*., 2008; El-Din, 2011). Internal pathology induced by this route of infection occurred within 6-7 dpi (El-Din, 2011). No such pathology was noted in the current study, despite high internal viral loads. The involvement of the gills in KHV entry is, however, also contested as KHV-specific transcripts were not found in experimentally infected carp early in the infection (Dr. Sven Bergmann pers. com.). Although the detection of KHV DNA in peripheral blood leukocytes by 6 hpi is earlier than the expected time interval for completion of KHV DNA synthesis of >8 hpi, which has been demonstrated *in vitro* (Ilouze *et al*., 2012b). Therefore, replication may not have been necessary for entry into the bloodstream.

Role of skin as portal of virus entry

Carp skin has recently been proposed as the major portal of entry of KHV using a recombinant virus expressing luciferase as the challenge isolate, with subsequent bioluminescent imaging (Costes *et al*., 2009; Fournier *et al*., 2012). Strong signals were recorded in the skin 24 h post challenge with this model by bath immersion and signal intensity increased during the challenge, however, no signals were observed internally until later infection stages (Fournier *et al*., 2012). In the current study strong signals were observed by ISH in the skin mucus throughout the first day of infection, however, KHV DNA could only be detected in gills and not in the skin of fish in the acute KHVD tank during the first 2 hpi, suggesting no uptake of virus via the skin in these fish. Positive signals by ISH were first

associated with skin tissue after 6 hpi. KHV DNA was also detected at low levels in the skin of some fish in a recent study after 6 hpi by real-time PCR (Adamek *et al*., 2013), in agreement with the current study where 1000 gen eq. can be detected in skin biopsies after only 1 hpi in the peracute KHVD tank, although KHV-specific transcripts of the TK gene were not detected until 12 hpi in the previous study (Adamek *et al*., 2013). *In vitro*, DNA replication of KHV has been shown to occur within 4-8 hpi and the presence of RNA transcripts were observed in the same study after only 1 hpi (Ilouze *et al*., 2012b). Furthermore, the TK gene is an early gene and transcripts for this gene are found prior to DNA synthesis after only 2 hpi (Ilouze *et al*., 2012b). Therefore, if KHV replication first occurs within the skin, the presence of transcripts would also be expected after 6 hpi in synchrony with the presence of viral DNA. However, Adamek *et al*., (2013) did not detect TK transcripts until >6 hpi, which suggests that the skin may be a secondary site of infection, at least from these samples. High copy numbers found in skin biopsies at early infection stages may constitute residual virus from skin mucus. Detecting the presence of KHV DNA within skin epithelium was unsuccessful by ISH, possibly due to over-digestion of some sections during processing. There may therefore have been positive epidermal cells that were missed during analysis. It is possible that the viral DNA detected at these early stages is due to attachment and entry of the virus through the skin. However, based on the detection of KHV in blood leukocytes in this study after 6 hpi, it is less likely that KHV replication occurred in the skin prior to dissemination through the blood to the internal organs as previously proposed (Costes *et al*., 2009; Fournier *et al*., 2012). However, considering the effective anti-viral properties of the mucus of healthy carp, it is more likely that the skin represents a portal of entry following damage to the epidermal and mucosal layers through which, as reported by Raj *et al*. (2011), removal of the protective carp mucus and epidermal

cell layers enhances KHV transmission through the skin. Raj *et al*. (2011) also demonstrated the neutralising activity of mucus against KHV and inhibition of KHV attachment to epidermal cells. The skin mucosal layer of fish, which has effective anti-microbial properties (Nagashima *et al*., 2003; Esteban, 2012) may not only be able to trap pathogens and immobilise them (Cone, 2009; Esteban, 2012), but by doing so pathogens may subsequently be removed from the skin by the water current (Mayer, 2003 cited in Esteban, 2012). Mucin fibres form a mesh, which often contain pores that are too big to prevent small sized capsid viruses from penetrating mucosal barriers (Cone, 2009; Lai *et al*., 2009). However, larger enveloped viruses may be effectively trapped, which was demonstrated for HSV-1, with a diameter of ~180 nm (Lai *et al*., 2009), and this may therefore also constitute an effective system of carp mucus against the large 180-230 nm infectious KHV virion (Hedrick *et al*., 2000), making the skin a less likely portal of entry. Continuous secretion of mucus during KHV infections may result in the skin patches (and sandpaper texture) (Adamek *et al*., 2013) and lesions characteristic of KHVD (Hedrick *et al*., 2000; Antychowitcz *et al*., 2005). Such lesions may provide ideal secondary sites for virus entry when this effective anti-viral mucosal barrier can be breached.

Infection via the skin does appear to be a realistic route of infection for KHV following injury and/or in the absence of mucus as virus replication has been observed in the epidermis by TEM after 3 dpi (Costes *et al*., 2009), although this does not conclude that KHV targets skin epithelial cells specifically as the virus is able to use receptors found on many different cell types permitting multiple organ tropism, which has also been demonstrated extensively in other studies using TEM (Bretzinger *et al*., 1999; Hedrick *et al*., 2000; 2005; Perelberg *et al*., 2003; Choi *et al*., 2004; Miyazaki *et al*., 2008; Cheng *et al*., 2011; El-Din, 2011; Matras *et al*., 2012). Although no virus particles were detected by TEM in the current study. The skin

may therefore constitute a secondary site of replication following systemic infection through the blood. In the study by Fournier *et al*. (2012), KHV was detected in the skin after 2 dpi in fish infected through oral inoculation, thus it is likely that the virus was transported through the blood to the skin, although the authors suggest virus particles shed from the infected fish entered the skin externally. In similar studies on pathogenesis, mechanically-induced wounding resulted in increased bacterial loads at the site of injury in studies using *Aeromonas hydrophila* in crucian carp (Chu and Lu, 2008), similar to the study carried out by Raj *et al*. (2011) for KHV in *C. carpio*. This was in contrast to the unwounded control group, whereby minimal bacterial loads were detected in the muscle and the majority of bacteria were instead found in the gills as early as 2 hpi (Chu and Lu, 2008). As the strong signals obtained by bioluminescence occurred after 24 hpi (Costes *et al*., 2009; Fournier *et al*., 2012), it is possible that KHV effectively replicates and is then shed via the skin following systemic infection, which based on the results of the current study, can be rapid. Replication in skin epithelial cells has been demonstrated after only 12 hpi by molecular methods (Adamek *et al*., 2013). If secondary infection occurs in the epidermis followed by subsequent excretion and removal by mucus, this may explain the increased viral loads detected in the skin by bioluminescence in previous studies (Costes *et al*., 2009; Fournier *et al*., 2012) and by real-time qPCR by Adamek *et al*. (2013) and in the current study. This would then also explain the significantly greater concentrations of KHV DNA in the mucus at the later stages of the acute KHV infection in the current study, i.e. > 5 dpi. Pathogenesis studies with alloherpesviruses, e.g. CCV, revealed significantly lower viral loads detected in skin tissue and in tank water using isolates with disrupted TK genes compared to the wild type virus indicating that the virus was effectively shed via the skin (Kancharla and Hanson, 1996). Strong bioluminescence signals associated with the skin have been observed in the early

stages of the infection with other fish viruses, most predominantly in the fins (Harmache *et al*., 2006). For example, the rhabdovirus, IHNV, was found to accumulate in the fins after 8 hpi as well as within mucus of both naturally and experimentally infected rainbow trout (La Patra *et al*., 1989a; b). Since the latter study demonstrated infectious virus in the skin mucus, not only after bath exposure to the virus, but also ip challenge, the mucus may contain excreted virus from secondary replication within the skin, which may explain the strong signals observed in the skin by bioluminescence for IHN-infected trout (Harmache *et al*., 2006) and KHV-infected carp (Costes *et al*., 2009; Fournier *et al*., 2012) and the high DNA viral loads found at later stages in both mucus and skin tissue by Adamek *et al*. (2013) and in the current study. Gilad *et al*. (2004) did not measure KHV DNA concentrations in the skin, but reported high viral loads in the mucus, also at very early stages post infection (> 1 dpi), which supports the hypothesis of virus excretion via the skin into the mucus.

The skin has been shown to be an important organ, and possibly a target organ, during early KHV pathogenesis, especially as replication has been demonstrated in skin epithelium (Costes *et al*., 2009; Fournier *et al*., 2012; Adamek *et al*., 2013) and once carp are infected, the protective mucosal barrier and other aspects of skin immunity are affected, possibly immunomodulated by KHV, resulting in increased infection of the skin by secondary facultative pathogens (Adamek *et al*., 2013). This disruption also likely enhances KHV infection through which the skin then becomes an ideal portal for the virus, replicating and shedding progeny virus into the mucus and surrounding water.

Role of gut as portal of virus entry

Low viral loads were initially found in the gut of fish in the acute KHVD tank 4 hpi, and in blood leukocytes after 6 hpi in both tanks, while all other organs were negative by real-time

qPCR. Using ISH it was possible to detect KHV DNA in the gut and in the blood vessels of the spleen, kidney and liver after only 1 hpi, while the brain was negative. Furthermore, KHV DNA could be detected in gut contents by ISH in some sections during the first day of infection, suggesting that the intestine could represent a possible portal of entry for the virus following ingestion of virus contaminated food and/or water. The intestine has previously been proposed as a possible portal of entry for KHV (Perelberg *et al*., 2003; Gilad *et al*., 2004; Dishon *et al*., 2005; Ilouze *et al*., 2006a; 2011; El-Din, 2011; Lee *et al*., 2012), and transcripts of the KHV TK gene have been detected in the intestinal epithelium (Syakuri *et al*., 2013). Fournier *et al*. (2012) suggested that the pharyngeal peridontal mucosa is a portal of entry following oral inoculation, based the presence of bioluminescence signals, but not in the intestines within the first 24 hpi. However, it is likely that the pharyngeal peridonatal mucosa represents a site of virus entry following mucosal damage, i.e. by mastication during food processing, as the authors discussed (Fournier *et al*., 2012), similar to the entrance of virus through the skin by wounding (Raj *et al*., 2011). Considering that all other internal tissues were negative by ISH during this narrow window of 2 hours following virus exposure, and during the first 8 hpi by qPCR, the gut may represent a portal of entry and site of initial replication after which the virus is disseminated through the blood by leukocytes. Although the early detection of KHV DNA in blood leukocytes after 6 hpi, again suggests that KHV is translocated to the blood before DNA synthesis within infected cells has been completed. This finding is in agreement with ISH analysis undertaken on archival tissues of KHV infected carp in Korea where strong signals were detected particularly in mucus and goblet cells of the intestinal epithelium (Lee *et al*., 2012). Positive detection of KHV DNA in macrophages of the intestine and melanomacrophage centres of the spleen and kidney, led Lee *et al*. (2012) to hypothesise that the virus spreads via macrophages after phagocytosis of

infected intestinal epithelial cells, which then migrate to the tissues via the blood causing a systemic infection. The fact that KHV is detected in the blood vessels of various tissues during the first day of infection, may explain the lack of detection by qPCR from DNA extractions because of the limited virus copy numbers. Early KHV viraemia and dissemination via the blood corroborates earlier findings of DNA detection in blood after 1 dpi (Pikarsky *et al*., 2004), and multiple tissue tropism of the virus via the blood after 1 dpi (Eide *et al*. 2011a; Fournier *et al*. 2012). A number of studies have isolated infectious virus from blood of infected fish (Kempter *et al*., 2009; Eide *et al*., 2011a; b; Dong *et al*., 2013; Matras *et al*., 2012). This presence of KHV in blood leukocytes may explain the negative results for KHV detection in gills and gut by bioluminescence until much later in the infection (i.e. no signals were evident in the gut following oral inoculation until 6 dpi) (Fournier *et al*., 2012). Thus, bioluminescence may lack the sensitivity to detect low levels of virus in internal; organs compared with molecular tests, such as the TaqMan real-time PCR used in the study by Gilad *et al*. (2004) and the current study.

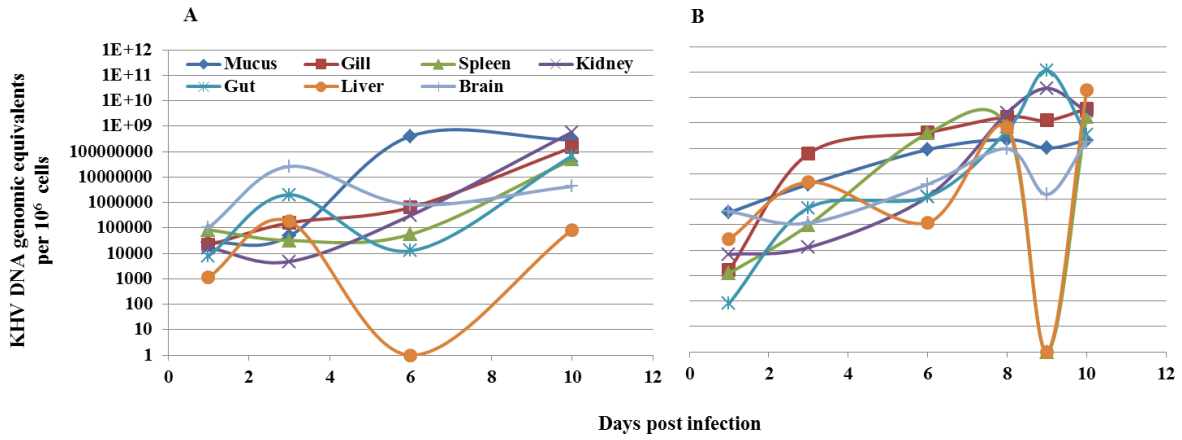
Later stages of KHV infection (> 5 dpi)

The strong bioluminescent signals detected in tissues later in the infection by Fournier *et al*. (2012), are in agreement with the increased viral loads detected after 4 dpi in the current study and in the study of Gilad *et al*. (2004). After 1 dpi a dramatic reduction in viral DNA was seen in gill mucus (<10 gen. eq.) in fish from the peracute KHVD tank, whereas a large increase in copy numbers after 4 dpi in gill mucus occurred in fish from the acute KHVD tank (>100,000 gen. eq.). Concentrations of viral DNA also increased in the gills of fish from the acute KHVD tank, although more gradually with no more than 100 gen. eq. detected

during the first 4 dpi, which may further explain the negative results reported by Fournier *et al*. (2012) using bioluminescence.

Within these first 4 dpi ISH signals were still limited in most sections, with gills always producing strong signals while the brain remained negative. After 5 dpi there was a massive increase in viral DNA observed in all tissues by qPCR, which was significantly higher than concentrations in tissues of peracute and early acute (< 5dpi) infection fish. Elevated signal abundance in ISH sections was also evident. Positive signals were observed in blood vessels, while a large number of gill epithelial cells, interstitial kidney tissue, splenocytes and liver hepatocytes, which corresponded to as much as 400,000 KHV gen. eq. in the kidney from 5-10 dpi and up to 50,000 copy numbers in all other positive tissues. Such findings corroborate the TEM results of Miyazaki *et al*., (2008) with virus particles found throughout all tissues and the brain, consisting of expanded blood vessels, possibly resulting in the neurological disturbances caused by KHVD. This trend was similar to that reported by Gilad *et al*. (2004) (Fig. 4.24) with higher concentrations of virus in tissues after 6 dpi at water temperatures between 18°C - 23°C. High viral loads were found in skin mucus, kidney tissues as well as the particularly high concentrations in intestines, in fish challenged in the study by Gilad *et al*. (2004) (Fig. 4.24). This supports the hypothesis of Dishon *et al*. (2005) that the intestine represents a portal of entry for KHV, but also acts as a site for virus excretion (Ilouze *et al*., 2006a; 2011). Dishon *et al*. (2005) reported the presence of KHV DNA, antigen and infectious virus particles in the faeces and secreted intestinal products of carp from 5-8 dpi. A recent study, where low KHV copy numbers were found in intestinal tissue after just 3 dpi and increased with time, reported on the inflammation and up-regulation of claudin proteins associated with tight junctions of epithelial cells in the gut mucosa, which was thought to be a

carp response to KHV-induced enteritis (Syakuri *et al*., 2013), possibly at the initial site of infection.



Data graphically illustrated is taken from the publication by Gilad *et al*. (2004)

Fig. 4.24 Graphical representation of KHV viral DNA concentrations in experimentally challenged fish in the study by Gilad *et al*. (2004). Two of the temperatures used during the challenge period of the trial are depicted, which are within the range most commonly associated with KHV outbreaks. (A) KHV DNA concentrations reported during the first 10 dpi at 18°C (B) KHV DNA concentrations reported during the first 10 dpi at 23°C. Graphs are presented on a logarithmic scale.

Based on the results of the current and other studies, the skin may not be the major portal of entry, as stated by Costes *et al*. (2009), and the gut, and possibly the gills, may still play a part in the early pathogenesis of KHV. Nonetheless, due to their role in pathogenesis, all these tissues may represent important sampling targets for KHV diagnostics

4.4.2 Detection of KHV during the early infection stages

The sensitivity of a number of diagnostic methods was subsequently investigated to determine the influence that KHV pathogenesis has on acute phase diagnostics and what sampling techniques and virus targets are most suitable for detection. As fish only harbour a

low level viraemia at this stage, the sensitivity of cell culture isolation is limited for KHV detection, despite being described as the most sensitive technique for diagnosis and surveillance for all other viral fin-fish diseases (OIE, 2012), particularly during potentially latent infections. However, even during clinical disease, problems have been reported on the isolation of KHV by cell culture (Sano *et al*., 2004; Matras *et al*., 2012; Yuasa *et al*., 2012b), which can only be achieved during a short window of KHVD in moribund fish, i.e. between 7-11 dpi (Matras *et al*., 2012). For example infectious virus was not detectable in CCB cells following inoculation with carp kidney and brain homogenates of infected fish after 3 dpe, whereas detectable levels were observed in gill, kidney and brain after 7 dpe (Yuasa *et al*., 2012b). In another study infectious virus was not detected from infected carp faeces extracts until 7-8 dpe (Dishon *et al*., 2005). The lack of detection at this very early stage in the infection was in spite of the high and rapid mortality rates that were observed (Dishon *et al*., 2005; Yuasa *et al*., 2012b) and as Bergmann *et al*. (2009a) stated: "isolation of KHV by cell culture methods is difficult even during an acute phase of infection", requiring a high titre for successful growth, highlighting the challenges of early KHV diagnosis. In the current study difficulties were also experienced with detecting KHV at an early stage of infection despite the considerable virulence of the isolate.

The focal localisation of ISH signals in KHV positive tissues possibly explains the false negative PCR results during earlier stages of infection. Despite intense signals in gill filaments during the first 4 dpi, relatively low copy numbers were detected and some samples were negative. Similar outcomes have been reported for other aquatic herpesvirus infections, e.g. in Atlantic cod (Marcos-Lopez *et al*., 2011) where focal infection of gill filaments observed by ISH did not always correspond to positive results by PCR. These erroneous negative results from the early stages of infection demonstrated the high sensitivity of the

ISH method, even compared to the highly sensitive real-time-qPCR (Gilad *et al*., 2004). ISH has been used in a number of studies to detect KHV DNA in infected tissues and blood leukocytes of carp (Bergmann *et al*., 2006), other potential reservoir fish species (Bergmann *et al*., 2006; Kempter *et al*., 2009) or in archival material (Lee *et al*., 2012), and appears sensitive enough to detect even minimal copy numbers (i.e. <5) during immediate stages post infection making it an effective confirmatory test. An advantage of ISH is the ability to examine pathological effects of the infection however the tissue architecture can be lost during the digestion process, sometimes making it impossible to distinguish the cell type infected. This was apparent in the kidney, spleen, liver and particularly skin tissues in the current study despite strong signals on some sections. Gregory (2002), when developing ISH for ISAV detection, suggested that different assay conditions may be needed for different tissues.

Antigen detection by IHC was not very successful using fixed tissues from fish undergoing acute clinical KHVD (5-7 dpi), despite harbouring high virus loads. Few of the MAbs tested provided positive signals, however, three MAbs detecting a capsid antigen (20F10), an antigen of ORF62 (10D10) and ORF68 (7C6) provided some promising staining, and were thus predominantly used. Ascites fluid (Asc. 10D10) recognising a KHV antigen, expressed by ORF62 (Aoki *et al*., 2011), produced abundant signals by IHC in the gills and kidney, although this did not correspond with the focal signals of infected epithelium detected by ISH, and the negative control fish also exhibited abundant signals suggesting a lack of specificity. However, focal signals, associated with the vessels and hepatocytes of the liver parenchyma were observed, while the negative control tissue remained negative. Pikarsky *et al*. (2004) successfully used IHC with rabbit anti-KHV serum to screen fish challenged with the virus by immersion. They found specific positive signals in the kidney after only 2 dpi,

which were associated with the renal interstitium and increased during the course of the 10 day challenge, similar to the signals obtained in the current study by ISH. However, cross-reactive signals with uninfected gill tissues were also reported. It is possible that the MAbs generated to the virus are specific for a virus antigen that is conserved with cellular antigen of the host. Herpesviruses may present host-like epitopes that assist in immune evasion (Vider-Shalit *et al*., 2007). Recent studies have demonstrated that KHV expresses an IL-10-like homologue, which it may utilise for evasion of the host immune response (Van Beurden *et al*., 2011a; Sunarto *et al*., 2012). Therefore viral antigens, such as the one encoding the tegument protein of ORF62 (Michel *et al*., 2010b), containing a cysteine protease region recognised by MAb 10D10 (Aoki *et al*., 2011) may be a conserved epitope with host cell proteins. This represents another problem with using KHV antigens for diagnosis. As the tegument of herpesviruses may include trace amounts of cellular-derived proteins (Loret *et al*., 2008; Newcomb and Brown, 2009; Michel *et al*., 2010b; Van Beurden *et al*., 2011b), they may not be ideal protein targets for diagnostic MAb development, which holds true for the KHV virion, which harbours at least 18 host cell proteins (Michel *et al*., 2010b). The highly abundant signals remained after IFAT. Thus, extensive staining within kidney tubular epithelial cells, which did not correspond with interstitial staining by ISH, were not associated with endogenous peroxidases in the host tissue. KHVD-induced nuclear changes have been reported in tubular epithelial cells previously (Hedrick *et al*., 2000), but the staining in the current study appeared too sporadic to be specific for infected cells. However, differential signal intensity was noted within hepatocytes of the liver. These signals may represent genuine expression of KHV ORF62 antigen in these cells during clinical disease, which may make the liver a useful target tissue using these MAbs. Extensive pathology and the presence of viral particles in the hepatic parenchyma, has been reported in a number of

studies (Hedrick *et al*., 2000; Pikarsky *et al*., 2004; Miyazaki *et al*., 2008; Cheng *et al*., 2011), thus this may be a useful diagnostic target at later stages of infection. Signals obtained with the other 2 MAbs detecting a capsid antigen and an antigen encoded by ORF68, were inconsistent and often inconclusive. Despite large concentrations of KHV DNA from 5-7 dpi, detection of antigens may still not be a sensitive enough method for early stage diagnostics. *In vitro* studies of KHV induced anti-IFN activity using flow cytometry indicated that KHV glycoprotein antigen could not be detected in infected cells until 5 dpi (when 40% of the infected cultured cells exhibited virus-associated fluorescence) (Adamek *et al*., 2012). Furthermore, rabbit anti-sera generated against the envelope protein encoded by ORF 81 enabled detection of KHV in infected gills and kidney after only 8 dpi (Rosenkranz *et al*., 2008), thus detection limits, with regards to time post-infection, may also be associated with the virus target antigen. The expression of sufficient protein for detection may therefore take a number of days after KHV begins to replicate in internal tissues. Highly abundant viral proteins in infected cells may be the most effective target.

Antigen masking may also have affected results from IHC screening, however, antigen retrieval procedures were also undertaken, but to no avail, and MAb 7C6 has previously been successfully used to detect viral antigen in fixed tissues (Aoki *et al*., 2011). Aoki *et al*. (2011) suggested that this MAb could be used to detect KHV directly, i.e. through the use of lateral flow technology. This could perhaps be used with blood samples, from which virus has been successfully isolated through co-cultivation of infected isolated leukocytes or infected blood on cell cultures (Kempter *et al*., 2009; Dong *et al*., 2013). However, as mentioned previously, the lag time before high titre infectious virus is obtained from blood or faeces samples (Dishon *et al*., 2005; Matras *et al*., 2012) restricts the

usefulness of such assays, i.e. diagnosis during clinical KHVD, after which point fish may be untreatable, which was highlighted by the peracute disease in the current study.

Fixed tissue imprints or blood smears may prove useful for detecting virus antigens. Tissue imprints of kidney, brain and liver were previously used for detecting KHV antigen early after experimental challenge (Pikarsky *et al*., 2004). Viral DNA was detected in leukocytes after only 6 hpi in the current study, and increased viral loads occurred within the early stages of infection in the acute KHVD tank, thus replicating virus present in the leukocytes may be detectable on smears by IFAT. A number of other studies have detected not only viral DNA (Pikarsky *et al*., 2004; Kempter *et al*., 2009; Eide *et al*, 2011a; b), but also infectious virus particles (Bergmann *et al*., 2010c; Dong *et al*., 2013) in infected carp blood. Smears fixed with alternative fixatives to cross-linking formalin, may minimise antigen masking, making this non-lethal diagnostic approach more feasible.

No virus particles were detected by TEM, confirming the difficulty in detecting virus particles in infected cells, which requires a virus concentration of 10^6 (OIE, 2012). TEM is an expensive technique, which has been useful for characterising the virus and various infected cell types (Hedrick *et al*., 2000; 2005; Miyazaki *et al*., 2008), but is not a useful diagnostic procedure even for detecting KHV during acute stages of infection.

Single or low copy numbers of DNA from other herpesviruses have been successfully detected using ISH (Teo and Griffin, 1990), which in principle, may be useful for detecting KHV DNA in persistent or potentially-latent infections of fish. The carp surviving infection in the current study, sampled after 70 dpi, appeared healthy. This fish harboured barely detectable levels of KHV-DNA in skin mucus by qPCR, and may have been persistently or latently infected. An antibody titre of 1/10,000 and neutralising antibody titre of 1/45 were

measured from the surviving carp by serology. The tissues of this individual have not yet been analysed by ISH, which could provide clues to where the virus is located during a persistent/latent infection. More significantly, detection of KHV DNA in the peripheral blood leukocytes of this fish by ISH, similar to the method applied in goldfish by Bergmann *et al*. (2010c), could enable this carp to be diagnosed non-lethally as infected (based on viral nucleic acid detection) as well as exposed (based on anti-virus antibody detection). PCR remains the method of choice for early stage KHV detection, however, determining the most sensitive assay and sampling method is vital.

4.4.3 KHV PCR diagnostics by lethal and non-lethal sampling

The present study highlights the importance of molecular methods for KHV detection as antibody-based diagnostics on tissue sections does not appear to be reliable, at least during the earliest stages of KHV infection. However, a large number of assays gave negative reactions within the first 4 dpi. The high viral loads in the mucus detected in the current study (up to 80,000 gen. eq.) have been reported previously (Gilad *et al*., 2004), suggesting that mucus may be an effective sample for early KHV detection, at least during acute or clinical disease. Early detection of the virus at this stage enables infected individuals to be identified and eliminated from the population, or at least may prevent the trade and exportation of sub-clinically infected fish. However, even when clinical signs, that are non-pathognomonic for the virus, such as enophthalmos, had occurred from 3 - 4 dpi, many of the PCRs still lacked the sensitivity to detect KHV in tissues. Temperature has recently been shown to influence the sensitivity of molecular methods for KHV detection using single round Gilad PCR (Gilad *et al*. 2002) during early infection stages (Matras *et al*., 2012). In the current study, by using a

hyper-virulent isolate of KHV, it was possible to demonstrate the challenges facing reliable KHV diagnosis, even by molecular methods, during acute and fatal KHVD.

A large number of false-negative results were observed between the various tissues using the alternative PCRs within the first 4 dpi in fish from both peracute and acute KHVD tanks. This suggests that pooling tissue samples, especially from different organs, as practiced in some diagnostic laboratories, may result in a dilution of the virus, enhancing the chances of false negative results, which is in agreement with previous studies (Bergmann *et al*. 2010a, Matras *et al*. 2012). Viral loads of < 5 gen eq. were detected in the kidney in the current study up to 4 dpi, which is considerably less than the mean loads after 3 dpi measured in kidney tissues of KHV-challenged carp in the study performed by Rakus *et al*. (2012). This difference may be associated with the susceptibility of carp to KHV between the studies, and this in turn may also influence the results obtained. In contrast, there were fewer false-negative samples with biopsies used between 5-10 dpi, when a huge increase in viral copy numbers (regardless of the PCR used) and abundant ISH signals were observed in all organs. All fish from the peracute KHVD tank had died by this time, and all acute KHVD fish exhibited morbidity with mortalities occurring from 7 dpi. An exception was the fish sampled at 9 dpi, which harboured much lower viral loads in all tissues, possibly representing an early survivor or a more resistant individual. Such fish may lead to false negative diagnosis with less sensitive PCR methods, even during relatively late stages of infection, as noted with the discrepancies between results observed for various tissues using a TK nested (CEFAS, Unpublished) and Gilad nested PCR (Bergmann *et al*., 2006). Due to the expense of testing, few fish were analysed per time point in this study, but false negative diagnosis of individual carp, such as the fish sampled after 9 dpi, can be detrimental. Indeed, the spread of KHV in the past may have been attributed to individual carriers, i.e. through carp shows in which

valuable koi from various geographic regions are held together in the same exhibition tank prompting efficient disease transmission (Hedrick *et al*., 2000; Haenen *et al*., 2004).

The rapid attachment of virus to carp mucus allowed detection of KHV in swab samples prior to the onset of pathology, with 80-100% of mucus swabs testing positive with the TK gene PCR (Bercovier *et al*. 2005), the nested TK gene PCR (CEFAS unpublished 2007), single round glycoprotein gene PCR (Bergmann *et al*. 2010b), semi-nested PCR (Bergmann *et al*. 2010a) and real-time PCR (Gilad *et al*. 2004) within 1-4 dpi from both peracute and acute KHVD tanks. It could be argued that the bath inoculation challenge model resulted in high DNA concentrations from the inoculum attaching to the mucus enabling efficient detection by PCR. Alternative challenge models should therefore be investigated in the future, i.e. by oral intubation or ip, to confirm the effectiveness of non-lethal KHV detection using mucus swabs. However, in the field, even detection of attached virus in the mucus provides a valuable diagnostic sample regardless of whether virus has been internalised. In the current study, not only were there always many viral copy numbers in the mucus, but the viral load increased in the mucus during the course of the challenge (reaching up to 1 x 10^7 gen. eq.). Excretion of virus via the skin has previously been demonstrated for another alloherpesvirus, channel catfish virus (CCV) (Kancharla and Hanson, 1996), so it may be possible that KHV is excreted effectively through the skin into the mucous providing a valuable sampling target, possibly explaining the increased concentrations from 5000 gen. eq. after 8 hpi to > 50,000 gen. eq. in 7/8 fish until 10 dpi. As explained in Section 4.4.1, the mucus is important in carp protection against KHV (Raj *et al*., 2011), thus even detecting DNA from neutralised, hence inhibited, non-infectious virus particles provides a useful indication of exposure, eliminating the lag phase before replicated virus can be detected in internal organs by lethal sampling.

Dishon *et al*. (2005) used faeces from infected carp for DNA extraction, with positive results only obtained by 5-7 dpi by their PCR. By this point, the fish may have already become sick and died, as these authors reported 96 % mortality within 8-10 dpi. However, non-invasive, early detection could provide an opportunity to intervene in the infection cycle e.g. by raising the water temperature to levels which inhibit viral replication (Dishon *et al*. 2005), which could be applied to fish testing positive for KHV DNA in their mucus but with no signs of the disease, similar to that seen in the current study. Early detection could help prevent the transfer of infected fish to KHV-free zones, as unregulated fish movements is likely to have contributed to the KHV epidemic, as reported for other fish diseases (Hedrick, 1996).

The semi-nested PCR produced positive signals at very early stages of infection, even from lethally-obtained samples. The one tube semi-nested PCR minimises the risk of contamination and, as also noted by Bercovier *et al*. (2005) for single round PCR, unlike real-time PCR, no expensive reagents and specialised machinery are required, which are not always available in diagnostic labs. Furthermore, this assay has been shown previously (Bergmann *et al*. 2010a) and in the current study to be at least as sensitive as real-time PCR, although this has not yet been confirmed statistically. In diagnostic labs that do not require a high throughput of samples, semi-nested PCR may therefore be a useful alternative. The results obtained from the single round PCR detecting the TK gene were particularly noteworthy as this assay is a listed diagnostic method in the OIE manual (OIE 2012), and is regarded as one of the most sensitive molecular assays for the detection of KHV DNA during acute disease and following recovery (Hedrick *et al*. 2005). Moreover, TK PCR has been successfully used to detect KHV DNA in goldfish (*Carassius auratus*), a potential transmission vector (Bergmann *et al*. 2010c, El-Matbouli & Soliman 2011). However, this

PCR detected KHV DNA in only 4 of the 14 carp gill biopsies from the two tanks during the first 4 dpi in the current study, and kidney and spleen samples (other organs recommended for sampling by the OIE) were negative. The nested TK gene PCR detected KHV DNA in a few instances where the TK PCR without the nested step was negative. However, using non-lethal sampling, 11/14 gill swabs, 13/14 skin swabs and 12/14 fin base swabs were positive for the same fish using the TK PCR. The discrepancy between gill tissue and mucus samples during the first 4 dpi was not associated with inhibition of the assay as positive controls were successfully amplified and the later stage positive detection from gill samples was obtained by the same DNA extraction protocol. This discrepancy may have been associated with the intake of KHV through the gills and into the blood. Indeed, despite strong signals observed by ISH, these were often focal and infected cells may be missed by DNA extraction procedures. Thus, the application of this PCR for diagnostics should be undertaken on mucus samples as well as kidney and spleen tissues. Previous studies using the TK gene PCR tend to have been carried out on tissue samples taken following the onset of clinical disease, where the assay has proved very sensitive (Bercovier *et al*. 2005, Meyer *et al*. 2011). In the current study gill and kidney tissue, as well as nearly all swabs taken between 5-10 dpi were positive for viral DNA. Thus, false-negative results from the earlier stages of infection are likely to be associated with the low virus copy numbers in tissues, i.e. during acute viraemia, which were below the limit of detection of the TK gene PCR. Although the TK gene encoded by ORF 55 is specific for KHV (Bercovier *et al*. 2005), instability of this gene has previously been highlighted (Bergmann *et al*. 2010a, Kielpinski *et al*., 2010; Meyer *et al*. 2011, Fuchs *et al*. 2011). KHV TK nucleotide sequence variants have been considered to possibly be associated with adaptations of the virus to European waters (Kielpinski *et al*., 2010), which has resulted in difficulties in detecting KHV using the TK PCR (Meyer *et al*., 2011).

In a recent study, gill swabs were used for the detection of viral DNA in carp undergoing a persistent infection (Bergmann & Kempter 2011). It was possible to re-activate the virus by stressing the fish with repeated netting. Between 10-1000 KHV DNA gen. eq. were detected by real-time PCR 3 days after netting, suggesting that alternative PCRs with similar detection limits, e.g. TK PCR (10-100 gen. eq.; Bercovier *et al*. 2005, Bergmann *et al*. 2010a) as well as the more sensitive nested, semi-nested and real-time PCRs (1-5 gen. eq.; Bergmann *et al*. 2010a) should be able to detect viral DNA in persistently infected, stressed fish, using non-lethal sampling. The one carp surviving the challenge in the acute KHVD tank tested positive for the virus at 70 dpi (even without stress-induction) using mucus swabs and the nested TK gene PCR, single round glycoprotein PCR, nested Gilad PCR and real-time PCR, supporting the potential of non-lethal sampling for the detection of persistent carriers, which maybe continuously shed low levels of virus.

Although the nested Gilad PCR is a highly sensitive method (Bergmann *et al* 2006, 2010a), false negative results have been reported with this assay from samples of experimentally challenged and naturally infected carp (Bergmann *et al*. 2010a; Pokorova *et al*. 2010). The latter may possibly be attributed to fish undergoing an early stage of infection (Pokorova *et al*. 2010). Another problem, which may arise with KHV detection based on the region of viral DNA targeted by the Gilad PCR primer sequence (Gilad *et al*., 2002; 2004; Bergmann *et al*., 2006) is that it is situated in a non-coding region of KHV DNA with no defined function, thus may not be conserved in newly emerging strains (Haenen *et al*. 2004, Bercovier *et al*. 2005). However, all the fish in the current study were infected with the same virus isolate and KHV was detected more often using the Gilad primers at later stages of the infection. Although variations in genes encoding glycoproteins of KHV have been reported between geographically distinct isolates (Han *et al*., 2013) the target of the glycoprotein gene

PCR and semi-nested PCR used in this study, encoded by ORF56, is 100% identical to other published glycoprotein sequences (Aoki *et al*., 2007; Bergmann *et al*. 2010a) and may be a suitably conserved region of the viral genome for KHV diagnostics.

Viral protein has been detected using immunofluorescence on fixed leukocyte preparations (Kempter *et al*. 2009), but the results of the present study suggest early detection of KHV in leukocytes is also very difficult even at a nucleic acid level. According to a previous study, 1 mL of blood was required for the detection of KHV in leukocytes of potentially latent infected koi (Eide *et al*. 2011a), making it an unsuitable method for sampling small fish, while Kempter *et al*. (2009) used 200–300 µL of blood from small KHV-infected fish for separating leukocytes and subsequently detecting KHV by different PCRs, suggesting that this method could be applied for early detection with the most sensitive PCRs.

Carp skin, which has been demonstrated to harbour high levels of KHV by bioluminescence after only 1 dpi (Costes *et al*., 2009) and qPCR after 3 dpi (Adamek *et al*., 2013), has also been used as a potential biopsy for non-lethal sampling by fin clipping fish to detect both early and latent stages of KHV infections by real-time PCR (Adamek *et al*. 2011). Skin samples may therefore be an additionally useful lethal sample for early KHV detection as skin biopsies also enabled detection of KHV-positive fish after 3-6 dpi in a previous study (Matras *et al*., 2012) and more often than OIE recommended spleen, kidney and gut samples in the current study, however, screening from skin still produced many false-negative results during early infection stages, especially when compared to less destructive mucus swabs. Additionally, there was no advantage using lethal samples of liver and brain for early virus

detection, which concurs with a recent study demonstrating that the virus is only detected in the brain at later stages post infection (Matras *et al*. 2012).

4.4.4 Concluding remarks

In conclusion the results of this study highlight some of the constraints with detecting KHV during the early stages of infection, which can be attributed to characteristics of its pathogenesis through which high viral loads are not present within internal organs until later stages in the infection (i.e. > 5 dpi), but may be present and detectable in the mucus. The gills or gut may represent portals of entry, but were still not effective biopsies for early stage detection. As isolation of infectious KHV particles is a timely procedure, and can only be achieved during clinical disease, alternative approaches are required for early detection. Antigen detection does not appear to be a reliable method, even when using highly specific MAbs, and detection of virus particles by TEM is not always possible, even during acute infection with a highly virulent virus isolate. Serum antibodies are not detectable during these early stages, thus highly sensitive molecular based approaches are required, especially considering the high mortality rates induced by this 'short-lived' disease making serological diagnostic approaches more suitable for detecting previously exposed carp. Swabs, predominantly from skin, seem to be a useful approach for virus detection at this stage, prior to the onset of clinical KHVD, particularly during acute viraemia and acute subclinical infections. It may be possible to use non-lethal sampling to detect viral DNA with most of the PCR methods currently used by diagnostic laboratories, including the PCR detecting the TK gene, as recommended by the OIE. However, the results of this and previous studies indicate that the use of more sensitive methods such as the glycoprotein gene PCR (Bergmann *et al*. 2010b) and semi-nested PCR (Bergmann *et al*. 2010a) should also be considered. Although

ISH is highly sensitive, a non-lethal and rapid detection system is preferred for detecting acute KHV infected fish.

Chapter 5

Antigen expression and characterisation during the early stages of Koi herpesvirus infection in vitro

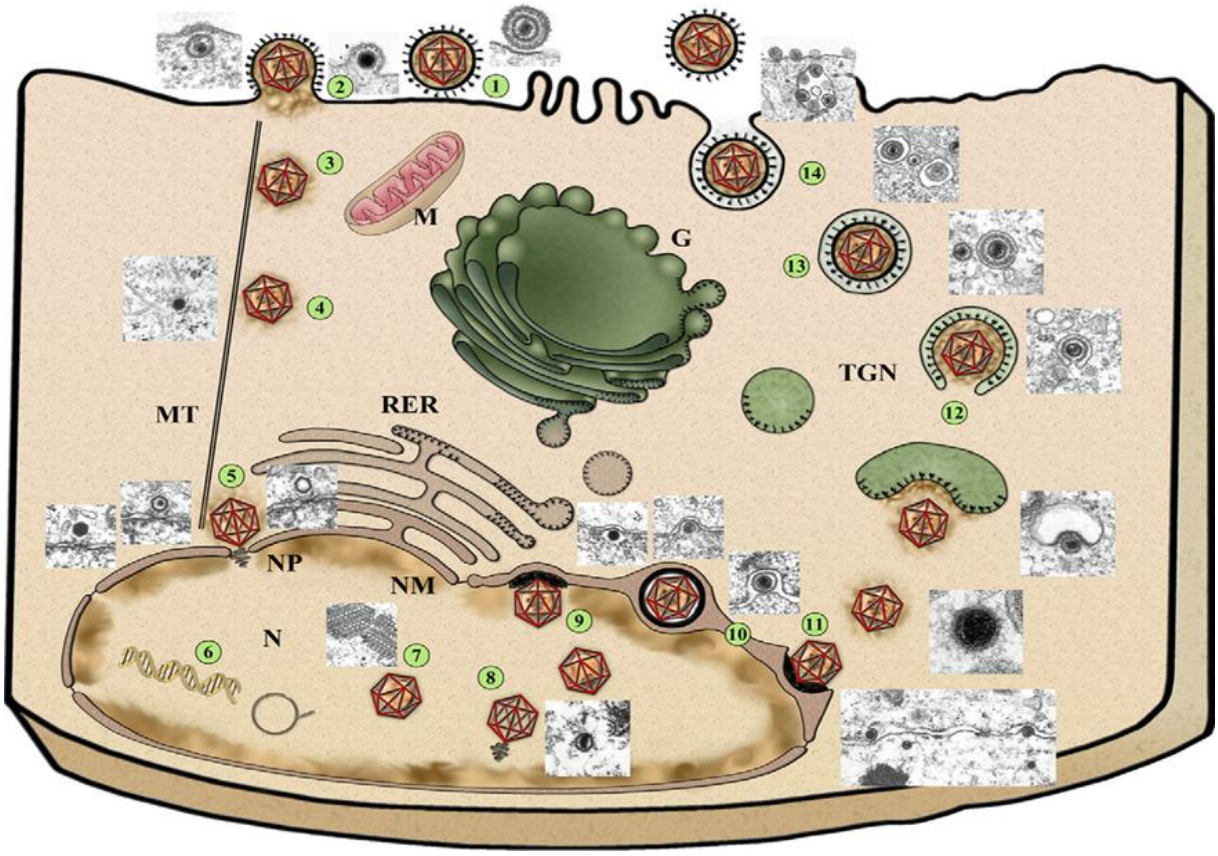
5.1- Introduction

5.1.1 The herpesvirus infectious cycle

Analysis of herpesviruses during the infectious cycle can provide an insight into the various structural proteins of the virus and give indications as to which stages of infection these may be important in with regards to virulence and antigenicity. Differences in the characteristics of these antigens may be useful for DIVA approaches to vaccination, especially where a specific antigen may be indicative of infection.

The infectious stages of the virus and proteins associated with this have been reviewed in detail for mammalian herpesviruses by Mettenleiter *et al*. (2009), and the virus morphogenesis at these stages is similar to that for KHV both *in vivo* (Miyazaki *et al*., 2008) and *in vitro* (Miwa *et al*., 2007), which are illustrated in Fig. 5.1. Some of the proteins involved in different stages of replication have also been characterised by immunogold TEM staining (Gilbert *et al*., 1994; Van Drunen Little-van den Hurk *et al*., 1995; Granzow *et al*., 1997; 2001; 2004), enabling these to be linked to their role in herpesvirus maturation. Infection of a cell occurs following attachment of an enveloped infectious virion to the cell plasma membrane (1) (Rey, 2006), which penetrates the phosopholipid bilayer of the cell membrane, i.e. via surface receptor, proteoglycan heparan sulphate (Mocarski *et al*., 2007) (2), recognised by glycoproteins of the virus envelope, which provide a broad cell tropism. Viral entry depends on cell type, where envelope glycoprotein B (gB) and a complex of glycoprotein L and H (gL-gH) facilitate attachment and penetration of epithelial cells, whereas fusion of the virion within an endocytic vesicle initiates infection of B lymphocytes via the endocytic pathway (Mocarski *et al*., 2007). Glycoprotein B (gB) is highly conserved amongst herpesviruses (Pereira, 1994), and the gene encoding it has proven useful for

assigning herpesviruses to particular sub families (Knipe *et al*., 2001 cited in Coberley *et al*., 2002). The envelope glycoproteins involved in cell attachment and penetration constitute the main targets for vaccine development (Pereira, 1994; BenMohamed *et al*., 2003). In bovine herpesvirus-4 (BoHV-4), murid herpesvirus-4 (MuHV-4) and human simplex virus-1 (HSV-1) exposure of epitopes facilitating fusion to the cell receptor, i.e. glycosaminoglycans (GAGs), is thought only to occur at the cell surface and possibly after endocytosis (Spear and Longnecker, 2003; Machiels *et al*., 2011). This strategy masks the epitope from neutralising antibodies thus evading the immune response of the host (Machiels *et al*., 2011).



After Mettenleiter (2008) cited in *Mettenleiter et al*. (2009)

Figure 5.1 Schematic diagram of herpesvirus infectious cycle within the infected cell. Numbers 1-14 indicate typical herpesvirus infection stages and associated virus morphogenesis, which are also presented in TEM micrographs. Stages of infection indicated numerically are described in the text. MT = Microtubule; RER = Rough endoplasmic reticulum; M = Mitochondrion; NP = Nuclear pore; N = Nucleus; NM = Nuclear membrane; TGN = Trans Golgi Network; G = Golgi body

The naked nucleocapsid is subsequently released into the cytosol (3), after fusion between the virus envelope and the cell membrane via gB, gH-gL, and sometimes gM-gN (Mocarski *et al*., 2007), initiating migration to the cell nucleus via microtubules facilitated by pUL25 (Kaelin *et al*., 2000; Ren *et al*., 2001; Pasdeloup *et al*., 2009) (4) whereby the capsid terminates at a nuclear pore (5) (Newcomb and Brown, 2010; Abaitua *et al*., 2012). Capsid formation, packaging of the viral genome and primary envelopment involve mostly conserved proteins of the herpesviridae (Mettenleiter *et al*., 2009).

The viral DNA is released through the nuclear pore into the nucleus (6) (Jovasevic *et al*., 2008) where transcription and replication take place (7). The concatemeric replicated viral genome is cleaved into unit-length during encapsidation (8) (McVoy *et al*., 2000), then cleaved into a pre-formed capsid (9) (Mettenleiter *et al*., 2009). Capsid formation occurs after a cascade of transcript expression events following the production of immediate early, early and late mRNA transcripts. Late proteins are imported back into the nucleus for capsid particle assembly surrounding the genomic DNA (Spencer *et al*., 1998; Mettenleiter, 2004) and the scaffolding of the capsid is secured via UL26 and UL26.5 (Dougherty and Semler, 1993; Yu *et al*., 2005).

The mature nucleocapsid buds at the inner nuclear membrane (INM) of the nuclear envelope (9) (Campadelli-Fiume and Roizman, 2006) resulting in fusion between the nuclear envelope and the primary enveloped virion in the perinuclear space (10) (Klupp *et al*., 2000; Granzow *et al*., 2001; Skepper *et al*., 2001; Mettenleiter *et al*., 2009). Intranuclear movement of capsids is thought to facilitate efficient contact with the INM for budding via actin filaments (Forest *et al*., 2005 cited in Mettenleiter *et al*., 2009) and two major proteins involved in primary envelopment, pUL31 and pUL34 (Klupp *et al*., 2000), work in synchrony during primary envelopment forming the nuclear egress complex (NEC) whereby the absence of either one prevents successful primary envelopment (Mettenleiter, 2002; 2004;

Mettenleiter *et al*., 2009). The primary enveloped virions then fuse with the outer nuclear membrane (ONM) (11) and the nucleocapsid is released into the cytosol for tegumentation and final maturation during secondary envelopment (12) (Granzow *et al*., 2001; 2004; Newcomb and Brown, 2009), which involves many proteins that are not conserved within the herpesviridae (Mettenleiter, 2002; 2004; Mettenleiter *et al*., 2009). Secondary envelopment occurs by budding of the intracytosolic capsid into a vesicle of the trans-golgi network (TGN) (12) (Klupp *et al*., 2001). Viral glycoproteins present in the TGN are involved in the envelopment of the mature virion within the cell vesicle (13), which is then transported to the cell membrane. The vesicle carrying the secondary enveloped virion fuses with the plasma membrane and releases the mature enveloped virion to the extracellular space (14) (Mettenleiter *et al*., 2009). The variations in conservation of herpesvirus proteins involved in the initial nuclear stages of infection and the non-conserved proteins involved in the later cytosolic stages of infection are suggested to be associated with differences in ancestry, with the large double-stranded DNA bacteriophages (McGeogh *et al*., 2006), and the envelope proteins of the hepesviridae family of mammals, avians and reptilians being considered to be much more closely related in terms of evolutionary distance compared to the alloherpesviridae of fish (van Beurden *et al*., 2011b).

5.1.2 Protein composition of the herpesvirus virion

Virions of herpesviruses contain more proteins than any other virus group and have large genomes (Flint *et al*., 2009), generally encompassing around 40 genes encoding for structural proteins. Some of these proteins execute similar functions in the replication cycle in the various families, but with the recent wide application of mass spectrometry in virological study, differences have been noted in the constituents of the proteins that make up the capsid, tegument and envelope of the virion. The genome of the most well characterised

herpesviruses, HSV-1, encodes for 8 capsid proteins, 13 envelope proteins and 23 tegument proteins (Loret *et al*., 2008), although recently the proteome of Pseudorabies virus (PrV) was extensively analysed revealing 47 structural proteins (Kramer *et al*., 2011). Published proteomic data on fish herpesviruses has been undertaken for Ictalurid herpesvirus-1 (IcHV-1) (Davison and Davison, 1995), Anguillid herpesvirus-1 (AngHV-1) (van Beurden *et al*., 2011b) as well as KHV (Michel *et al*., 2010b). However, there are great differences between KHV and even the most closely related herpesvirus, AngHV-1, with KHV, according to the most recent analysis, consisting of 3 capsid proteins and 13 envelope proteins, but only 2 tegument proteins (Michel *et al*., 2010b) compared to 7 capsid proteins, 11 envelope proteins and 22 tegument proteins in AngHV-1 (Van Beurden *et al*., 2011b), while 22 KHV proteins are still not allocated to a structural virion role (Michel *et al*., 2010b). In fact only 9 of the 40 structural proteins of KHV were found to be homologous to IcHV-1 and the amphibian ranid herpeviruses, RaHV-1 and RaHV-2 (Michel *et al*., 2010b; Van Beurden *et al*., 2011b). The most abundant protein of KHV virions is encoded by ORF92, an orthologue of the major capsid protein of IcHV-1, RaHV-1 and RaHV-2. The protein encoded by ORF66, is also highly abundant in KHV virions, with a sequence homology similar to that of the capsid triplex protein ORF42 of AngHV-1 (Michel *et al*., 2010b; van Beurden *et al*., 2011b). The largest protein detected in KHV virions is the tegument protein encoded by ORF62 (Michel *et al*., 2010b), which is an ovarian tumor-like cysteine protease domain (Aoki *et al*., 2009; 2011), also found in AngHV-1 ORF83 and IcHV-1 ORF65 (Van Beurden *et al*., 2011b). This tegument protein is also considered to be a homologue of the large tegument protein, UL36, conserved in all mammalian and avian herpesviruses (Michel *et al*., 2010b). The major envelope protein of KHV is thought to be encoded by ORF99, which is homologous to the major envelope glycoproteins of IcHV-1 ORF46, RaHV-1 ORF46 and RaHV-2 ORF72 (Michel *et al*., 2010b). To date the most immunogenic of the KHV structural proteins is

considered to be another major envelope protein, encoded by ORF81 (Rosenkranz *et al*., 2008), which is homologous to the major envelope glycoprotein encoded by ORF59 of IcHV-1 (Davison and Davison, 1995). Nonetheless, the antigenic characteristics and biological function of many of the proteins, with regards to the replicating virus, are yet to be elucidated. Molecular applications have enabled characterisation on the basis of gene expression (Ilouze *et al*., 2012a; b); however, use of monoclonal antibodies (MAbs) may provide useful information on expression characteristics of the final folded product as they focus on specific epitopes of the virus and thus various structural proteins.

5.1.3 Use of MAbs for studying virus protein characteristics and pathogenesis

The antigenicity and role of many of the 40 structural proteins (Michel *et al*., 2010b), from the 156 encoded for by KHV (Aoki *et al*., 2007), have yet to be determined. The envelope proteins are the most immunogenic of these for aquatic (Rosenkranz *et al*., 2008; Hansen *et al*., 2011) mammalian (Pereira, 1994; Franti *et al*., 2002), reptilian (Coberley *et al*., 2002) and avian herpesviruses (Fuchs *et al*., 2007). However, alternative structural proteins have been found to be immunogenic in their respective hosts such as the tegument (Gibson and Irmiere, 1984 *cited in* Van Drunen Little-van den Hurk *et al*., 1995; Van Drunen Little-van den Hurk and Babuik., 1986; Van Drunen Little-van den Hurk *et al*., 1995), and capsid or nucleocapsid proteins (Crabb and Studdert, 1990; Pau *et al*., 1998; Corchero *et al*., 2001; Coberley *et al*., 2002; Mebatsion *et al*., 2002; De Paschale and Clerici, 2012) as well as some non-structural proteins (Kaashoek *et al*., 1996).

MAbs provide a unique specificity for individual viral components recognising a single epitope of the protein of interest, and benefiting over the application of polyclonal antibodies or antisera, which lack such specificity and may contain sub-populations of antibodies of different classes with varying affinity and avidity (Cancel-Tirado *et al*., 2004;

Siddiqui, 2010). MAbs are derived from a single B cell clone (Nelson *et al*., 2000) and bulk stocks can be produced en-mass and utilised for biological analyses of viral antigens, but also for developing diagnostic tests, therapeutics, targeted drug delivery systems or even treatments (Siddiqui, 2010). The precision of MAbs in detecting a single site, not an average of determinants like polyclonal antibodies or anti-sera (Benjamin *et al*., 1984), enables antigenic structures to be delineated in great detail, which has contributed to epitope mapping of some fish viruses such as VHSV (Fernandez-Alonso *et al*., 1998) and Nodavirus (Costa, 2005; Costa *et al*., 2007). However, before such advances can be made (on the epitope scale) for KHV, determining and characterising the immunogenic antigens of this complex group of molecules is required not only of the virus envelope, but also for internal proteins, which may comprise properties useful for DIVA strategies.

MAbs have been used to identify specific antigenic determinants of different virus proteins, which has enabled characterisation of cross-reacting and neutralising antigens of viruses that exhibit vast heterogeneity between isolates, including aquatic birnaviruses such as infectious pancreatic necrosis virus (IPNV) (Caswell-Reno *et al*., 1986). In other important aquatic viruses, such as ISAV, MAbs have enabled identification and characterisation of the vital viral surface protein, haemagglutinin esterase (HE) (Falk *et al*., 1998; Krossøy *et al*., 2001).

Important characteristics of antigen involvement in infection, serotype cross-reactivity, replication, neutralisation and antibody-dependent enhancement (ADE) mechanisms have also been identified by utilising MAbs binding to various structural and non-structural proteins for mammalian viruses: Porcine reproductive and respiratory syndrome virus (PRRSV) (Cancel-Tirado *et al*., 2004), Dengue virus (DV) (Kao *et al*., 2001), Sindbis virus (SBV) (Flynn *et al*., 1990) and avian viruses, e.g. Avian influenza virus (AIV) (Yewdell *et al*., 1983). The use of MAbs and mono-specific anti-sera has also contributed to

the identification of many herpesvirus proteins and their functions by the use of immunogold TEM, immunoblotting, immunoprecipitation and immunofluorescence (Giugni *et al*., 1992; Gilbert *et al*., 1994; Granzow *et al*., 1997; 2004; Kaelin *et al*.; 2000; Klupp *et al*., 2000; Skepper *et al*., 2001; Fuchs *et al*., 2007; Pasdeloup *et al*., 2009). However, very few studies have utilised MAbs for studies on KHV, and those have focused on diagnostic test development (Aoki *et al*., 2009; 2011), diagnosis (Kempter *et al*., 2009), major glycoprotein characterisation (Rosenkranz *et al*., 2008), protein affinity purification (Gotesman *et al*., 2013) and screening of recombinant mutants (Costes *et al*., 2008; 2009). There has been no emphasis on their application for investigating aspects of the virus biology and pathogenesis. Attempts were made in Chapter 4 to analyse the expression of various structural proteins of KHV, recognised by MAbs, in the tissues of experimentally infected carp using IHC and IFAT. This approach was largely unsuccessful. Therefore, the expression of KHV proteins recognised by the same MAbs could be analysed in infected cultured cells *in vitro*.

Microtitre plate immunofluorescence (IF) procedures were previously applied for IPNV and ISAV to improve the sensitivity of virus titration in cell culture and antigen could be detected as early as 16 hpi and 1 dpi, respectively (Falk *et al*., 1998; Espinoza and Kuznar, 2002). Kao *et al*. (2001) used flow cytometry with MAbs to detect DV in infected cell cultures which allowed earlier virus antigen detection after only 16 hpi compared to 26 hpi by IFAT. A recent method developed for quantifying Rotavirus (RV) infected cells used a microtitre plate IF procedure, which enabled determination of minimum inhibitory concentrations (MIC) of neutralising substances against this reovirus (Xijier *et al*., 2011).

DIVA vaccination strategies can possibly be developed to distinguish fish vaccinated with inactivated KHV vaccines by utilising structural proteins of the virus that will only induce antibody responses in fish when the virus is replicating, as demonstrated previously for inactivated AI vaccines (Lambrecht *et al*., 2007; Kim *et al*., 2010; Hemmatzadeh *et al*.,

2013). Thus, investigating the expression characteristics of antigens belonging to various virus structural proteins may provide valuable information on their cellular localisation and abundance. A semi-quantitative immunofluorescent approach mirroring that of Xijier *et al*. (2011) was evaluated in the current study for this purpose.

5.1.4 Aims

The aim of this study was to identify structural proteins of KHV with differential expression kinetics. Although extensive work is now ongoing to investigate the role of KHV proteins, limited information is available regarding the structural and functional roles that many of these proteins play in virus infection and pathogenesis. The current study was therefore conducted to examine expression properties of different structural protein antigens of KHV, which could provide useful information for their application in DIVA diagnostics. Two novel IF methods were developed in parallel to quantify intracellular antigen abundance during the course of the KHV infectious cycle *in vitro*. One approach utilised 96 microtitre plates with infected cell cultures that could be measured spectrophotometrically. The second approach utilised confocal microscopy and image analysis of infected cell cultures grown on cover slips. Furthermore, in order to determine any relationships that could be drawn between antigen expression and virus morphogenesis or viral DNA loads, TEM and real-time qPCR were performed, respectively, on additional synchronous infected cultures.

5.2 – Materials and Methods

5.2.1 Monoclonal antibody (MAb) production and screening

5.2.1.1 Hybridoma cell lines

Hybridoma cell lines producing 8 MAbs detecting different antigens of KHV were kindly provided by Dr. Malte Dauber (FLI, Germany). Two other hybridoma cell lines producing MAbs detecting antigenic proteins encoded by ORF62 and 68 of KHV (Aoki *et al*., 2011), as well as mouse ascites fluid and hybridoma cell culture supernatant also recognising these antigens, were kindly supplied by Professor Takashi Aoki (University of Marine Science and Technology, Tokyo, Japan) and Dr. Taesung Jung (Aquatic Biotechnology Center, College of Veterinary Medicine, Gyeongsang National University, Korea). The hybridoma cell lines were cultured and MAbs prepared as described in Section 2.1.1.

5.2.1.2 Screening MAbs by ELISA

The eight purified MAbs were concentrated and diluted to the same concentrations in order to determine MAbs with highest affinity to purified virus and suitable concentrations for further analysis. Hybridoma supernatant and mouse ascites fluid detecting KHV ORF62 and 68 recombinant proteins were also tested by ELISA using dilutions from 1/10 to 1/10,000.

Ninety-six well microtitre ELISA plates (Immulon-4 HBX, Thermo Fisher Scientific, Germany) were coated with 50 µL well^{-1} of sucrose gradient purified KHV dissolved in TNE buffer (Section 2.5.2) or BSA (bovine serum albumin, Sigma-Aldrich, USA) at 0.4 µg well^{-1} diluted in 0.05M coating buffer (carbonate-bicarbonate, pH 9.6, Sigma-Aldrich, USA) and incubated at 4°C overnight. The following day the plates were washed 3 x with low salt wash buffer (LSWB, 0.02M Trisma base, 0.38M NaCl, 0.05% Tween 20, pH 7.3) and were post-coated with 250 µL well^{-1} of 10% skimmed milk powder (SMP) w/v (Marvel, UK) for 3 h at room temperature (RT) (i.e.22^{o}C) in order to block non-specific binding sites. The plates

were washed 3 x again with LSWB before adding 100 µL of purified KHV MAbs at concentrations of 10 µg mL^{-1}, 5 µg mL^{-1} and 1 µg mL^{-1}, diluted in phosphate buffered saline (PBS, 0.02M phosphate, 0.15M NaCl, pH 7.2) or mouse ascites fluid and hybridoma cell culture supernatant to recombinant proteins of ORF62 and ORF68 of KHV. After 1 h incubation with MAbs the plates were washed 5 x with high salt wash buffer (HSWB, 0.02 M Trisma base, 0.5 M NaCl, 0.1% Tween-20, pH 7.7) with a 5 min incubation on the last wash and 100 µL goat anti-mouse IgG antibodies conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich, UK) were added to the wells and incubated for 1 h at RT. The plates were washed again with HSWB as described above and the assay was developed by the addition of 100 uL $well^{-1}$ of chromogen (42mM 3'3'5'5'-Tetramethylbenzidine dihydrochloride) diluted 1/100 in substrate buffer (0.1 M citric acid, 0.1 M sodium acetate, pH 5.4, 0.033% H_2O_2). The reaction was stopped after 10 min with the addition of 50 µL 2M H_2SO_4 and the plates were read at 450 nm on a spectrophotometer (Bio Tek Synery HT instrument, Gen5 program, Fisher Scientific, Leicestershire, UK).

5.2.1.3 SDS-PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis)

The SDS-PAGE was undertaken according to the methods described by Laemmli (1970) with modifications. Koi herpesvirus that had previously been purified (Section 2.5.2) was diluted to 0.1 mg mL^{-1} in TN buffer (10mM Tris, 10mM NaCl, pH 7.4). Uninfected Common Carp Brain cells (CCB) that had previously been cultured for 4 days (Section 2.2.2.2) were lysed and used as negative controls. One hundred microlitres of both purified KHV and lysed CCB cell suspension were combined with 100 µL of 2 x SDS sample buffer (0.5M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.2M dithiothreitol, 0.02% bromophenol blue). Reduced proteins were boiled for 2 min and then centrifuged (Microlite, Thermo IEC, US) for 2 min at 16,000 x *g* to pellet debris from the samples.

Two 12.5% polyacrylamide gels were prepared with 10 mL acrylamide solution (Pro-Pure Next Gel™ 12.5 % kit, Amresco, US), 6 µL N,N,N'N'-Tetramethylethylenediamine (TEMED) (Fisher, UK) and 10% ammonium persulfate (Sigma-Aldrich, St. Louis, US). The amount of sample applied to each gel depended on the size of wells used. One hundred microlitres of reduced sample $well^{-1}$ was used for large single well combs while only 10 µL $well^{-1}$ was used for small multi-well combs. Precision Plus Protein™ Kaleidoscope™ Standards (BioRad, US) were included in each gel. Polypeptides were then separated by gel electrophoresis using 1 x running buffer (Pro-Pure™ x 20 Running buffer, Amresco, US) at 175 volts for 75 min using the Hoefer SE250 mini-vertical gel electrophoresis unit (Hoefer, US). Gels that were not used for transblotting were stained separately with either Coomassie Blue R-250 solution (Fisher Scientific, UK) (0.25 % w/v in 40 % methanol, 10 % acetic acid and 50 % dH_2O) or were stained with silver using either Silver stain Plus™ (BioRad, US) or ProteoSilver™ silver stain kit (Sigma-Aldrich, US) according to the manufacturer's instructions. Silver staining provides increased sensitivity over coomassie blue staining with detection limits of 0.1 ng BSA mm_2^{-1} (Rabilloud *et al*., 1994).

5.2.1.4 Western blot with MAbs

Western blot was undertaken using 10 MAbs to KHV against purified KHV and CCB cell lysate proteins. Polyacrylamide gels containing separated proteins were transferred to nitro-cellulose membranes (Amersham™ Hybond™ ecl, ge Healthcare, UK) by applying 60 volts for 30 min in transblot buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) using a wet blotting apparatus (Fisher brand, UK) according to the manufacturer's instructions. After blotting, successful transfer was evident from the presence of Precision Plus Protein™ rainbow markers and the membranes were blocked overnight at 4°C. Two different blocking methods were compared in order to determine differences between specific epitopes recognised by the MAbs and non-specific binding. One membrane was blocked with

100 mL 1 x RotiBlock in H_2O (Roth, Germany) and another was blocked with 2% SMP (Marvel, UK) in Tris buffered saline (TBS, 0.02M Trisma base, 0.5M NaCl, pH 7.5) and the remainder of the procedure used skimmed milk blocking in the diluent similar to the method for screening sera according to Adkison *et al*. (2005). The following day, membranes were washed 3 x with TBST (TBS containing 0.1% Tween-20) for 5 min wash per wash. The membrane was cut into strips and each strip was incubated with a different MAb. Purified MAbs were diluted to 20 µg mL^{-1} in either TBS or TBS containing 1% SMP and hybridoma supernatant or ascites fluid were diluted 1/10 in the same buffers. Eight hundred microlitres of each diluted MAb was incubated with membranes containing both purified KHV and CCB cell lysate. After 1 h incubation at RT, the membranes were washed 3 x for 5 min with TBST and 800 µL goat anti-mouse IgG biotin (Sigma-Aldrich, UK) diluted 1/200 in either TBS or TBS +1% SMP. After 1 h incubation at RT the membranes were washed again 3 x 5 min with TBST before adding 800 µL streptavidin-horseradish peroxidise (Streptavidin-HRP, Vector Labs, US) diluted 1/200 in TBS or TBS 1% casein. After the final incubation, membranes were washed 3 x TBST for 5 min per wash, followed by a 1 min wash with TBS without Tween. Staining was then developed using the 4 CN peroxidase substrate system (2-C: KPL, US) according to the manufacturer's instructions. The reaction was stopped with ultrapure H_2O after 5-10 min.

5.2.1.5 Indirect fluorescent antibody test (IFAT) on tissue culture sections

(a) Cell culture on glass cover slips and virus inoculation

Round 1.6 mm^2 glass cover slips (Fisher Scientific, UK) were sterilised in 70% ethanol then flamed over a Bunsen burner before being placed in 12 well tissue culture plates (Nunc, Denmark). Koi Fin cells (KF-1) were cultured in 25cm^2 tissue culture flasks Section 2.2.2.1 until 100% confluence had been obtained. Cells were typsinised, counted and split as described in Section 2.3.2. Approximately 0.4-0.5 x 10^6 cells $well^{-1}$ were seeded on the cover

slips and cultured in 2 mL MEM medium (Invitrogen, UK) containing 10% foetal bovine serum (FBS), 2mM L-glutamine (Invitrogen, UK) and 1% non-essential amino acids (NEAA) (Invitrogen, UK) in a 22°C incubator containing 4% CO_2 for 24-48 h.

The following day the spent medium was removed and the monolayers were inoculated with 0.2 mL of $10^{3.84}$ $TCID_{50}$/mL KHV (approximate multiplicity of infection (MOI) 0.002) with isolate H361 for 2 h at 20°C. After the absorption period, the cells were resupplemented with MEM containing the same supplements but reduced foetal bovine serum concentration to 2%. The virus infected cultures were incubated at 22°C for 9 days at which point CPE and plaque formation were evident. The medium was removed and the monolayers were washed twice with PBS before fixing the cells with cold (-20°C) 100% acetone (Fisher Scientific, UK). Cells were fixed for 15 min before removing acetone and air drying for 30 min. The fixed infected cultures were stored at -20°C until ready for processing.

(b) IFAT with MAbs on fixed KHV infected cell cover slips

Fixed cells were rehydrated for 5 min with 1 mL PBST (0.01M PBS, 0.05% Tween-20). Cover slips were removed and placed into fresh 12-well plates and the cells were washed 3 x PBST for 2 min. After the last rinse, PBST was completely removed and the cells were covered with 1 mL 5% SMP diluted in PBST and incubated for 30 min at 37°C. Cells were then washed 4 x PBST for 2 min before the addition of 1 mL of purified MAbs at 20 µg mL^{-1} or hybridoma supernatant and ascites fluid diluted 1/10 in PBS. The MAb preparations were added to both KHV positive and mock infected cells to determine specificity of signals. Additionally, non-KHV specific MAbs of the same isotype detecting a different virus (i.e. ISAV) and a blank (PBS) were added to KHV positive cells as primary antibody controls. The MAb hybridoma supernatants detecting ISAV were kindly provided by the Aquatic Vaccine Unit, University of Stirling, Stirling, Scotland. After 1 h incubation at RT, MAbs were removed and the cells were washed 4 x PBST for 2 min before the addition of 1 mL

1/100 goat anti-mouse IgG conjugated to fluorescein isothyocianate (FITC) (Sigma-Aldrich, USA) and incubated for 1 h at RT. Wells were finally washed again with 4 x PBST for 2 min and mounted onto slides (Solmedia) with DAPI (Vectashield, Vector, UK) and sealed with nail varnish. All slides were kept in the dark until analysis by a fluorescent microscope (Olympus BX50, Japan) or confocal microscope (Leica TCS SP2 AOBS confocal laser scanning microscope (CLSM, Germany)) using Leica confocal software (version 2.6.1) at 40 x magnification.

5.2.2 Expression of antigens detected by MAbs

IFAT was undertaken on cells cultured on 96-well tissue culture plates in a KHV time trial during the first 7 days of infection in order to determine the expression characteristics of different KHV antigens *in vitro*.

5.2.2.1 Preliminary investigation with cell and viral associated fluorescence

(a) Cell confluence associated DAPI signal

In order to determine the most suitable sensitivity setting for DAPI fluorescence emitted from cells and to assess the relationship between DAPI fluorescence and monolayer confluence, a preliminary investigation was undertaken on a black immunofluorescence 96 well plate (Greinar Cellstar®) seeded with increasing number of CCB cells. Cells were seeded from 8 x 10^3 cells well^{-1} to 3 x 10^4 cells well^{-1}. The cells were cultured overnight in conditions as described in Section 2.2.2.2, washed with DPBS and fixed with cold (-20°C) methanol (Fisher Scientific, UK). Fifty microlitres of DAPI mountant diluted at various concentrations in PBS was added to the wells and incubated for 10 min at RT. Excess DAPI was removed, the plate was washed and then read on a Synergy HT spectrophotometer (Fisher Scientific, Leicestershire, UK) at various sensitivity settings using the Gen 51.10 program.

(b) Viral antigen expression associated FITC signal

A second preliminary experiment was undertaken to assess the feasibility of using the anti-KHV MAbs for analysing and quantifying viral antigen expression using an immunofluorescence technique. A 96-well immunofluorescence plate was seeded with 2 x 10^4 CCB cells $well^{-1}$ and inoculated with KHV at higher MOI of approximately 0.02. The cells were washed and fixed after 3 dpi and 12 dpi and an IFAT procedure was undertaken with MAbs 10A9 and 20F10 to determine the change in fluorescent signal. Non-KHV specific MAbs detecting ISAV and mock infected cells screened with a pool of 10A9 and 20F10 were also included. The procedures undertaken were similar to those described in Section 5.2.2.2., which follows.

5.2.2.2 96-well IFAT investigation of KHV antigen expression

(a) Cell culture and virus inoculation on CCB and KF-1 cells

Virus MOI was determined independently for each cell line as the seeding density for each culture varied for effective KHV virus production. This was achieved by culturing cells in a spare $25cm^2$ tissue culture flask seeded with the same number of cells as that of the entire 96-well plate, trypsinising the cells following the same incubation period as the experimental plates, and then performing a cell count (as described in Section 2.2.3). The CCB and KF-1 cells were cultured as described in Section 2.2.2. Nine x $25cm^2$ culture flasks of CCB and KF-1 cells at passage 80 and 110, respectively, were cultured to full confluence. The cells from each cell line were pooled together after trypsinisation and subcultured into culture vessels prepared for the infection time trial. Cells were seeded at 2 x 10^4 cells $well^{-1}$ for KF-1 cells and 1.5 x 10^4 cells $well^{-1}$ for CCB cells. Both cell lines were inoculated at an MOI of approximately 0.1 from a KHV virus stock of $10^{4.4}$ $TCID_{50}$ mL^{-1}, passage 17. Dilution of virus was prepared in HBSS, 2% FBS and mock infected cells received only diluent.

(b) Experimental design and fixation time points

Seven black immunofluorescence 96-well tissue culture plates (Greinar Cellstar®) were seeded for each cell line. Three plates were used for analysing KHV antigens expressed during the first day of infection (0–24 hours post infection, hpi) and three were used for analysis from 2–10 dpi. One plate was mock infected and fixed at various times over the course of infection to analyse non-specific binding of MAbs to cell derived proteins. One clear 96-well tissue culture plate was also prepared for each cell line for scoring CPE progression over the course of infection and for undertaking a back titration assay. One 12-well plate was also used, seeded with 2 x 10^5 CCB cells $well^{-1}$ for determining virus titre in Plaque Forming Units (PFU) by plaque assay as described in Section 2.4.2.

The 96-well plates that were to be analysed by IFAT for detection of KHV antigens recognised by MAbs, were prepared for mock infection and KHV infection in different columns of the plate (Fig 5.2.).

Columns of the positive wells of the plate were prepared for screening of different MAbs including MAbs to other viruses as controls. Columns containing mock infected cells were prepared to provide a comparison of DAPI signal of uninfected cells over the course of infection and FITC signal of pooled MAbs to uninfected cells over the course of infection.

Before initiating the time trial, medium was removed from the first row of wells and the cells were washed twice with DPBS then fixed with cold (-20°C) methanol. These cells were designated as time point ‘0 hpi’ cultures. Medium was then removed from all remaining wells on the plate and 100 µL of KHV at an MOI of 0.1 was added to the monolayers of positive wells. Mock infection controls in column 1 and 2 received only HBSS, 2% FBS. The time trial was initiated from this point on.

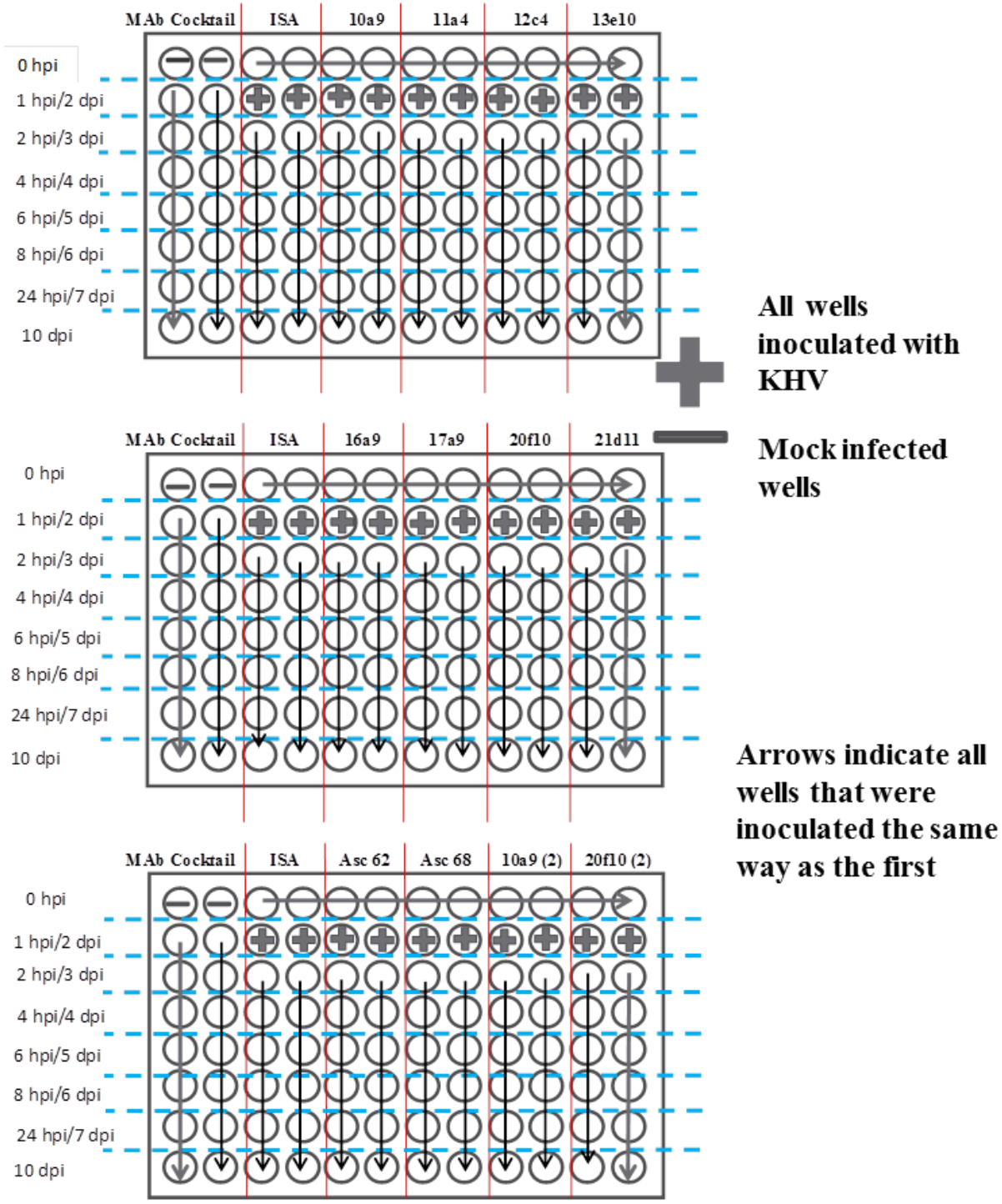


Figure 5.2 Schematic diagram of 96-well tissue culture plate layout for immunofluorescence quantification of koi herpesvirus MAb-antigen binding. Time post fixation of wells is indicated on the left of the plates and the MAb screened for those wells is indicated above the plates. Note that the MAbs 10A9 and 20F10 were run in duplicate on two separate plates for each cell line

The cells were incubated at 20°C and after 1 hpi the second row of wells of the first set of plates (first day of infection) were cleared of culture medium, washed twice with DPBS and fixed with cold methanol. After 2 hpi the procedure was repeated for the third row of wells on the same set of plates.

Cells were washed twice with DPBS and culture medium containing 2 % FBS was resupplemented to all other cells after 2 hpi and the plates were returned to 20°C. The wash and fixation procedure was repeated for rows 4, 5, 6 and 7 after 4, 6, 8 and 24 hpi, respectively, on the same set of plates. After 2 dpi the procedure was repeated for fixation of cells on the second set of plates on the second row of wells. Rows 3, 4, 5, 6 and 7 were washed and fixed after 3, 4, 5, 6 and 7 dpi, respectively. The final row on each set of plates was fixed after 10 dpi so as to ensure that antigen detection by MAbs was similar for both sets of plates at an advanced stage of infection.

The two mock infected plates were prepared for screening by culturing the same density of cells as positive plates with fixation of cells at 0 hpi, 4 hpi, 24 hpi, 3 dpi and 7 dpi.

The same inoculum was also inoculated onto clear 96-well culture plates seeded with KF-1 cells and CCB cells. However, this plate was inoculated with 5-fold serial dilutions of virus on pre-formed monolayers so as to assess (1) the progression of CPE over the course of infection and (2) to check the titre of the original inoculum. A plaque assay was also performed on a 12-well plate with CCB cells. The assays were performed and titres determined as described in Section 2.4.2.

(c) IFAT on 96-well tissue culture plates

In order to quantify the relative fluorescence emitted by MAbs detecting different antigens of KHV, IFAT was performed with MAbs at standardised concentrations which had been

previously optimised for successful detection of KHV on infected cell cultures under fluorescence microscopy (Section 5.3.1.3).

Plates were thawed from -20°C, rehydrated and washed 4 x 2 min with 300 µL $well^{-1}$ PBST. The cells were then blocked with 250 µL $well^{-1}$ 5% SMP in PBST for 1 h at RT. The eight affinity-purified MAbs were diluted in PBS to 20 µg mL^{-1}. Mouse ascites fluid containing MAbs against recombinant proteins of ORF62 and ORF68 were diluted 1:10 in the same diluent. After blocking non-specific binding sites, the plates were washed 4 x 2 min with 300 µL PBST.

One hundred microlitres of the MAbs, ascites fluid and hybridoma supernatant were added to the wells as illustrated in the template (Fig 5.2) and incubated for 1 h at RT. The plates were washed again 4 x 2 min with PBST and 100 µL Goat anti-mouse IgG FITC-conjugated MAbs diluted 1/100 in PBS were added to the wells and incubated for 1 h at RT. The plates were then washed again with 4 x 2 min with PBST and kept in the dark at 4°C until the fluorescence was read spectrophotometrically.

The FITC was measured using a Synergy HT spectrophotometer (Fisher Scientific, Leicestershire, UK) with Gen 51.10 program for data acquisition at a sensitivity setting of 120 (sensitivity of fluorescence detection). Filter settings were applied at wavelengths of 485/20 excitation and 528/20 emission.

Once fluorescence by FITC had been measured on all plates, 50 µL DAPI mountant (Vectashield, Vector, UK) diluted 1:10 in PBS was added to all wells and incubated at RT for 10 min. Excess DAPI was removed from the wells by 4 x 2 min washes with PBST and the plate was read a second time spectrophotometrically. The same program was used at a sensitivity of 120 but with filter sets of 360/40 excitation and 460/40 emission.

(d) FITC: DAPI quantification for determining specific KHV antigen detection

Initially, analysis was undertaken using FITC to determine the increased antigen expression of the MAb target in virus infected cells and DAPI to analyse the increase and/or decrease in cell confluence between control cells and virus infected cells. However, high cell densities increased autofluorescence, thus it was difficult to distinguish specifically between noise and antigen detection between MAbs with fluctuating cell confluence. Therefore a ratio of FITC to DAPI signal was used to determine specific virus detection and to establish a method to assess relative increase or decrease in viral antigen expression.

Relative fluorescence intensities were analysed as a ratio of FITC to DAPI to indicate infection and the abundance of antigen protein detected. The formula utilised by Xijier *et al*. (2011) for quantifying structural proteins of RV by fluorescent labelling with MAbs was applied here as follows:

$$^{V}\mathbf{FITC/DAPI} - {}^{B}\mathbf{FITC/DAPI}$$

Where
V = Measurement ratio of the presence of virus
B = Blank control (0 hpi fixation)
(*After Xijier et al*., 2011)

(e) Statistical analysis of MAb-antigen binding of interest

A number of tests were used to compare significant differences between antigen expression by FITC/DAPI fluorescence over time and between different MAbs. Student t-test was used for determining significant differences between means of FITC fluorescence of 2 MAbs (which were of particular interest) during preliminary trials associated with virus infection over time. Non-parametric data obtained during the trial (relative expression) was analysed using a Kruskal-Wallis test to determine significant differences by analysis of medians of

FITC:DAPI ratios (relative antigen expression) during early infection (1-24 hpi) and late infection (2-7 dpi) for MAbs 10A9 and 20F10. Differences between expression measured by MAbs 10A9 and 20F10 during the early infection stages and then the later infection stages were also evaluated with this test. The Minitab 16 statistical software package was used for all analyses and graphical illustrations were compiled from data using Microsoft Excel, 2010.

5.2.3 Expression of glycoprotein and capsid antigens *in vitro*

5.2.3.1 Preparation of CCB and KF-1 cells for KHV inoculation

Prior to initiating the experiment, suitable cell seeding densities were determined in various culture vessels in order to inoculate cells with KHV during active cell growth at the required confluence.

Cells and virus were cultured and maintained as described in Section 2.2.2. A suitable MOI was also established prior to the trial for analysing viral morphogenesis and antigen expression. A spare culture flask of cells was harvested 24 h after seeding for estimating cell density of each monolayer for both cell lines prior to inoculation so that the MOI could be determined. All KF-1 cell cultures were inoculated with KHV at an MOI of 0.01 and CCB cells at an MOI of 0.02 and inoculations were undertaken as described previously in Section 2.3.2.

(a) Culture of cells on 12-well plates for IFAT analysis

KF-1 cells and CCB cells were seeded onto sterile cover slips in 12-well tissue culture plates as described previously (Section 5.2.1.5). A total of 7 x 12-well plates were prepared for both cell lines. Suitable seeding densities were determined for each cell line independently in order to obtain 50 % confluence for KF-1 cells and 70 % confluence for CCB cells. KF-1 cells were seeded with 0.36 x 10^6 cells $well^{-1}$ and CCB cells with 0.14 x 10^6 cells $well^{-1}$. The cells were cultured for 24 h at 20°C prior to initiation of the time trial.

(b) Culture of cells in 25cm^2 tissue culture flasks for real-time PCR analysis

Twenty-eight x 25cm^2 tissue culture flasks for each cell line were required for viral DNA quantitation by real-time PCR. Fourteen of these were used for negative controls and fourteen for positive KHV infection. KF-1 cultures were seeded at 1.7 x 10^6 cells flask^{-1} and CCB cultures were seeded at 0.7 x 10^6 cells flask^{-1}. Cells were cultured for 24 h at 20°C prior to initiating the trial.

(c) Culture of cells in 75cm^2 tissue culture flasks for TEM analysis

Nine x 75cm^2 tissue culture flasks for each cell line were required for analysis of viral morphogenesis by TEM. Two flasks from each cell line were used as controls for the start and end of the experiment. KF-1 cultures were seeded at 5 x 10^6 cells flask^{-1} and CCB cultures were seeded at 2 x 10^6 cells flask^{-1}. Cells were cultured for 24 h at 20°C prior to initiating the trial.

Cells were observed for CPE and images captured using an inverted phase-contrast ULWCD 0.3 microscope (Olympus, Japan) attached to a Infinity X U-CMAD3 camera (Olympus, Japan).

5.2.3.2 KHV infection, sampling points and fixation of cells

(a) Infection and sampling of 12-well plates

Old medium from 12-well tissue culture plates was removed and 6 of the 12 wells were inoculated with 0.2 mL KHV while the other 6 were inoculated with diluent only (HBSS, 2% FBS) to serve as mock infected controls. The cells were incubated at 20°C for 2 h for viral adsorption to the monolayer. After 1 h, the inoculum was removed from 1 plate of each cell line, cells were washed twice with 1 mL DPBS and fixed with 500 µL 100% cold acetone (-20°C). The cells were fixed for 15 min at RT before air drying for 30 min at RT. After 2 x washes with DPBS 1.8 mL of MEM media containing 2% FBS was resupplemented to the

monolayers of all other plates after 2 hpi and the plates were incubated at 20°C with 4% CO_2. The fixation procedure was repeated for randomly selected plates sampled at time points of 4 and 8 hpi then 1, 3, 5 and 7 dpi. All fixed plates were stored at -20°C until processing.

(b) Infection and sampling of 25cm^2 culture flasks

One 25cm^2 culture flask was sampled from each cell line at time point 0 to serve as experimental negative controls. Following inoculation of 1 mL KHV, the trial was initiated and cells were sampled after 1 hpi. Sampling was undertaken by scraping the monolayer into suspension and centrifuging at 2,000 x *g* for 10 min at 4°C in order to pellet the cells. A slow speed centrifugation was undertaken so as not to rupture intact infected cells as analysis of cell associated virus DNA in pellets and DNA in the supernatant was undertaken separately. The supernatant was removed and dispensed in to separate bijoux. The pelleted cells were washed by re-dissolving the pellet with 10 mL DPBS then centrifuging a second time at 2,000 x *g* for 10 min at 4°C to re-pellet the cells. Excess supernatant was removed by inverting the tube over an ethanol wipe for 1-2 min. Both supernatant and pellet were subsequently stored at -70°C until DNA extractions were undertaken. The same procedure was undertaken for 1 positive and 1 negative randomly sampled 25 cm^2 culture flask for both cell lines at 2, 4, 6 and 8 hpi then 1, 2, 3, 4, 5, 6, 7 and 8 dpi.

(c) Infection and sampling of 75cm^2 culture flasks

Old medium was removed from all flasks and monolayers were inoculated with 3 mL KHV for an adsorption period of 2 h at 20°C. After 1 hpi, the inoculum was removed from 1 positive and 1 negative flask of both cell lines and the monolayers were washed twice with 10 mL DPBS. Cells were fixed with 6 mL 2.5% glutaraldehyde (Sigma-Aldrich, UK) by completely submerging the monolayer with fixative, scraping the cells into suspension using a rubber policeman then immediately centrifuging 3 mL of the suspension in 2 x 12 mL centrifuge tubes at 2000 x *g* for 10 min at 4°C to fix cells into a pellet. Slow speed

centrifugation was again vital to the procedure to prevent rupture to the cells and thus loss of ultrastructural architecture when visualised by TEM. Excess gluteraldehyde was decanted off and the fixed pellets were retained. Fresh 2.5% glutaraldehyde was added to the pellets which were subsequently fixed for 2-4 h or overnight at 4°C. The fixative was removed and 2 mL cacodylate buffer rinse was added to the pellets, which were flicked into suspension using a wooden applicator to ensure the pelleted cells had fixed. Fixed pellets were stored at 4°C until processed. The procedure was repeated for randomly selected positive flasks at 4 and 8 hpi then 1, 3, 5 and 7 dpi. A mock infected negative control, from the same original stock of cells used for infection, was also sampled for each cell line after 7 dpi.

5.2.3.3 DNA extraction

Centrifuge tubes containing virus infected cell pellets and bijoux containing KHV infected cell supernatants from the time course were thawed from -70°C at RT. The DNA was extracted directly from KHV infected supernatant, but pellets were first processed by re-dissolving at RT for 1-2 min in fresh MEM media containing 2% FBS. The pellets were then sonicated by 4 x 30 sec blasts in ice cold water using a Kerry sonicator (Kerry Ultrasonics Ltd., UK). The dissolved, sonicated pellets were centrifuged at 2,500 x *g* and the supernatant was used for DNA extraction.

Virus DNA was extracted using the NucleoSpin® RNA Virus, Viral RNA Isolation kit (Machery-Nagel, Germany) according to the manufacturer's instructions with the addition of proteinase K for extracting viral DNA. All buffers and reagent used were supplied in the kit except for Proteinase K (Machery-Nagal, Germany) and ethanol (96-100%). Briefly, virus was lysed by the addition of 600 µL of buffer RAV1 containing carrier RNA to 150 µL of each sample in a 1.5 mL Eppendorf tube. Twenty microlitres of Proteinase K (20 µg mg/mL^{-1} stock) was added to each lysis mixture. The mixture was aspirated then vortexed (Vortex Whirlmixer, Fisherbrand, UK) for 10-15 sec. The sample mixture was incubated for 5 min in

a 70°C water bath then centrifuged at 11,000 x *g*. The supernatant was then removed and combined with 600 µL ethanol and vortexed for 10-15 sec.

For binding viral DNA, silica columns were placed into 2 mL collection tubes and loaded with 700 µL of lysed sample. This was centrifuged at 8,000 x *g* for 1 min. The residual solution was loaded onto the virus column and the centrifugation was repeated. The flow through was discarded and the Nucleospin virus column was placed into a new collection tube.

The membrane was then washed and dried by the addition of 500 µL Buffer RAW, to the NucleoSpin ® RNA virus column and centrifuged for 1 min at 8,000 x *g* to remove contaminants and PCR inhibitors. A second wash was undertaken with 600 µL buffer RAV3 to the column which was also centrifuged at 8,000 x *g* and the flow-through discarded. Ethanolic buffer RAV3 was removed completely after a final wash of 200 µL with buffer RAV3 followed by centrifugation for 5 min at 11,000 x *g*. Viral DNA was finally eluted from the column after the addition of 50 µL Buffer RE preheated to 70°C. DNA quality was checked and quantity measured using a Nanodrop® ND-1000 spectrophotometer (Labtech International). All DNA was stored at -20° until used for PCR.

5.2.3.4 Real-time qPCR

Real-time TaqMan qPCR was undertaken at the Friedrich Loeffler Institut using the method according to Gilad *et al*. (2004) with modifications according to Bergmann *et al*. (2010a). Real-time PCR was run on all positive and negative samples obtained from the trial from both cell lines following the same methods described previously in Section 4.2.2.5.

5.2.3.5 TEM processing and visualisation

Glutaraldehyde fixed cell pellets were post-fixed in 1 % osmium in cacodylate buffer in closed vials for 1 h at RT. The pellets were then washed for 3 x 10 min in distilled H_2O.

Thorough rinsing of cacodylate buffer was important at this stage as 'En-bloc' staining was undertaken with uranyl acetate and sodium cacodylate is incompatible with uranyl salts. 'En-bloc' staining of pellets was undertaken with 2 % uranyl acetate in 30 % acetone in the dark for 1 h then the pellets were dehydrated through an acetone series of ascending concentrations. Dehydration was undertaken in 60 % acetone for 30 min, 90 % for 30 min, 100 % for 30 min then incubation in fresh 100 % acetone for 1 h.

Pellets were then infiltrated with agar low viscosity resin (ALVR) on a rotator (Taab, UK). The pellets were first incubated with ALVR diluted 1:1 in acetone for 45 min followed by 100% ALVR for 1 h and then into fresh ALVR for another h. The pellets were finally embedded in block moulds and polymerised in an oven at 60°C overnight. Ultrathin sections were cut for visualisation under a TEM microscope as described in Section 4.2.5.

5.2.3.6 IFAT on glass cover slips and confocal microscopy

The IFAT was conducted in a similar manner to the method described in Section 5.2.1.5.(b). Only MAbs 10A9 and 20F10 were subjected to fluorescence quantification by image analysis. The KHV infected and mock infected cells of both cell lines received 250 µL of 20 µg mL^{-1} purified MAbs dissolved in PBS after rehydration and blocking. The cells were then incubated with goat anti-mouse IgG conjugated to FITC (Sigma-Aldrich, UK) as described previously. The cells on cover slips were finally mounted on to glass slides (Solmedia, Shrewsbury, UK) in 20 µg mL^{-1} propidium iodide (Sigma-Aldrich, UK) diluted in PBS and sealed with nail varnish (Avon, UK). All slides were kept in the dark at 4°C until visualised.

Confocal microscopy and image analysis were performed according to methods described previously for measuring apoptosis in Pancreas Disease alphavirus infected salmonid cells *in vitro* (Herath, 2010). Stained cells were observed using a Leica TCS SP2 AOBS confocal laser scanning microscope (CLSM) (Leica Microsystems, Milton Keynes,

UK) coupled to a DM TRE2 inverted microscope (Leica Microsystems, Milton Keynes, UK) and employing a X 63 oil/glycerol immersion objective, in conjunction with Leica confocal software (v. 621). Images were captured in the grey (transmission), red, green and blue channels using the relevant excitation and emission wavelengths for the respective dye, depending on the target (Table 5.1). In order to avoid cross-talk between channels, a sequential scanning configuration was used with images collected successively rather than simultaneously on 3 separate channels. At least 2 replicate images per culture well were captured, including positive, negative and control cells from each cell line at each time point post-infection. Replicate experimental cultures were taken for the KF-1 cell line, but not the CCB cell line. Stacks of 25 serial depth images (*z*-stacks) were taken from each sample of cells by scanning a frame area of 1024 × 1024 pixels ($x \times y$ μm) in the x, y plane. The stacks of images through cells had a total depth of 25 μm comprising 25 transects of 1 μm moving from the basal surface of the cell to the apical surface. Prior to image analysis, the grey channel from each image was removed and the stacks were collapsed to give a projection of maximal fluorescence intensity for the stack as a single 2-D image (Leica Maximum Projection). Where 3D imaging was performed, stacks of 30 images were scanned through cells and anaglyph stereo images created using the Leica confocal software.

Table 5.1 Properties of fluorescent dyes used to detect KHV antigens in infected cells

Target	Probe	Channel	Excitation Min	Emission Max	Laser line
KHV Antigen	FITC labelled MAb	Green	495	519	495
Nuclei 1	DAPI	Blue	405	411	405
Nuclei 2	Propidium iodide	Red*	535	617	535

*** Images were subsequently converted and displayed as blue.**

5.2.3.7 Quantification of viral fluorescence by image analysis and statistical significance of antigen expression

The Carl Zeiss KS 300 image analysis platform was used for image analysis employing a custom made macro script developed by Dr. James Bron (Institute of Aquaculture, University of Stirling, UK), which enables the quantification of a number of morphometric and densitometric features of the target fields (i.e. whole image) or individual objects (e.g. nuclei). The script gives an output of the measurements of the data for each image and processed images for subsequent visual interpretation and quality control.

One of the main advantages of the script is that it encodes a fixed series of operations with no user-interaction, which ensures consistency between measurements and removes user bias. The use of digital analysis in this context is also much faster than manual analysis, more accurate and allows improved inter-user repeatability.

For quantifying nuclear signal intensity, the nuclei were segmented from the background using a HLS colour segmentation function and the adjoining nuclei were separated from one another using a grain separation function and subjected to size thresholding in order to exclude noise. The final segmented areas were used as a field for densitometric measurements of nuclei and nuclear fragments enabling measurements of mean total nuclear fluorescence per section, which represented 1 of the 5 parameters used in the study. The separation function was used in a similar manner for isolating MAb-associated signals, also in the cytoplasm, in order to measure the level of virus antigens through use of MAb signal intensity as a proxy.

Quantification of fluorescence signals associated with nuclear staining (propidium iodide) and antibody-antigen complexes of KHV (FITC) was achieved using the macro described above to provide 5 parameters measured from the replicate scans (n = 2 per slide)

taken from individual sections of infected and non-infected cells. Data from the total cell area (CELL), the total nuclear area (NUC), the average intensity of nuclear fluorescence exhibited by the MAb (MND), the average intensity of the total cell fluorescence, including cytoplasm, exhibited by the MAb (MBD) and the whole field of cell fluorescence above the set sensitivity threshold (HIMAB) were used during the study. Three of these parameters: NUC, MBD and MND proved useful for quantification of virus-associated signal by allowing determination of difference in relative MAb fluorescence (MND or MBD) compared to nuclear fluorescence (NUC) (representing cell confluence) and finding the difference between infected samples and negative controls. This was similar to the approach used for the microtitre IFAT analysis (Section 5.2.2.2 (d)) although it employed the following formula with a negative control subtracted at every time point:

$$^{V}\mathbf{MND/NUC} - {}^{C}\mathbf{MND/NUC}$$

Or

$$^{V}\mathbf{MBD/NUC} - {}^{C}\mathbf{MBD/NUC}$$

Where
V = Measurement ratio of the presence of virus in infected cells
C = Measurement ratio of the presence of autofluorescence signals/noise in uninfected cells

5.3– Results

5.3.1 Monoclonal antibody characterisation

5.3.1.1 ELISA screening of MAbs

Eight monoclonal antibodies (10A9, 11A4, 12C4, 13E10, 16A9, 17A9, 20F10, 21D11) were successfully concentrated and purified after growth of the hybridoma in cell culture.

However, the original hybridoma supernatant and mouse ascites fluid for MAbs detecting recombinant proteins of ORF62 and 68 (10D10 and 7C6, respectively) were used in the study.

Despite 8 of the MAbs being diluted to the same protein concentration there were vast differences between mean absorbance when screened in ELISA against whole KHV virus. MAbs 10A9, 17A9 and 21D11 produced the highest mean absorbances (OD_{450nm}) against purified virus with consistently strong signals at all concentrations. All 8 of the purified MAbs successfully detected KHV with OD levels above the sensitivity threshold at concentrations as low as 5 µg mL^{-1}. However, absorbances produced by MAbs 11A4 and 20F10 were approximately half that of 10A9, 17A9 and 21D11. MAb 12C4 was the only negative MAb at a concentration of 1 µg mL^{-1} (Fig. 5.3 A). Hybridoma supernatant containing MAb 7C6 detecting the recombinant protein of ORF68 was only positive at the lowest dilution (i.e. 1/10 dilution), however, MAb 10D10 detecting recombinant protein of ORF62 was negative (Fig. 5.3 B). The reaction of both mouse ascitic fluid solutions, diluted 1/100, were below the sensitivity threshold (OD_{450nm} 0.18) of the ELISA (Fig. 5.3 C). Negative control PBS wells were also below the sensitivity threshold. MAbs to an alternative virus: ISAV had previously tested negative on this ELISA.

5.3.1.2 Screening of monoclonal antibodies by Western blot

As only low yields of KHV protein could be obtained from bulk purification of virus from cell culture supernatants, viral proteins separated by SDS-PAGE were stained with silver staining rather than Coomassie Blue in order to detect low levels of virus protein due to its greater sensitivity (*i.e.* as little as 0.1 ng) (Rabilloud *et al*., 1994).

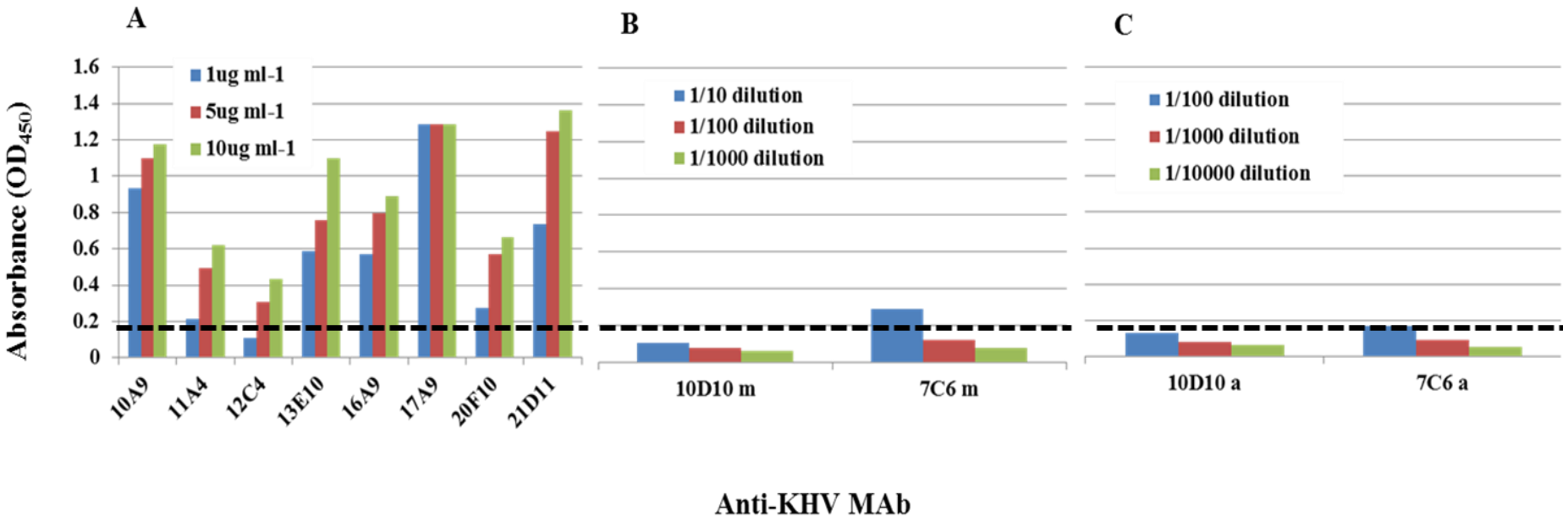


Figure 5.3 Reaction of MAbs and mouse ascites fluid with purified Koi herpesvirus (KHV) antigen by ELISA. (A) Mean absorbance values of purified MAbs produced by mice immunised with whole KHV (B) Absorbance values of hybridoma supernatant of MAbs produced by mice immunised with recombinant KHV proteins (C) Absorbance values of ascites fluid from mice immunised with recombinant KHV proteins. Bars represent the mean OD of replicates (*n*=2). Broken black bar indicates sensitivity threshold (cut off).

(a) SDS-PAGE separation and staining of purified KHV proteins

Purified KHV was serially diluted prior to performing SDS-PAGE so that a better contrast could be obtained from background staining to allow detection of the most prominent protein bands. Proteins from lysed uninfected CCB cells were also separated by SDS-PAGE and stained. More bands were obtained on the gel of the CCB cell lysates compared to the purified KHV proteins (results not shown). A number of bands associated with the purified KHV were not observed in the gel of the lysed CCB cells, however, and between 20 and 22 bands associated with the virus were detected on the gel (Fig. 5.4 A). A dominant band at approximately 130 kDa was noted from the purified KHV gel (indicated by arrowhead on Fig. 5.4).

(b) Western blot analysis of MAbs against purified KHV proteins

Western blot analysis of MAbs to KHV was replicated with and without intensive blocking in an attempt to eliminate non-specific binding and background signal so that the specific proteins recognised by different MAbs could be determined. Prior to sample blocking (addition of 2 % SMP w/v in the diluent) between 2 - 6 dominant bands were observed after incubating MAbs with the membranes containing purified KHV proteins, which were not observed with membranes containing uninfected CCB cell lysate (Fig. 5.4 C-D). MAbs against recombinant proteins of ORF62 and ORF68 were negative in Western blot analysis (result not shown). Proteins of approximately 250 kDa and 130 kDa were evident for all 8 of the purified MAbs, whereas proteins of 240 kDa and 150 kDa were distinctive for 6 and 7 of the 8 MAbs, respectively. A band of approximately 170 kDa was only present on membranes incubated with MAbs 13E10 and 21D11 and a band of 70 kDa was only present for MAbs 11A4 and 12C4. Notably, a dominant band of approximately 100 kDa was only present for MAb 20F10 (Fig. 5.4 C).

After additional blocking there was a clear reduction in signal and far fewer bands were obtained as a result. However, the highest molecular weight proteins were still clearly detected and all 8 purified MAbs produced intense bands at 250 kDa. Most interestingly, a dominant band of approximately 100 kDa could still be observed from MAb 20F10. There were also noticeable bands of approximately 150 and 75 kDa detected by MAbs 21D11. Other bands that could still be distinguished were a band of approximately 30 kDa recognised by MAb 16A9 and 150 kDa recognised by MAb 13E10 and MAb 11A4 (Fig. 5.4 B).

5.3.1.3 IFAT screening of and confocal microscopy of MAbs specific for KHV

To further verify the MAb recognition of KHV and the cellular localisation of the antigen, IFAT was undertaken on KHV infected KF-1 cells grown on coverslips. Cells were fixed at 7-10 dpi. All MAbs were positive by IFAT, however, different fluorescence intensity and localisation within the cell was observed with different MAbs. Most of the MAbs exhibited cytoplasmic signals only, which were usually diffuse (Fig 5.5), whereas MAb 10A9 produced signals more closely associated with the cell membrane (Fig 5.5 A). However, despite exhibiting intensive cytoplasmic staining, MAb 20F10 also produced very specific, diffuse nuclear signals (Fig. 5.5 G). MAb 7C6, recognising an antigen of ORF68, also exhibited both cytoplasmic and nuclear staining although to a lesser degree and the nuclear signals appeared more focal and granular (Fig. 5.5 I).

Weaker signal was noted with MAbs 10A9 and 13E10 and the most intensive staining was observed with MAb 17A9. The MAb 10D10 exhibited relatively weak specific signal compared to its negative control. Negative controls (mock infected cells screened with MAbs) sometimes displayed minimal sporadic non-specific staining and autofluorescence, but were always negative for specific fluorescence when compared to positive infected cells

(results not shown). Positive KHV infected cells screened with MAbs to ISAV were always negative.

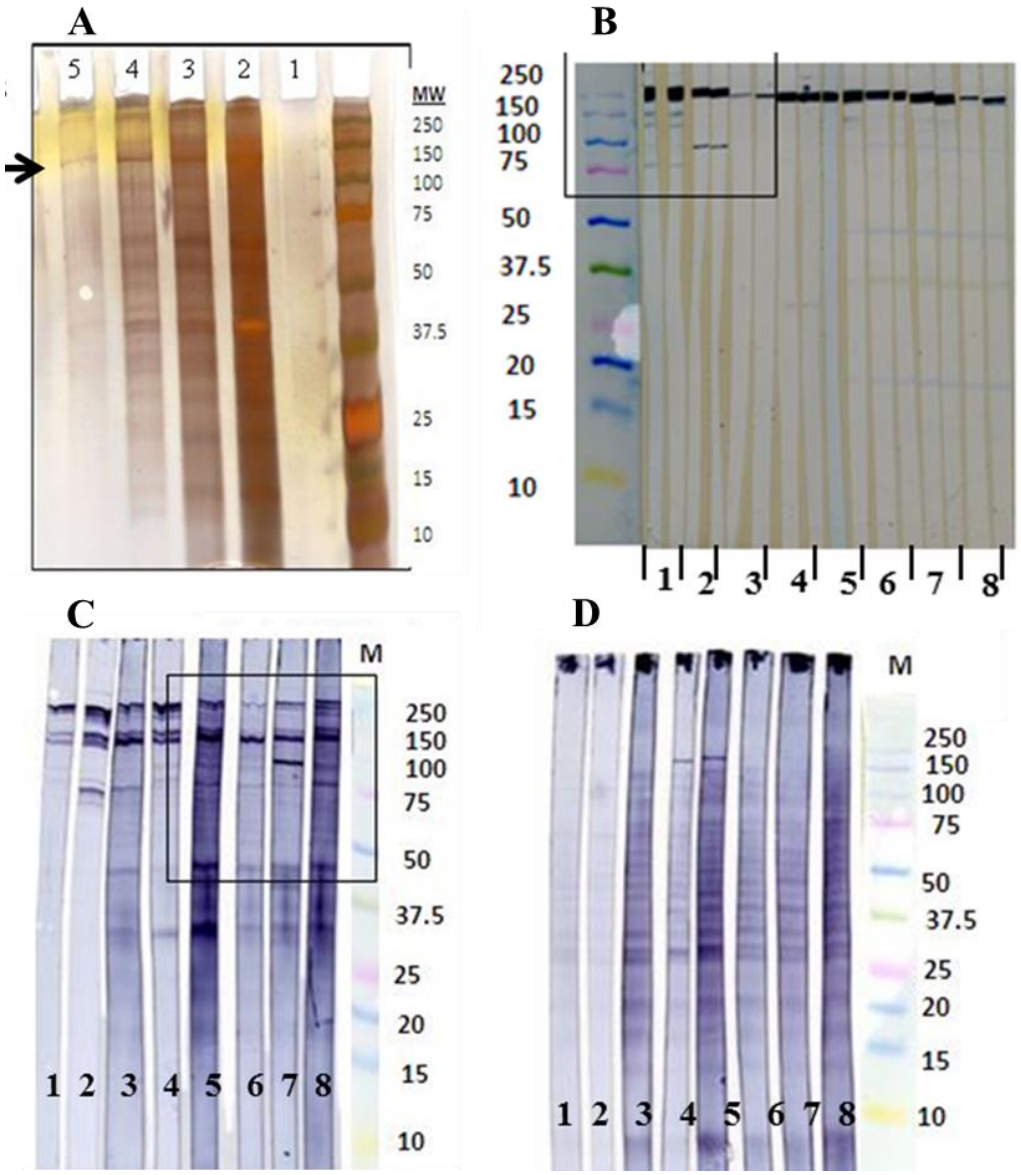


Figure 5.4 SDS-PAGE and Western blot analysis of sucrose gradient purified Koi herpesvirus (KHV) and CCB cell lysate using the panel of MAbs. (A) SDS-PAGE of sucrose gradient purified KHV, American isolate H361, after 2-fold dilution of viral proteins (Lanes 2-5). Lane 1 – Diluent only, 2 – KHV protein of 0.5 mg mL^{-1}, 3 - 0.25 mg mL^{-1}, 4 - 0.125 mg mL^{-1}, 5 - 0.0625 mg mL^{-1}. Arrowhead = dominant KHV protein at 130 kDa; (B) Western blot (WB) analysis of 8 purified MAbs screened against purified KHV with diluent blocking MAbs: Lane 1 – 21D11, 2 – 20F10, 3 – 17A9, 4 – 16A9, 5 – 13E10, 6 – 12C4, 7 – 11A4, 8 – 10A9; (C) WB analysis of 8 purified MAbs screened against purified KHV without diluent blocking (D) WB analysis of 8 purified MAbs screened against CCB cell lysate without diluent blocking. C and D MAbs: Lane 1 – 10A9, 2 – 11A4, 3 – 12C4, 4 – 13E10, 5 – 16A9, 6 – 17A9, 7 – 20F10, 8 – 21D11

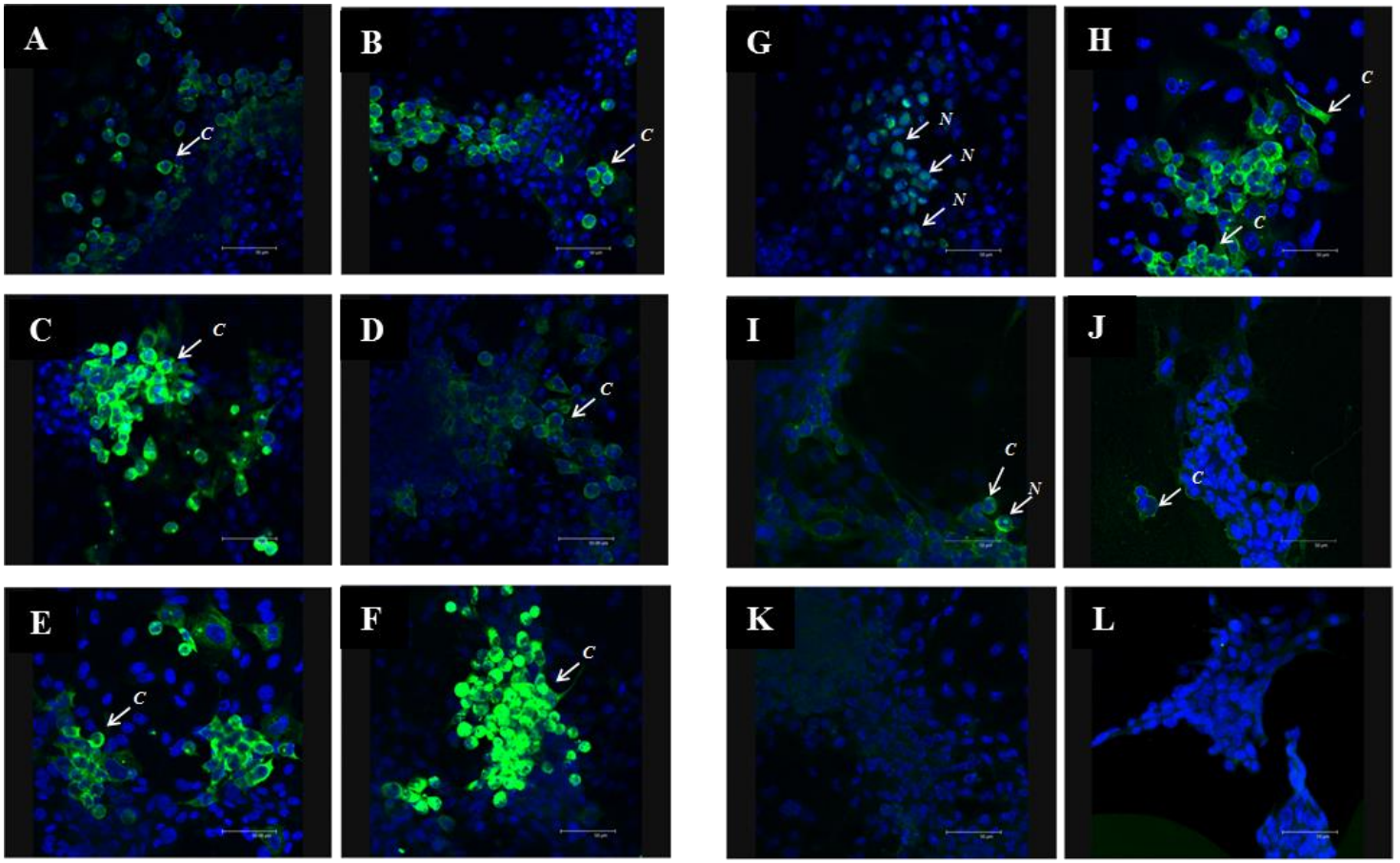


Figure 5.5 Confocal micrographs of Koi herpesvirus (KHV) infected KF-1 cells screened by IFAT using different monoclonal antibodies to the virus. (A) MAb 10A9 (B) MAb 11A4 (C) MAb 12C4 (D) MAb 13E10 (E) MAb 16A9 (F) MAb 17A9 (G) MAb 20F10 (H) MAb 21D11 (I) MAb 7C6 (J) MAb 10D10 (K) ISA MAb control (L) PBS control. Images captured by a × 3 zoom. Blue = DAPI (nuclei), Green = FITC (KHV) A, B, E-L: 10 dpi; C & D: 7dpi. *N* = Nuclear staining, *C* = Cytoplasmic staining. All micrographs show an overlay of green and blue channels.

Characteristics of all the MAbs are summarised in Table 5.1 including the results from other Laboratories on neutralisation testing and antibody isotyping (Friedrich Loeffler Institut and IoA, University of Stirling) and characterisation (Aoki *et al*., 2009; 2011). Briefly, MAbs 11A4 and 17A9 have been found to have varying degrees of virus neutralising capacity and both recognise virus glycoproteins and MAb 10A9 is known to recognise an envelope glycoprotein antigen. MAb 12C4 is a primary envelope antigen and MAb 20F10 a capsid antigen (Dr. Sven Bergmann, pers. comm., unpublished data), while MAbs 7C6 and 10D10 recognise recombinant proteins expressed from sequences of ORF68 and ORF62, respectively (Aoki *et al*., 2009; 2011).

5.3.2 Early stage antigen expression of KHV *in vitro* determined by binding of MAbs

The panel of MAbs detecting various antigens of KHV were screened against KHV-infected cell cultures through a time course using immunofluorescence in order to evaluate the expression of these antigens. The first approach was intended to elucidate the most useful MAbs that could detect KHV antigens of interest during the early stages of virus replication. As there were a large panel of MAbs to be tested, a 96-well plate IFAT procedure was developed on cultured and infected cells, which could enable high throughput analysis of antibody-antigen binding, at various stages post-KHV infection.

5.3.2.1 Preliminary experimental proof of concept

(a) DAPI fluorescence of cells seeded at varying densities

As DAPI stains nucleic acids, the relative fluorescence obtained from DAPI stain should enable a relative determination of the cell confluence of cultures in the wells. There was a positive correlation between cell seeding density and DAPI fluorescence as expected.

Table 5.2 Summarised MAb characterisation: Current and previous work

Anti KHV MAb	Ig isotype	Antigen recognised	NT	WB MW of recognised peptides (kDa)	IFAT Cellular locality of antigen staining	ELISA Mean ($OD_{450\ nm}$) at 10µg mL^{-1}
10A9	U	Envelope glycoprotein (*a)*	U	250, 240, 150, 130	Cytoplasm	1.18
11A4	IgG1κ	Envelope – capsid (TM) (a)	++ *(a)*	250, 240, 150, 140, 130	Cytoplasm	0.62
12C4	U	Primary envelope (*a)*	U	250, 150, 130, 70	Cytoplasm	0.43
13E10	IgG	Unknown	- *(a)*	250, 240, 170, 150, 130	Cytoplasm	1.10
16A9	U	Unknown	U	250, 240, 150, 130, 40, 30	Cytoplasm	0.89
17A9	IgG2 κ	Glycoprotein	+ *(a)*	250, 130	Cytoplasm	1.29
20F10	IgG1κ	Capsid *(a)*	- *(a)*	250, 240, 150, 130, 100	Nucleus/ Cytoplasm	0.67
21D11	U	Unknown	U	250, 240, 170, 150, 130, 75	Cytoplasm	1.36
10D10	IgM	ORF62 Tegument *(b)*	U	- *(b)*	Cytoplasm	0.1** 0.13* *(b)*
7C6	IgG1	ORF68 Unknown *(b)*	U	140, 72, 70*(b)*	Nucleus/ Cytoplasm	0.29** 0.17* *(b)*

MW-molecular weight; NT-neutralisation test; TM – Transmembrane; U-Unknown

*** MAb hybridoma supernatant at 1/10 dilution**

**** Ascites fluid at 1/100 dilution**

***(a)* Research at FLI (Bergmann pers. comm.)**

***(b)* Aoki *et al*. (2009; 2011); tegument association of ORF62 reported by Michel *et al*. (2010b)**

A higher sensitivity setting provided a greater quantitative range between cells of different seeding densities (Fig. 5.6 A).

(b) FITC fluorescence of cells infected with KHV over different time periods: 3 days and 12 days post infection

There was a significant increase in FITC fluorescence observed in cells infected with KHV after 12 dpi compared with 3 dpi after screening with anti-KHV MAbs 10A9 (p=0.001) and 20F10 (p=0.002), but not with the alternative MAb detecting ISAV (p=0.126) or mock infected cells screened with pooled anti-KHV MAbs (p=0.651) (Fig.5.6 B).

5.3.2.2 Relative FITC fluorescence of MAbs on mock infected CCB cells fixed over the experimental time course.

All MAbs were screened on CCB cells that had been mock infected and sampled at 1, 3 and 7 dpi. The relative FITC:DAPI fluorescent ratio was calculated as described in Section 5.2.2.2 (d) and most MAbs showed either very little or no positive FITC:DAPI fluorescence on uninfected cells compared with infected cells, apart from minor background signals with ascitic fluid and major background with MAb 16A9 (results now shown).

5.3.2.3 Relative FITC fluorescence of MAbs on KHV infected cells over the course of infection

High background fluorescence was noted in KF-1 cells, with some fluorescence present with uninfected cells and cells stained with the ISAV MAbs. Fluorescence from controls in CCB cells was much lower throughout the time course. Positive fluorescent signals were seen towards the end of the trial, 7 dpi, with 6 of the 10 MAbs (11A4, 12C4, 13E10, 17A9, 20F10 and 21D11) in infected KF-1 cultures and with 8 of the 10 MAbs (11A4, 12C4, 13E10, 16A9, 17A9, 20F10, 21D11 and ASC 62) in infected CCB cultures at which point there was a high CPE with nearly all cells infected.

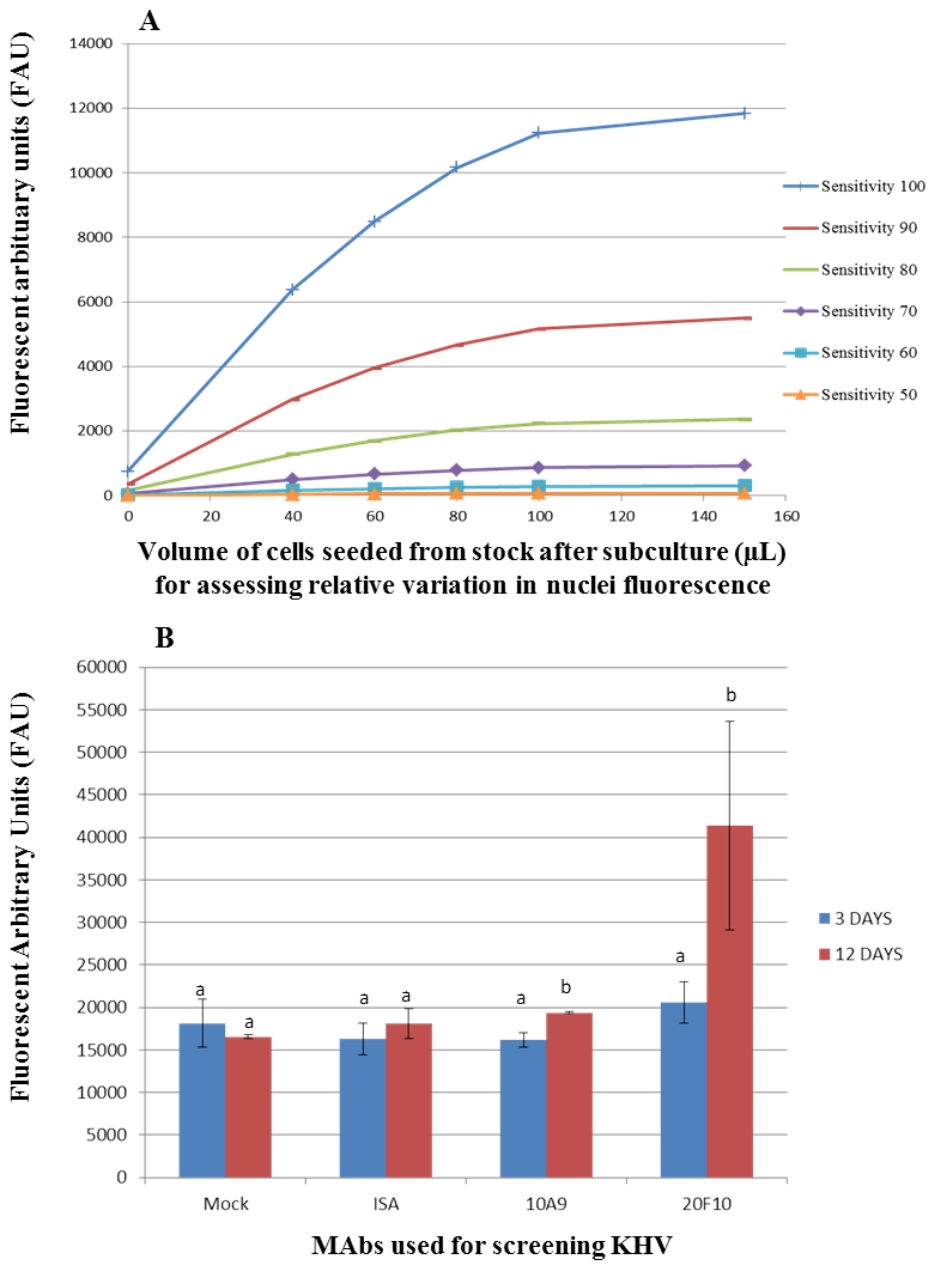


Figure 5.6 Preliminary investigation of cell and virus fluorescence: DAPI (nuclear) fluorescence emitted at increasing CCB cell confluence and koi herpesvirus (KHV) infected CCB cells with MAbs 10A9 and 20F10. (A) Decreasing volumes of cells seeded into wells equivalent to 4 x 10^3 – 3 x 10^4 cells $well^{-1}$ (Mean of replicate cell cultures, *n* = 2). Only results of 1/10 DAPI dilution shown. Fluorescence signals obtained at wavelength 360/460 nm. Relative sensitivity according to spectrophotometric detector thresholds is shown in arbitrary units to the right of the figure. (B) Preliminary screening of KHV infected CCB cells with MAbs 10A9 and 20F10. Comparison of FITC emission with MAbs 10A9 and 20F10 recognising KHV and an alternative MAb to ISAV screened at 3 and 12 dpi. Mean ±SE (*n* = 6 infected cell cultures). 2-sample t-test (≥95%). Different letters indicate significant differences.

However, strong signals were only obtained from MAb 20F10 in KF-1 cells and from MAbs 13E10, 20F10 and ASC62 in CCB cells at this late stage of infection. The specificity of MAb binding was more distinguishable in CCB cells as the non-specific binding observed in KF-1 cells had largely masked specific signals of bound MAbs (results not shown).

It should be noted that the mock control used in the current study consisted of a cocktail of all MAbs screened to uninfected cells, thus is not a direct indication of non-specific binding activity of every independent MAb and as a result background signals of mock infected cells may have been largely influenced by MAb 16A9 in KF-1 cells as noted in section 5.3.2.2 (results not shown).

5.3.2.4 Expression of KHV envelope glycoprotein and capsid protein in KF-1 and CCB cells determined by quantitation of MAb binding

As initial results of the antigen detected by MAb 10A9, a known envelope glycoprotein, and the antigen detected by MAb 20F10, a capsid associated antigen (Table 5.2) showed some noticeable differences in relative MAb fluorescence, the data for the binding of these MAbs to KHV were analysed further. The aim of this was to determine the expression of envelope glycoprotein (10A9) compared to capsid (20F10) proteins of KHV during viral infection.

DAPI fluorescence, and thus cell confluence, was relatively stable during the first day of infection in both cell lines with a slight increase noted in CCB cells, however fluctuations did occur during the first 2–8 hpi. From 2–10 dpi there was an obvious decline in cell nuclear fluorescence, particularly in KF-1 cells but also in CCB cells, which was more progressive towards the later stages of viral infection (Fig. 5.7 A-B and 5.8 A-B). However, no significant differences between cell nuclear fluorescence from the early stage of KHV infection (1-24 hpi) and the later stages of KHV infection (2-7 dpi) in either KF-1 cells (p=0.326) or CCB cells (p=0.439) was observed.

The expression of envelope glycoprotein and capsid antigens, measured by relative FITC:DAPI signals of MAbs 10A9 and 20F10, respectively, differed enormously throughout the time course of infection. There was a notable decrease in antibody binding of both MAbs in both cell lines after 2 hpi. This was followed by an increased binding to capsid antigen after only 6 hpi, as detected by MAb 20F10 in CCB cells, which was not observed until after 24 hpi in KF-1 cells (Fig. 5.7 C and 5.8 C). In contrast, binding to envelope glycoprotein antigen, as detected by MAb 10A9, was never observed during the first 24 hpi in CCB cells, but there was slight positive signal in KF-1 cells sampled at time point 24 hpi, comparable to capsid antigen binding by MAb 20F10 (Fig. 5.7 C and 5.8 C). There were no significant differences between glycoprotein and capsid antigen expression during the earlier stages in either CCB (p=0.082) or KF-1 cells (p=0.141).

Between 2–10 dpi there was a vast increase in capsid antigen expression indicated by the greater antibody binding of MAb 20F10 (Fig. 5.7 D and 5.8 D). Expression of capsid antigen at the later stages (2-7 dpi) was significantly greater in both KF-1 cells (p<0.001) and CCB cells (p<0.001) compared to the earlier stages (1-24 hpi). The initial increase occurred after 48 hpi in both cell lines but, notably, there was still very little expression of envelope glycoprotein antigen as indicated by minimal changes in fluorescence observed from cells screened with MAb 10A9. The expression of glycoprotein antigen was significantly greater in KF-1 cells (p=0.05), but not CCB cells (p=0.73) at later (2-7 dpi) compared to earlier (1-24 hpi) stages of infection. Between 4-6 dpi there was a notable plateau in capsid antigen binding by MAb 20F10 in KF-1 cells, which was paralleled by a slight decrease in signal from 5–6 dpi in CCB cells. After 6 dpi there was another rapid increase in fluorescence from MAb 20F10, and thus capsid expression, which decreased slightly again in CCB cells sampled after 10 dpi (Fig. 5.7 D and 5.8 D).

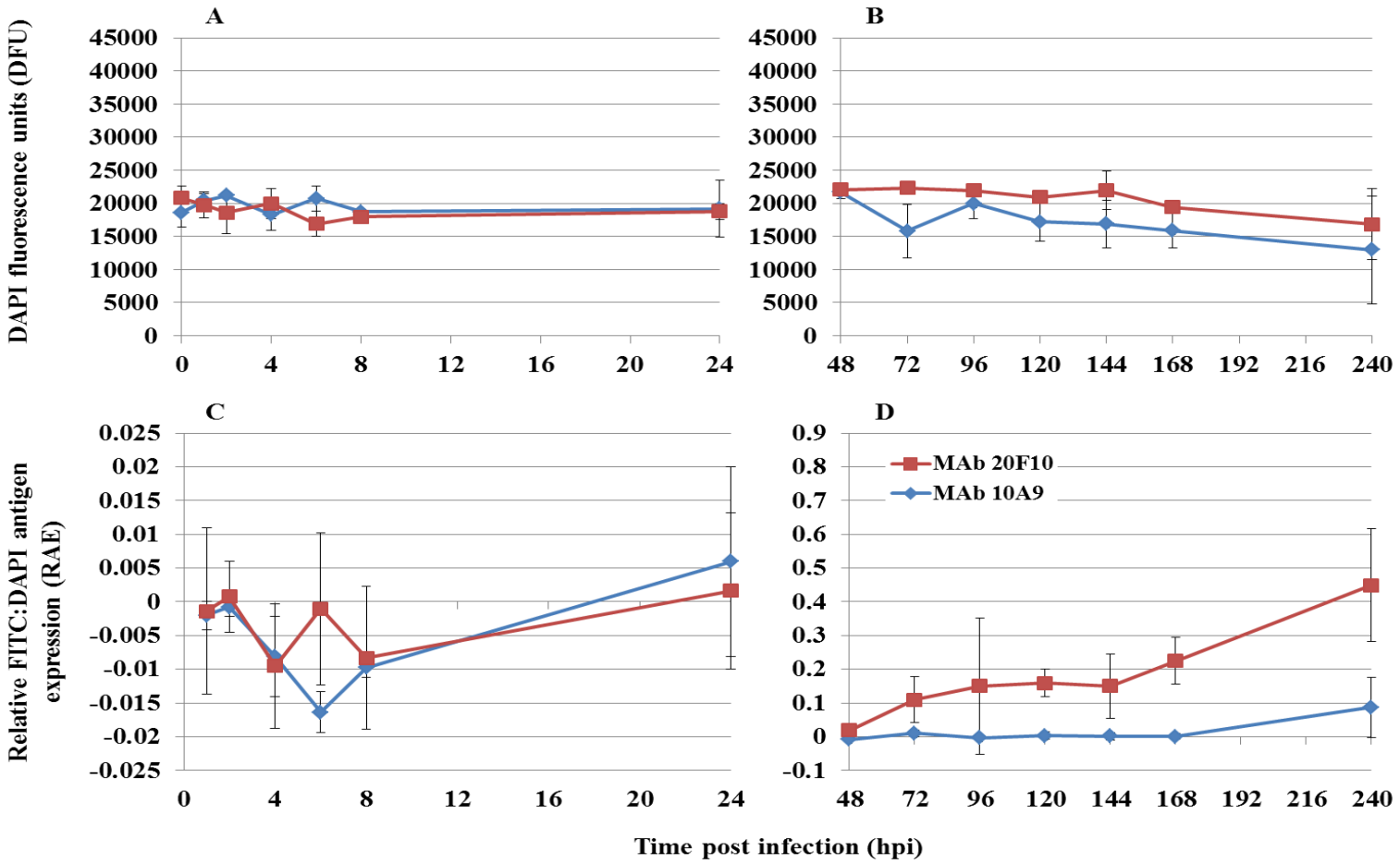


Figure 5.7 Graphical representation of fluorescence from MAbs 20F10 and 10A9 recognising Koi herpesvirus antigens over the course of infection in KF-1 cells. Graphs on the left are of cells analysed during the first day of infection, graphs on the right are of cells analysed over the following 2-10 dpi. (A and B) DAPI fluorescence of cells; (C and D) Difference of relative FITC to DAPI stain from blank wells at 0 hpi. Mean ± SE (n = 4 individual cell cultures)

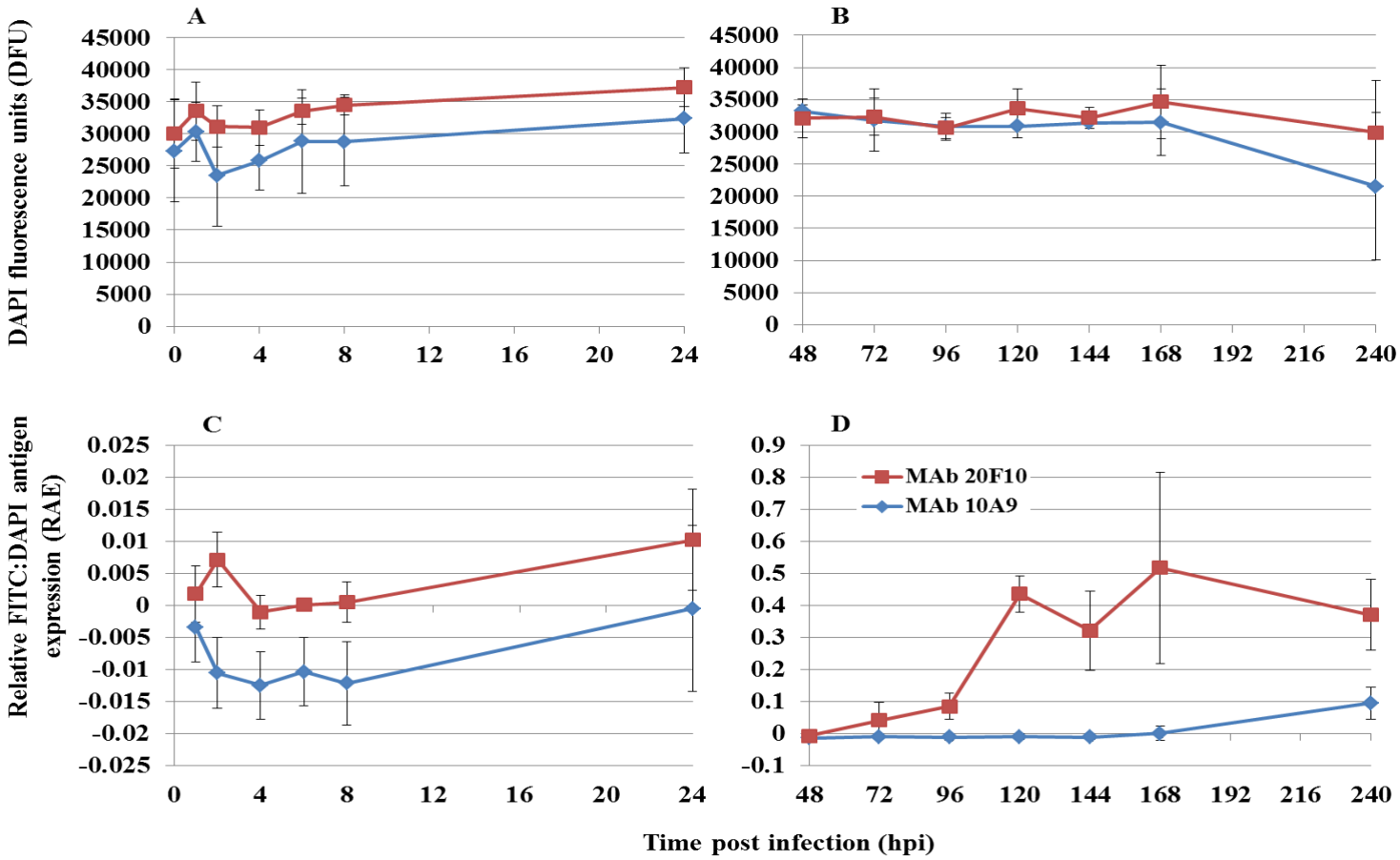


Figure 5.8 Graphical representation of fluorescence from MAbs 20F10 and 10A9 recognising Koi herpesvirus antigens over the course of infection in CCB cells. Graphs on the left are of cells analysed during the first day of infection, graphs on the right are of cells analysed over the following 2-10 dpi. (A and B) DAPI fluorescence of cells; (C and D) Difference of relative FITC to DAPI stain from blank wells at 0 hpi. Mean ± SE (n = 4 individual cell cultures)

There was no detectable increase in binding of MAb 10A9 to envelope glycoprotein antigen, suggesting minimal antigen expression or protein abundance, within the detection limits of the assay, until the final sampling point at 10 dpi where nearly all cells in all cultures were infected (Fig. 5.7 D and 5.8 D). Capsid antigen expression was significantly greater than glycoprotein expression in both KF-1 cells ($p<0.001$) and CCB cells ($p<0.001$) during the later (2-7dpi) stages of infection.

5.3.3 KHV morphogenesis and image analysis of envelope glycoprotein and capsid antigen expression during infection

Stages of virus morphogenesis were analysed by TEM in parallel to envelope glycoprotein and capsid antigen expression using a second novel IF approach with MAbs 10A9 and 20F10 by confocal microscopy and image analysis. This was achieved by infecting cell cultures on coverslips fixed at the same time points used in the time trial presented previously (Section 5.3.2.4). In an attempt to evaluate any relationships between virion formation, antigen expression and viral DNA load, real-time TaqMan qPCR (Gilad *et al*., 2004) was also performed on cell cultures infected simultaneously with the same virus isolate. The IF approach used in this experiment would also enable a degree of verification of the antigen expression trends observed using the 96-well microtitre plate procedure, where difficulties in interpretation of specific antibody binding were experienced due to non-specific binding of MAbs in the mock infected wells, particularly in the KF-1 cell line.

5.3.3.1 Cytopathic effect and viral DNA load

(a) Cytopathic effect of infected cells

A rapid cytopathic effect was obtained following inoculation with KHV, which began at 3 dpi, at which stage small plaques were evident particularly within CCB cell monolayers. By 4 dpi both CCB and KF-1 cultures exhibited cytoplasmic vacuolation and increased plaque

abundance. After 5 dpi there were many vacuolated cells as well as formation of syncytia and most cell nuclei appeared condensed and presented marginated chromatin. Cells became rounded and, after death, detached from the monolayer. After 5 dpi CPE progressed rapidly and more than 50% of cells from both cell lines were infected by 6 dpi with CPE becoming even more pronounced by 7 and 8 dpi (see Fig. 5.9, Table 5.3).

(b) Quantification of KHV DNA in cell pellets and supernatant

Real-time qPCR was undertaken according to the methods of Gilad *et al*. (2004) with modifications according to Bergmann *et al*. (2010a) from DNA extracted from cell culture supernatant and pelleted infected cells. Cycle threshold (Ct) values of positive controls from dilutions of plasmids bearing a KHV DNA insert of 484 bp derived from ORF89-90 (Gilad *et al*., 2002), in which the plasmid concentrations and fragment copy numbers were previously known (Bergmann *et al*., 2010a), were relatively consistent for all runs.

In the trial Cts ranging from were 22-23 for 1 ng KHV DNA and 29-30 for 10 pg KHV DNA. Subsequent to inoculation, there was a rapid absorption of virus to cells whereby the quantity of viral DNA obtained from cell pellets was equivalent to that of the original inoculum for both cell lines after only 1 hpi (Fig. 5.10).

Although an increase in viral DNA occurred in the supernatant from both CCB and KF-1 infected cell cultures, this was not detectable until 4 dpi. Infected KF-1 cell supernatant contained more viral DNA than CCB cells after 8 dpi. In CCB cells 500-fold less DNA copies (1×10^7) were measured from the supernatant after 8 dpi compared to that obtained from cell pellets (5×10^9), whereas 20-fold less was observed in KF-1 cell supernatant (5×10^7) compared to pellets (1×10^9) (Fig 5.10).

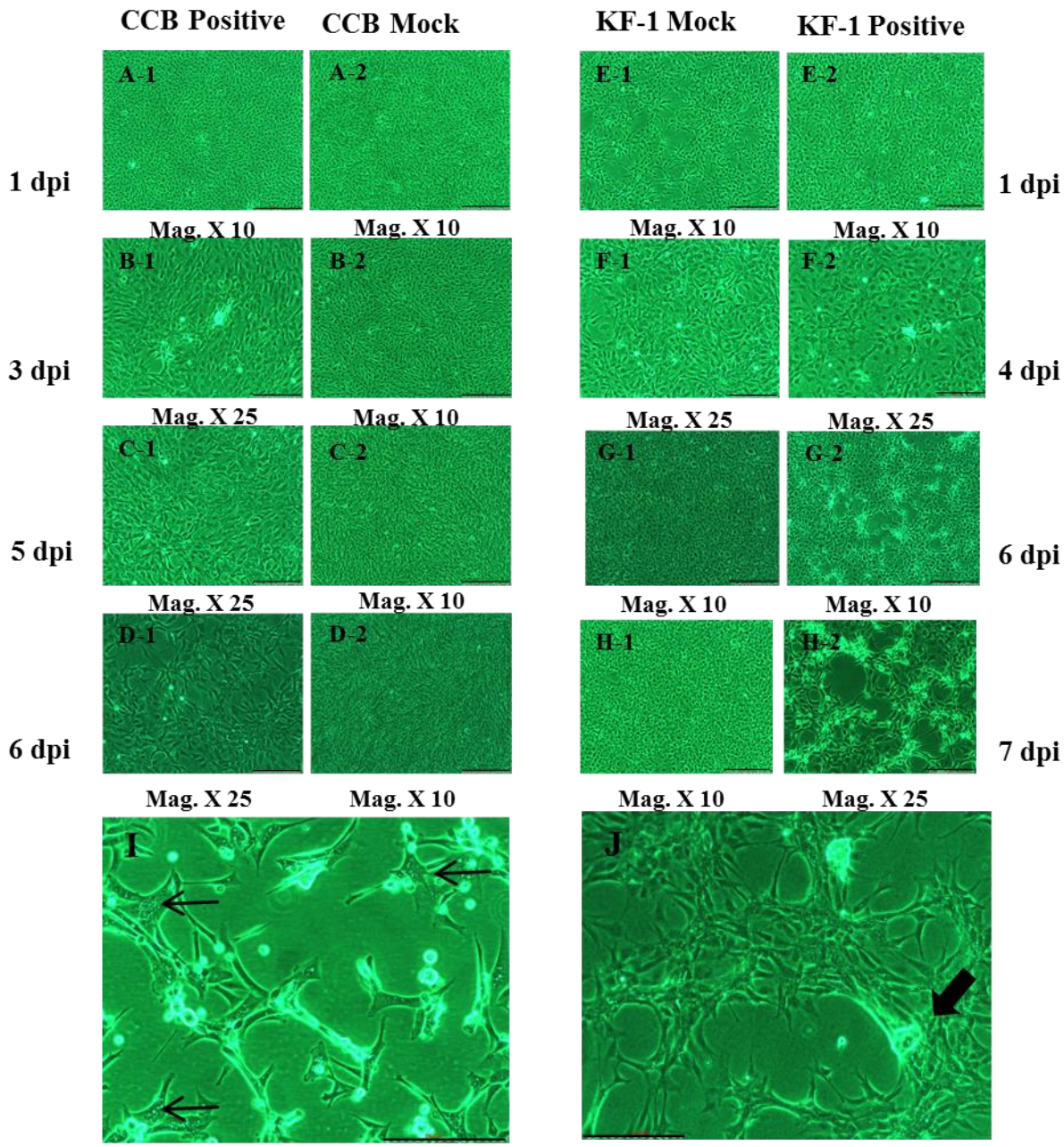


Figure 5.9. Cytopathic effect of Koi herpesvirus infected CCB cells and KF-1 cells during the 7 day time trial. A-D – CCB infected and mock control cells after (A) 1dpi, (B) 3dpi, (C) 5dpi and (D) 6dpi. E-H –KF-1infected and mock control cells after (E) 1dpi, (F) 4dpi, (G) 6dpi and (H) 7dpi.(I & J) High magnification of CCB cells after 7dpi, arrows show vacuolation in the cytoplasm (I) and formation of syncytia (J). Mag. x 25 Scale bar = 500 µm; Mag. x 10 Scale bar = 200 µm

Table 5.3 Scoring of cytopathic effect of Koi herpesvirus infected KF-1 and CCB cells during the first 10 dpi

CELL LINE	DPI								
KF-1	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**10**
CPE	-	-	-	(+)	(++)	(+++)	+(+++)	++	++(++)
CCB	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**10**
CPE	-	-	-	(+)	(+++)	+(+)	++	++(+)	++(++)

CPE scoring from (+) weakest to +++ severe

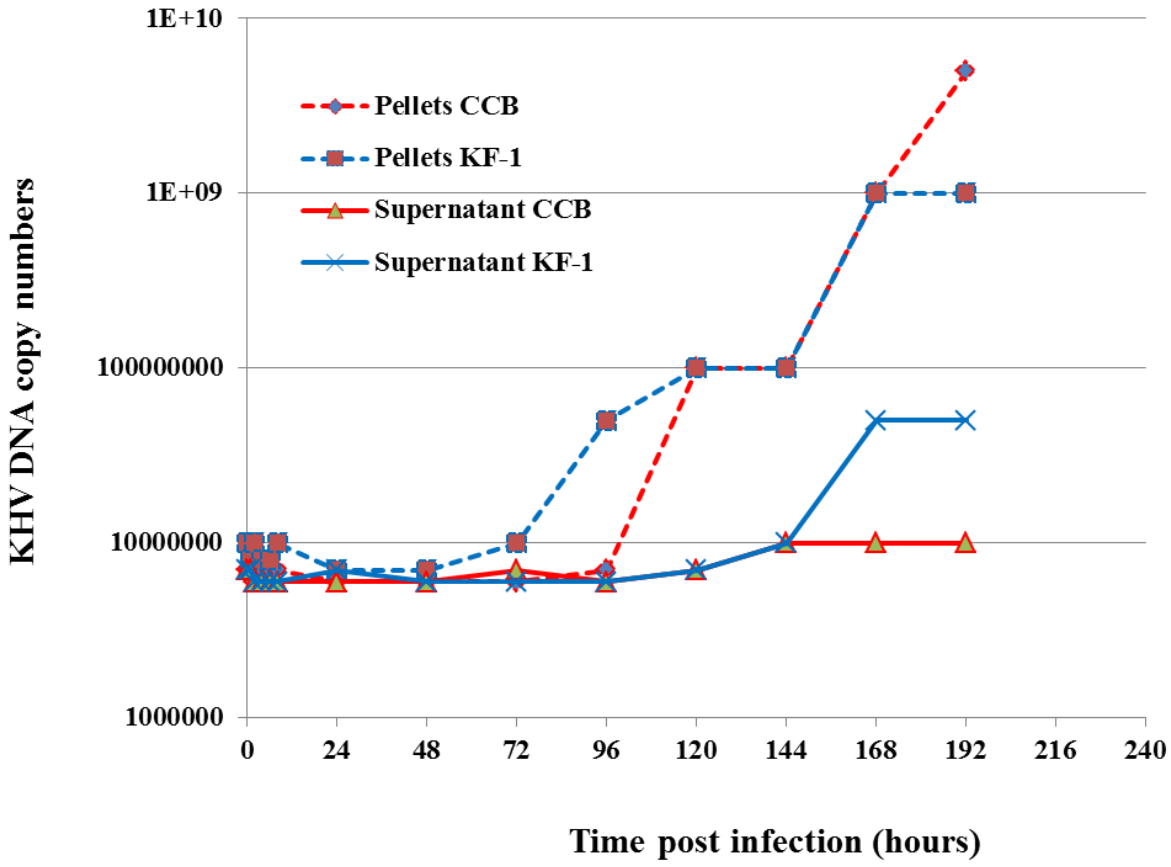


Figure 5.10. Real-time TaqMan qPCR results of Koi herpesvirus (KHV) DNA measured in KF-1 and CCB cells during the first 8 dpi. The results are expressed as KHV DNA genomic equivalents per sample DNA template extracted from pellets and supernatant of infected KF-1 and CCB cell cultures

In contrast to supernatant samples, viral DNA within the cell pellets increased dramatically after 3 dpi in both cell lines, particularly in CCB cells, whereby >800-fold increase in KHV DNA copy numbers were observed after 8 dpi (Fig. 5.10). Mock infected cell cultures were always negative (Not shown).

5.3.3.2 TEM analysis of KHV morphogenesis in infected cells

TEM micrographs of KHV morphogenesis were obtained from the *in vitro* time trial as well as from the preliminary TEM experiment (6 dpi). Virus particle sizes differed depending on the stage of morphogenesis. The mean sizes were as follows: immature capsids = 97.56 nm (SD 8.78), nucleocapsids = 114.12 nm (SD 12.13), primary enveloped virions = 138.32 nm (SD 18.43) and secondary enveloped mature virions = 167.97 nm (SD 31.38), which are all within the range of KHV particles reported in the literature.

(a) KHV in infected cells during the first 24 h post infection

One of the most notable observations was the presence of viral capsids within the nucleus of a number of infected cells after only 1 hpi. The capsids were either empty or consisted of a double arc structure (Fig. 5.11 A-B) and these were occasionally distributed throughout the nucleus (Fig. 5.11 D), but there were no mature capsids with electron dense cores. No capsids were observed in the nuclear membrane at this stage, and particles in the cytoplasm had not yet budded into cytoplasmic vesicles. Although no actual fusion to, or endocytosis at, the cell membrane was observed, empty coated pits were evident, extending intracellularly from the plasma membrane (Fig. 5.11 C) and putative cytoplasmic nucleocapsids could be observed in close proximity to these, possibly migrating towards the nuclear pores, however, no microtubules were observed near these structures (Fig. 5.11 C) and there were no capsids seen docked at the nuclear pores. Occasionally, empty and electron dense capsid-like structures were observed in a linear distribution in the cytoplasm (Fig. 5.11 E). By 4 hpi,

virus capsids were observed throughout the nucleus at various stages of maturation, often located at the periphery by the inner nuclear envelope (Fig. 5.11 F).

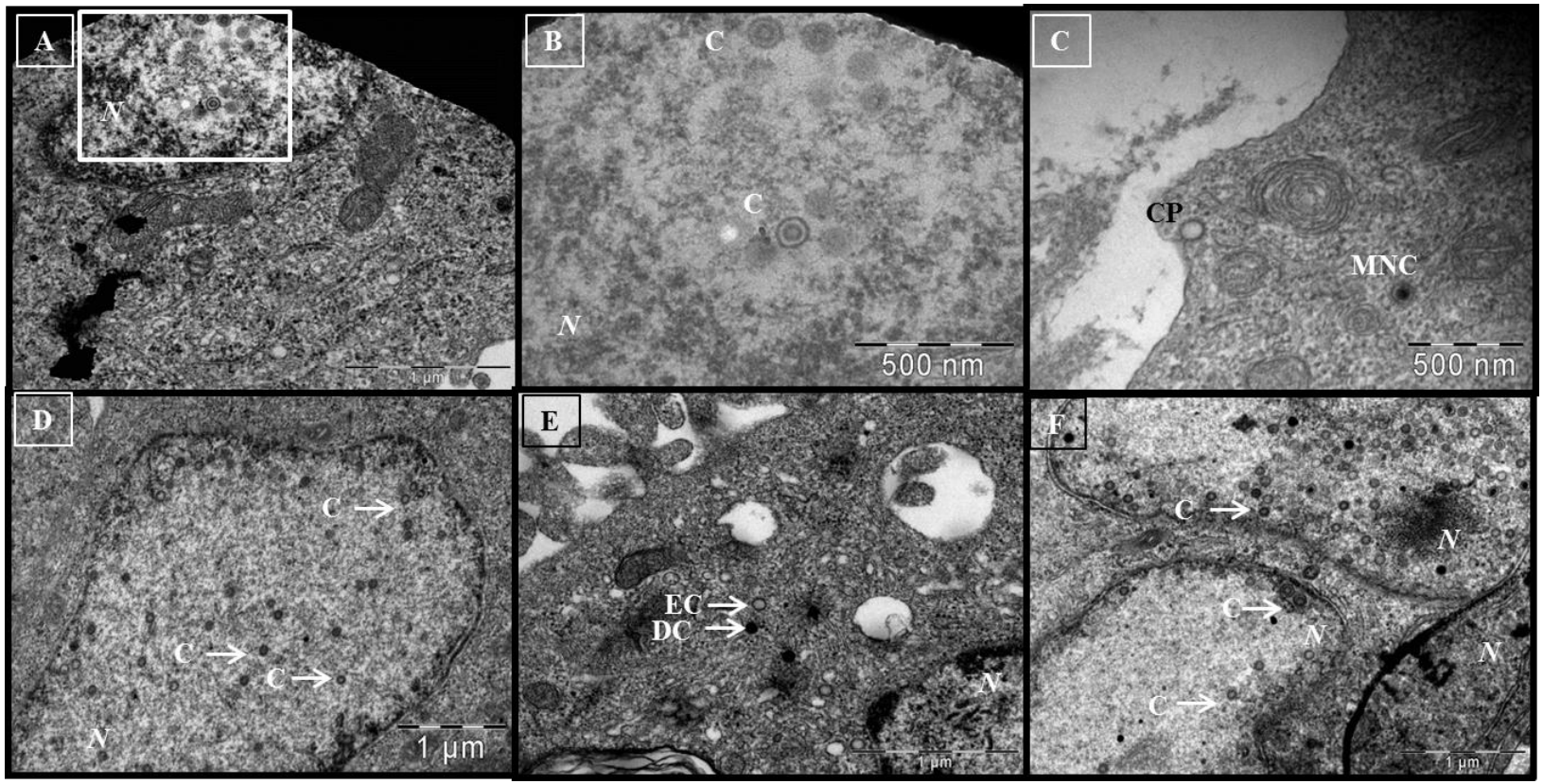


Figure 5.11 TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 1 hpi – 4 hpi. (A) Infected KF-1 cell 1 hpi (B) High magnification of nucleus shown in square of A (C) Cell membrane of infected KF-1 cells 1 hpi (D) Nucleus of infected CCB cells 1 hpi (E) Naked capsids within the nucleus of infected KF-1 cells after 1 dpi (E) Empty and electron-dense structures in a linear array of infected KF-1 cells. Note the close proximity to secondary lysosomes (F) 2 infected CCB cells in close proximity 4 hpi. Note the accumulation of capsids towards the periphery of the diffuse cell nucleus with varying degree of maturation. Primary envelope virions can also be observed. *N* = Nucleus; C = capsids; CP = Coated pit; MNC = putative migrating nucleocapsid; EC = Empty capsid-like structures; DC = Electron dense structures

Although many cells were uninfected and remained healthy (Fig. 5.12 A) the formation of paracrystalline arrays of capsids were found in both CCB and KF-1 cells and the nuclei of some cells had begun to express transluscent characteristics and chromatin margination (Fig. 5.12 B and C). Very seldom capsid particles could be seen within the nuclear envelope (Fig. 5.11 F). Naked nucleocapsids (without a secondary envelope) in the cytoplasm of infected cells had formed by 1 dpi, some were electron dense while others were empty (Fig. 5.12 D). Secondary enveloped particles could also be seen in the cytoplasm of some cells by 1 dpi, which still harboured large numbers of capsids in the nucleus (Fig 5.12 E).

(b) KHV virus in infected cells after 3-7 dpi

After 3dpi, although the number of infected cells had increased, there were still relatively few virus particles noted and many cells remained uninfected. Extracellular virions were observed at this stage from KF-1 cells but not CCB cells (Fig.5.13 C). Primary envelopment was clearly seen with up to three or four nucleocapsids contained within the perinuclear cisterna at one time (Fig. 5.13 D-E). Mature virions were observed in intracytoplasmic vesicles of CCB cells (Fig. 5.13 A-B) and capsids were observed at various stages of maturation, including more para-crystalline-like arrays in the nucleus of KF-1 cells (Fig. 5.13.F).

After 5-7dpi there were large numbers of virus particles in both cell lines at various stages of morphogenesis. After 5 dpi there were a greater abundance of cytopathic vacuoles with some containing internalised mature enveloped virus particles, while other virions had budded into cytoplasmic vesicles (Fig. 5.14 A). Mature virions were often observed associated with, and budding within, vesicles from the Golgi apparatus (Fig. 5.14 B) or from the cell membrane, which were sometimes in clumped extracellular aggregates (Fig. 5.14 C-D). The large vacuoles observed in the cytoplasm of infected cells of both cell lines (Fig. 5.15 A and C) often contained virus particles or lysed virus-like capsid structures.

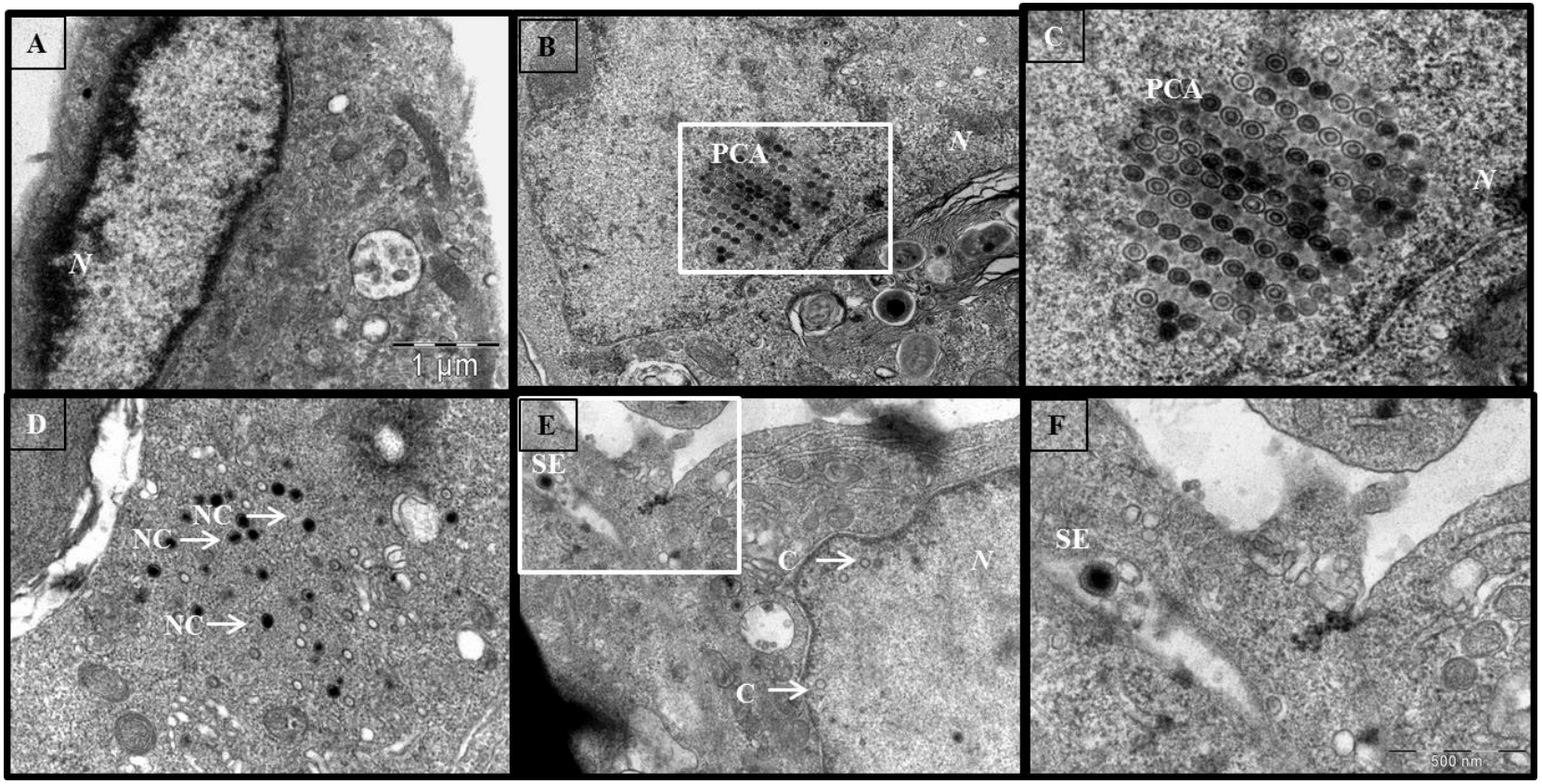


Figure 5.12 TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 4 hpi – 1 dpi. (A) Uninfected CCB cell (B) Infected KF-1 cells 4 hpi with paracrystalline formation of capsids in the nucleus (C) High mag. of capsids shown in square of B (D) Naked capsids within the cytoplasm of infected KF-1 cells after 1 dpi. Some capsids are mature (electron dense), while others are empty (E) Infected CCB cells after 1 dpi showing the formation of capsids within the nucleus and mature virions that have a acquired a secondary envelope in the cytoplasm (F) High mag. of the mature secondary enveloped virion of square shown in E. *N* = nucleus; PCA = Paracrystalline array of capsids; NC = Naked capsids; C = Capsids. Scale bars are not shown in B, C, D and E because of technical fault

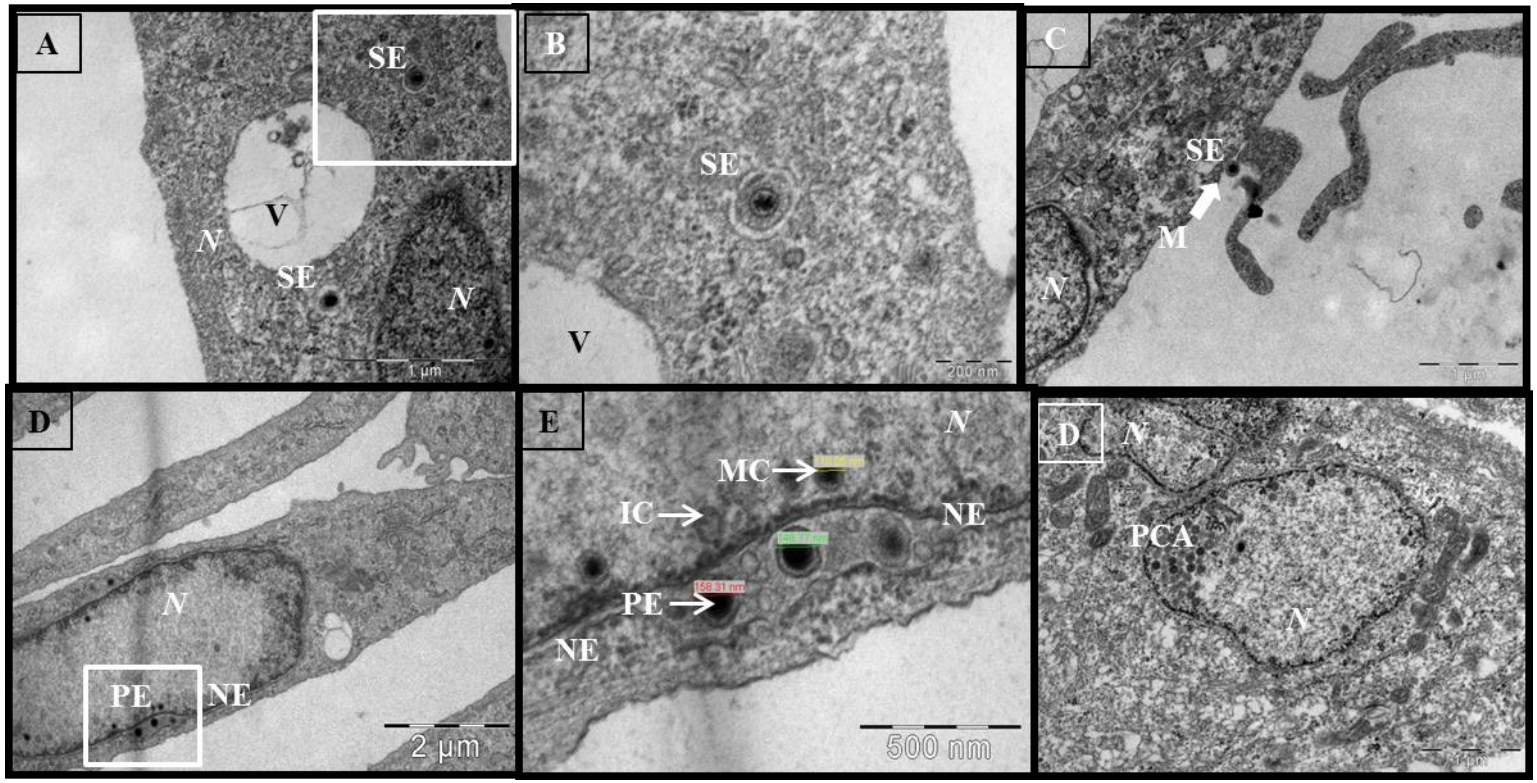


Figure 5.13 TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 3 dpi. (A) Infected CCB cells with mature secondary enveloped virions (B) High magnification of mature secondary enveloped virion (shown in square of A) within a vesicle in the cytoplasm (C) Infected KF-1 cells with budding infectious enveloped mature virion on cell membrane (D) Infected CCB cells containing capsids at various maturational stages in the nucleus and nucleocapsids budding through the nuclear envelope and acquiring a primary envelope (E) High magnification of square in D showing 3 primary enveloped virions within the nuclear envelope while smaller immature and mature capsids remain in the nucleus. (F) Infected KF-1 cell with paracrystalline array of capsids formed within the nucleus. *N* = Nucleus; SE = Secondary enveloped virion; V = Vacuole; PCA = Paracrystalline array of capsids; CPV = Cytopathic vacuole; M = Cell membrane. IC = Immature capsid; MC = Mature capsid; PE = Primary enveloped virion; NE = Nuclear envelope; M = cell membrane

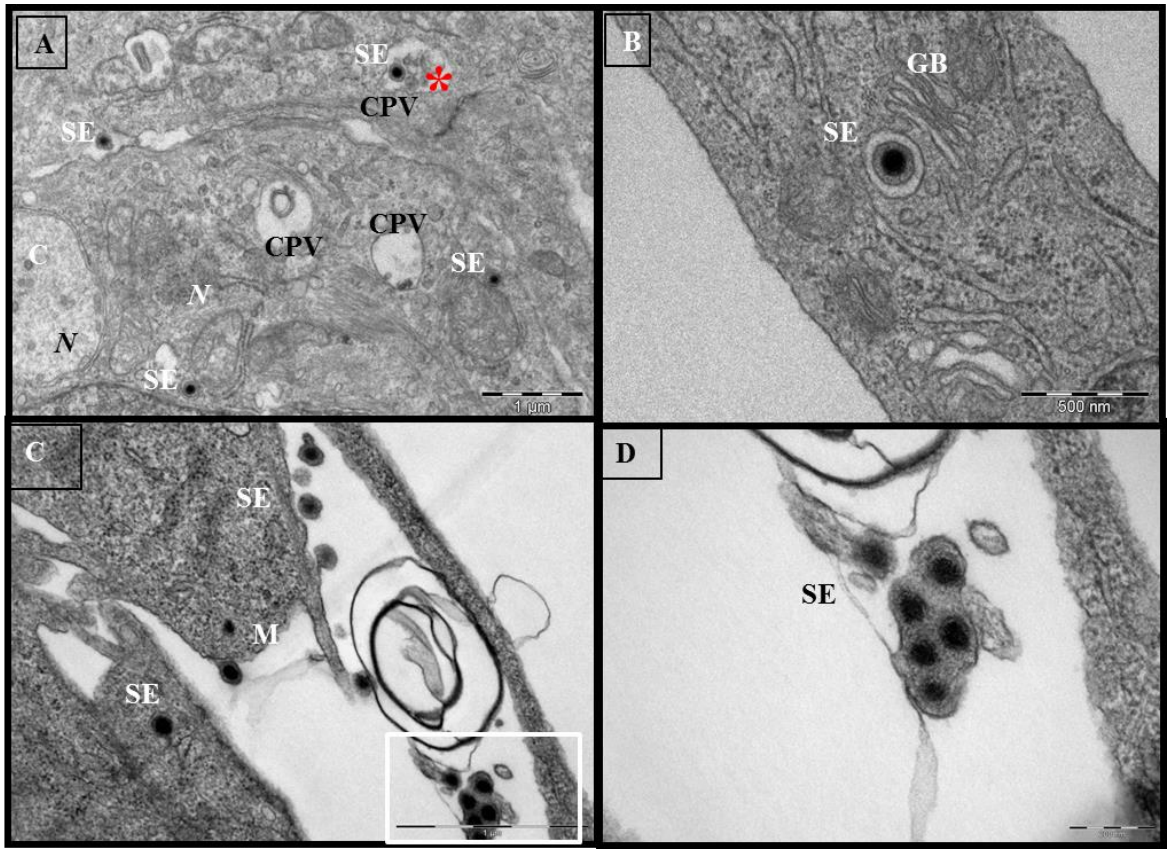


Figure 5.14 TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 5-7 dpi. (A) Low magnification of infected CCB cells 6 dpi with a number of cytopathic vacuoles and secondary enveloped mature virions budding from various membranous organelles. The red star (*) indicates secondary enveloped virion within a cytopathic vacuole. (B) Mature secondary enveloped virion within the cell cytoplasm, budding from golgi apparatus derived vesicle in CCB cells, 7 dpi (C) Infected KF-1 cells, 6 dpi with many mature secondary enveloped virions budding through the cell membrane (D) High mag. of square in C showing aggregates of extracellular, mature, infectious secondary enveloped virions (E) *N* = Nucleus; SE = Secondary enveloped mature virion; M = Cell membrane; GB = Golgi body

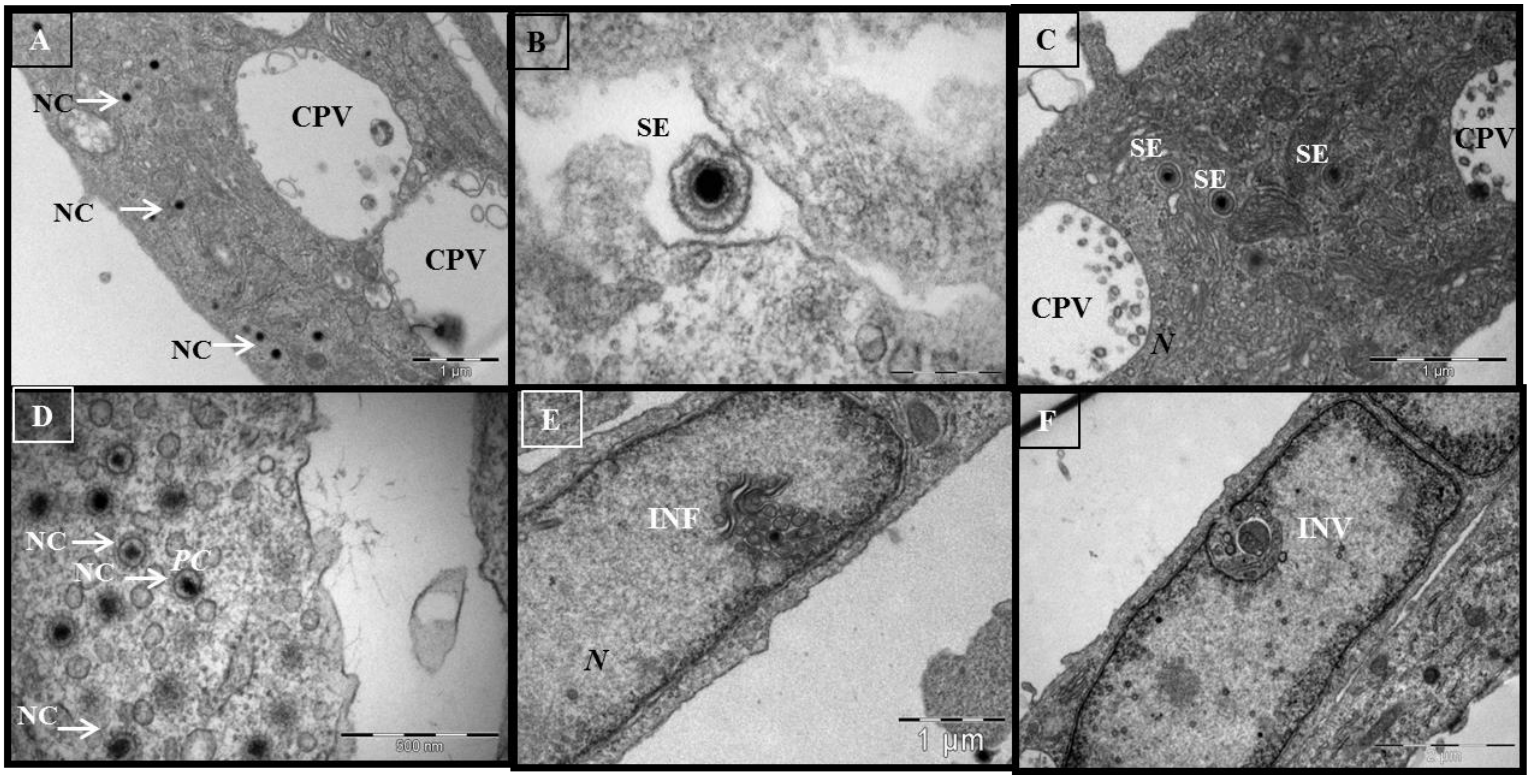


Figure 5.15 TEM micrographs of CCB and KF-1 cells infected with Koi herpesvirus 5-7 dpi. (A) Large vacuoles in the cytoplasm of infected KF-1 cells, 6 dpi – note the presence of several electron dense virus particles (B) High magnification of mature secondary enveloped virion in infected CCB cells, 6 dpi – note the defined layers: glycoprotein envelope with surface projections, tegument layer, capsid and electron dense core (C) Infected KF-1 cells, 7 dpi, containing secondary enveloped mature virions within cytoplasmic vesicles. Large cytopathic vacuoles are also evident containing cell debris (D) Clusters of naked/unenveloped capsids in the cytoplasm of infected CCB cells after 7 dpi Note the protruding core into the cytoplasm (E) Intranuclear folds of the inner nuclear membrane in CCB cells surrounded by capsids of various morphogenic stages after 7 dpi. (F) Infected CCB cell, 7 dpi exhibiting proliferation of the inner membrane of the nuclear envelope with vesicles containing virus particles forming within the nucleus *N* = Nucleus; NC = Naked capsids (Arrows showing mature nucleocapsids); *PC* = Protruding core; CPV = Cytopathic vacuole; SE = Secondary enveloped virions; INF = Intranuclear folds; INV = Intranuclear vesicle.

Clusters of naked nucleocapsids were often found in close proximity to these vacuoles, sometimes with protruding cores (Fig. 5. 15 A and D), and secondary enveloped mature virions within intracytoplasmic vesicles (Fig. 5.15 B and C). By this stage the compartments of the mature herpesvirus virion were clearly defined, including the projections of the glycoprotein envelope, amorphous tegument layer, capsid and dense core (Fig. 5.15 B). The nuclei were often deformed at late infection stages exhibiting a thickening and even re-duplication of the nuclear membrane giving rise to a number or irregular formations (Fig. 5.15 E-F). Some of the irregular nuclei consisted of inner nuclear vesicles (Fig. 5.15 F) or even folds, which were surrounded by nucleocapsids at various stages of maturation (Fig. 5.15 E).

5.3.3.3 Confocal analysis of capsid and glycoprotein antigen expression using MAbs through image analysis

(a) Confocal microscopy of KHV antigen expression during the course of infection

The fluorescence signal emitted from the MAbs detecting KHV envelope glycoprotein (MAb 10A9) and KHV capsid protein (MAb 20F10) indicated differences in the expression of these 2 virus epitopes as observed by confocal microscopy, which corresponded with results obtained in Section 5.3.2 using a 96-well IFAT procedure. Very weak signals were first noted from MAb 10A9 in KF-1 cells after 8 hpi (Fig. 5.16). Staining increased dramatically after 5 dpi with nearly all cells in analysed sections exhibiting cytoplasmic signals. In contrast, no obvious signals were observed visually for MAb 10A9 until 1 dpi in CCB cells when weak fluorescence was first observed. Intensive staining with MAb 10A9 could be observed only after 7 dpi in CCB cells (Fig. 5.17).

Early antibody binding was evident at a very early stage of infection, 4 hpi, in KF-1 cells when screened with MAbs detecting KHV capsid protein (MAb 20F10) (Fig. 5.16). However, the signal often appeared cytoplasmic despite initial characterisation of the MAb as

producing nuclear signals. The intensity of signals in KF-1 cells increased from 24 hpi and very obvious signals were observed after 1 dpi, which advanced further after 3 dpi (Fig. 5.16). CCB cells revealed a much lower degree of staining until 1 dpi, but by 3 dpi most cells expressed a high level of cytoplasmic fluorescence, this increase continuing in intensity to after 5 and 7 dpi (Fig. 5.17).

Despite initial attempts to seed all wells with the same density of cells, many cells did not adhere well to the glass cover slips used in the trial resulting in a variation in cell confluence during the course of infection. Negative controls, including an ISA MAb and cells screened only with diluent, were always negative although some obvious non-specific signal was occasionally observed, likely associated with debris and artefacts (i.e. autofluorescing cover slip adhesive) from processing.

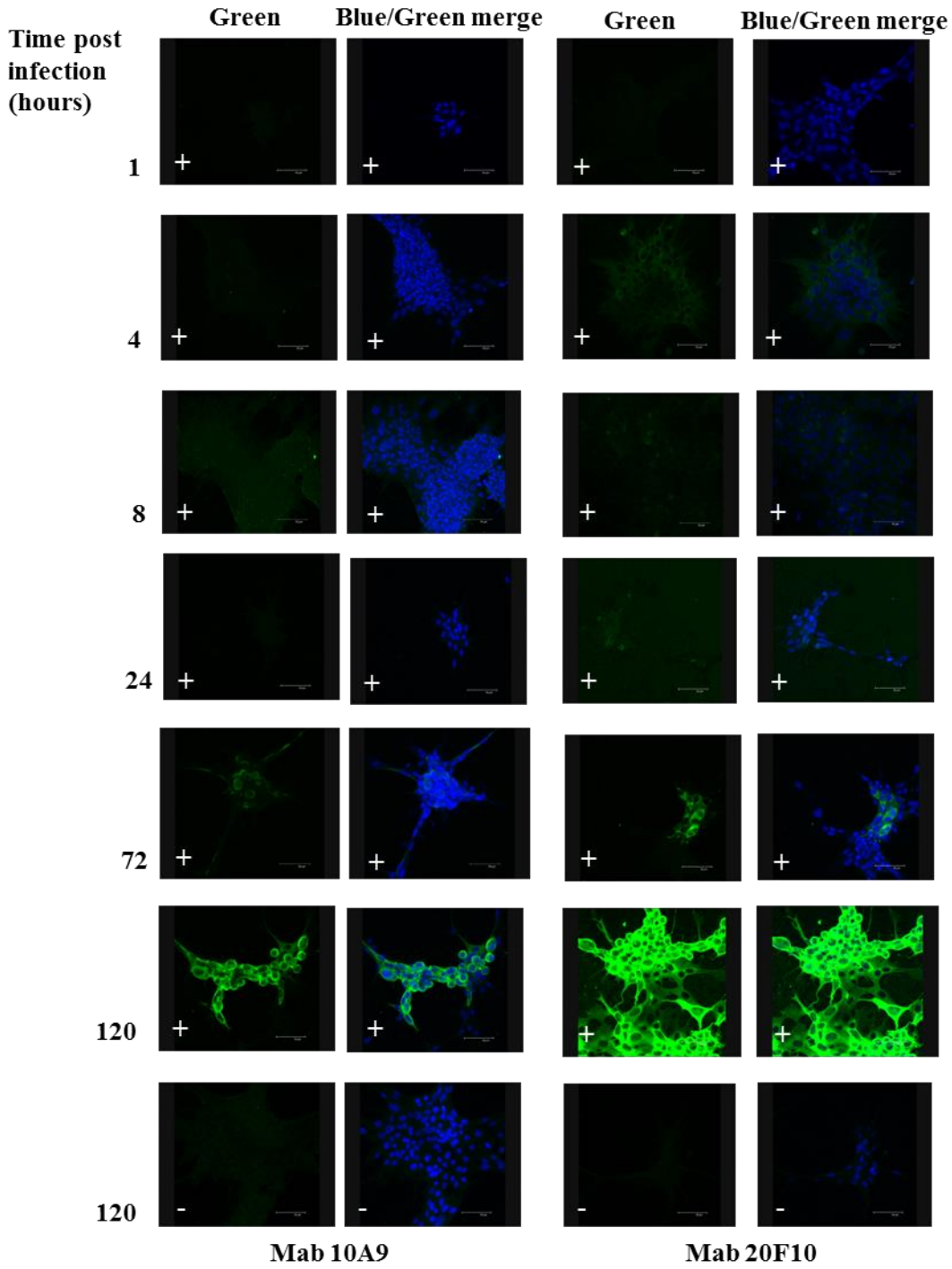


Figure 5.16 Confocal micrographs of FITC fluorescence signals from Koi herpesvirus infected KF-1 cells screened with MAbs 10A9 and 20F10 during infection. The time of sampling is indicated left of the micrographs. The filter channels used during scanning the sections is indicated above the micrographs, *i.e.* the green channel only shows virus signals for glycoprotein (MAb 10A9) or capsid (MAb 20F10) antigens and blue/green merge shows virus signal in relation to the cell nuclei (blue)

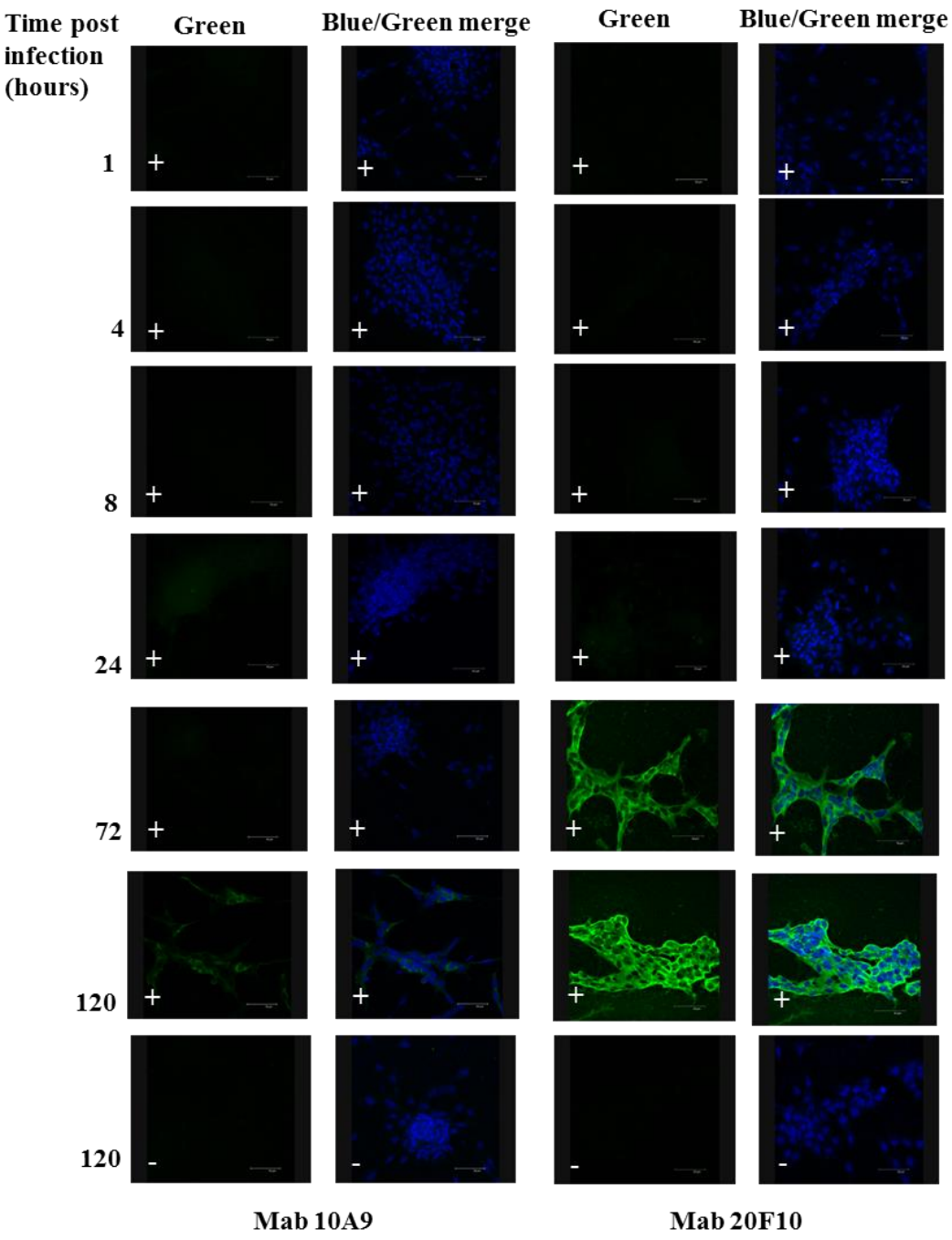


Figure 5.17 Confocal micrographs of FITC fluorescence signals from Koi herpesvirus infected CCB cells screened with MAbs 10A9 and 20F10 during infection. The time of sampling is indicated left of the micrographs. The filter channels used during scanning the sections is indicated above the micrographs, *i.e.* the green channel only shows virus signals for glycoprotein (MAb 10A9) or capsid (MAb 20F10) antigens and blue/green merge shows virus signal in relation to the cell nuclei (blue)

(b) Quantification and statistical differentiation of glycoprotein and capsid antigen expression through image analysis

(i) Transformation and separation of fluorescence into quantifiable data

Different parameters were successfully obtained from cultures scanned by confocal microscopy, which were subsequently transformed from DAPI and FITC fluorescence signals and utilised for image analysis (Fig. 5.18). The two parameters, HIMAB and CELL revealed a large degree of background autofluorescence of monolayers, i.e. signal above the set threshold of HIMAB or the total cell area fluorescence, in a number of sections caused by either contaminating debris or artefacts from processing such as the adhesive used during cover slipping. Therefore only NUC, MND and MBD were used to determine the relative specific fluorescence from anti-KHV MAbs detecting KHV antigens.

(ii) KHV glycoprotein and capsid antigen expression measured by image analysis

Capsid antigen expression was observed as early as 8 hpi in CCB cells and 4 hpi in KF-1 cells (Fig. 5.19 A and C) and an increase in expression was noted after 24 hpi in CCB and KF-1 cells. After 3 dpi there was a dramatic increase in capsid antigen expression of >10 fold in KF-1 cells (Fig. 5.19 D), which was more gradual in CCB cells up to 7 dpi (Fig. 5.19 B). The nuclear associated capsid expression and antigen abundance was much greater than that associated with the cytoplasm after 24 hpi (Fig. 5.19 A-D). Envelope glycoprotein antigen expression was also detectable after only 8 hpi in CCB cells and 4 hpi in KF-1 cells (Fig. 5.19 A and C), although no substantial increase was noted until 5 dpi in both cell lines. After this point there was high abundance within CCB cells, after 7 dpi, but the levels dropped in KF-1 cells after 5 dpi, possibly as a result of many cells having lysed by this stage in the KF-1 cell line (Fig. 9 H2). Notably, the levels of glycoprotein antigen at the later stage of infection in CCB cells had surpassed those of the cytoplasm-associated capsid antigen (Fig. 5.19 B).

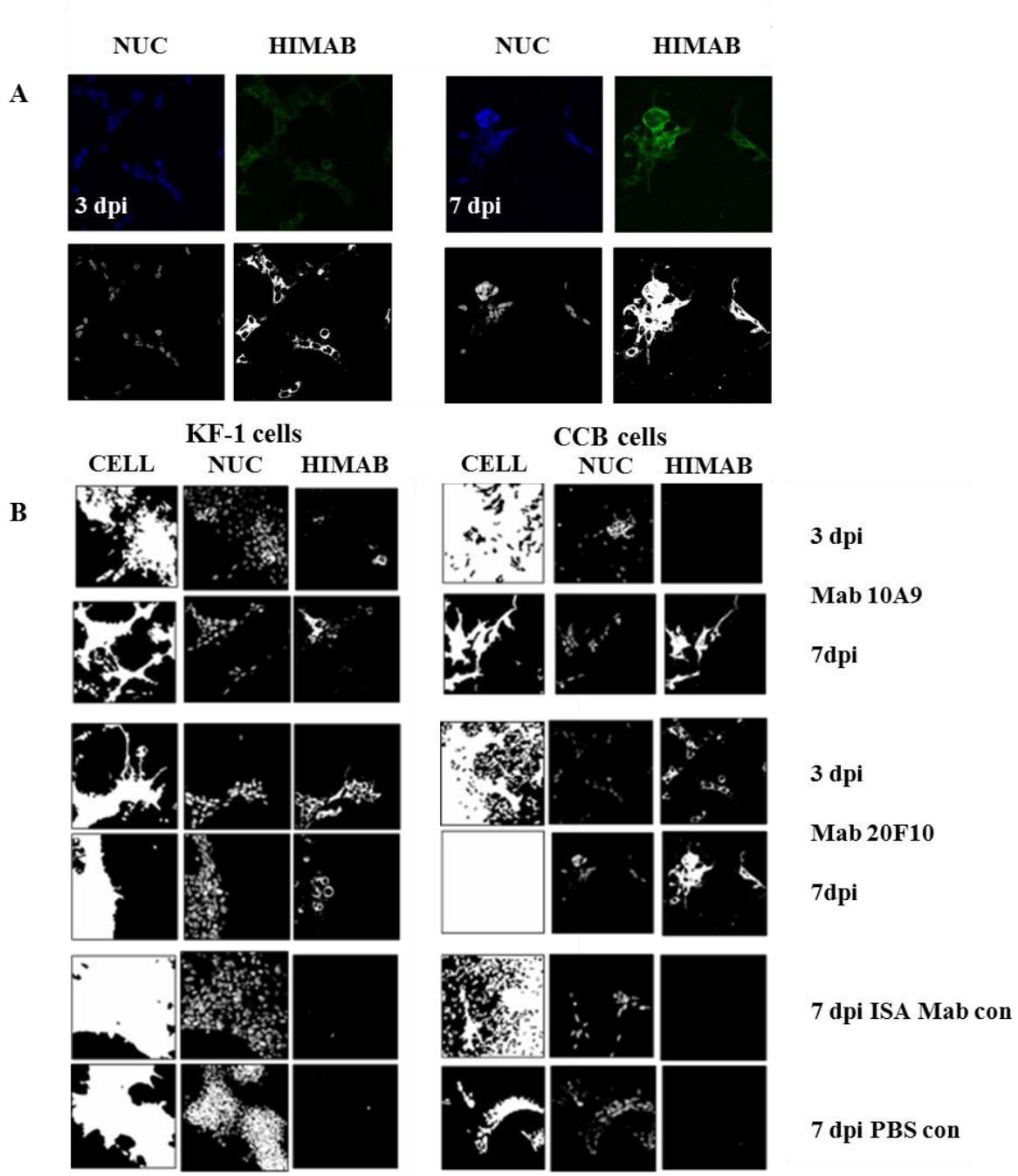


Figure 5.18. Transformation of confocal microscopy fluorescence for image analysis in Koi herpesvirus (KHV) infected KF-1 and CCB cells screened with MAbs 10A9 and 20F10. (A) Example for KHV infected CCB cells after 3 and 7 dpi screened with MAb 20F10 .The parameter converted for image analysis is indicated above each column. Top row = confocal micrographs, bottom row = image utilised for image analysis subsequent to transformation (B) Images of quantification parameters utilised by the image analysis macro. The parameter measured for image analysis is above the micrographs and above this is the cell line used. Time post infection is indicated on the right of each row as well as the MAb used for screening.

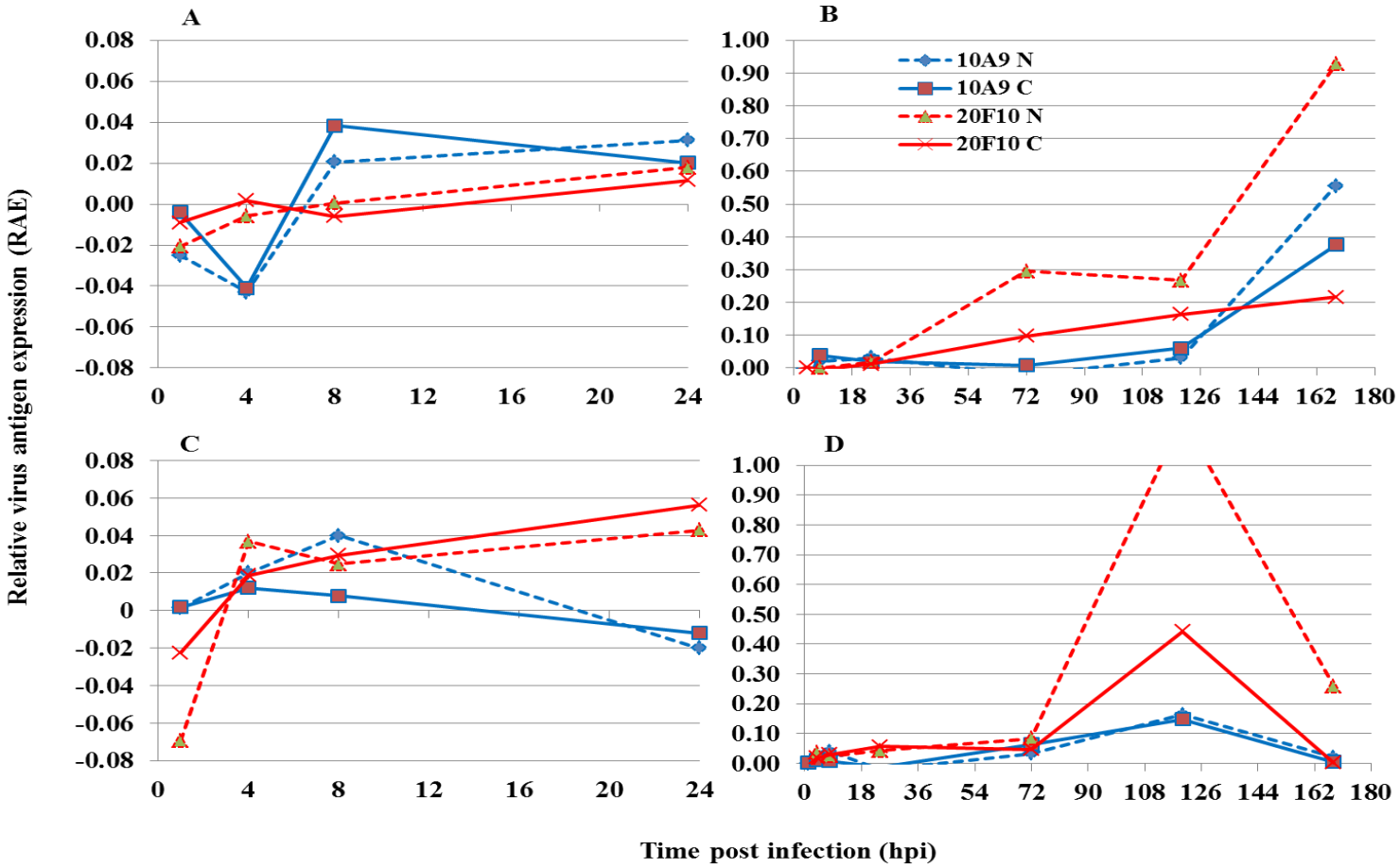


Figure 5.19 Koi herpesvirus antigen expression by differential fluorescence of control and infected cells *in vitro* using image analysis of capsid and envelope glycoprotein MAb binding. (A) KHV Infected CCB cells first 24 hpi (*n* = 1 cell culture); (B) KHV Infected CCB cells 1-7 dpi (*n* = 1 cell culture); (C) KHV infected KF-1 cells first 24 hpi (Mean *n* = 2 cell cultures); (D) KHV infected KF-1 cells 1-7 dpi (Mean *n* = 2 cell cultures).

As the same inoculum of virus was used to simultaneously inoculate all monolayers in the various vessels prepared later for TEM, real time qPCR, microtitre plate and confocal IFAT analysis, only limited replicates could be prepared due to logistical constraints. Therefore analyses of variance of medians between early (1-24 hpi) and later stage (2-7 dpi) nuclear (MND) and cytoplasmic (MBD) antigen expression were only attempted in infected KF-1 cells after pooling data from early infection stages (2-24 hpi) and later infection stages (2-7 dpi) (n=6) by using the Kruskal-Wallis test. There were no significant differences in nuclear signal (NUC) between early and later stages of KHV infection in either KF-1 (p=0.252) or CCB cells (p=0.509). There was significantly greater expression of both glycoprotein (MAb 10A9) (p=0.05) and capsid (MAb 20F10) (p=0.016) antigens at later stages of KHV infection compared to early stage infection in KF-1 cells. There were no significant differences, however, in antigen expression between the MAbs 10A9 or 20F10 at either an early or late stage of infection in KF-1 cells.

(iii) Differential antigen localisation and infected cell abnormalities highlighted by confocal microscopy

At later stages of infection, a high abundance of capsid antigen associated with both the nucleus and cytoplasm was observed, particularly in highly confluent areas of the cell monolayer (Fig. 5.20 A1). In contrast, the glycoprotein antigen was never expressed within the nucleus, but signals were often intense around the cell membrane (Fig. 5.20 C). In some cells capsid antigen signals were associated solely with the nucleus (Fig. 5.20 A2-A3). Strong signals with MAb 10A9 also revealed associated binding around the periphery of the nucleus and plasma membrane (Fig. 5.20 C2-C3) and the formation of giant cells (Fig. 5.20 C4-C5). Screening with other anti-KHV MAbs at this later stage of infection showed the extent of syncytium formation with some giant cells containing up to 20 nuclei (Fig. 5.20 B), and differential nuclear signals, to those expressed by the capsid antigen (Fig. 5.20 D).

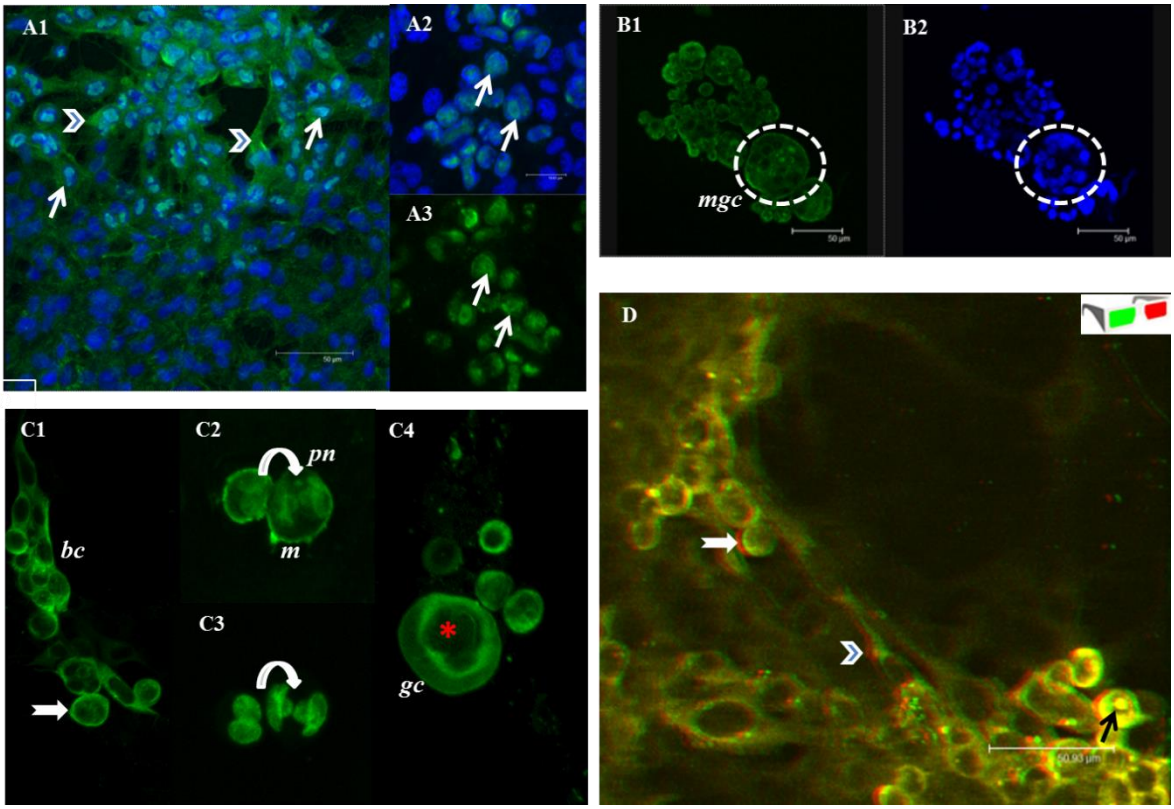


Figure 5.20 Confocal micrographs of Koi herpesvirus (KHV) infected KF-1 and CCB cells *in vitro* labelled with monoclonal antibodies (MAbs) to different virus antigens. (A1-A3) KHV infected cells labelled with MAb 20F10 detecting virus capsid (A1) Confluent region of KF-1 cell monolayer with cytoplasmic and concentrated nuclear signals of KHV, 7 dpi; (A2) High magnification overlay of capsid antigen signals in the nucleus; (A3) Virus signal only of cells shown in A2; (B1) Virus signal of multinucleated giant (*mgc*): infected CCB cells labelled by MAb 11A4 recognising KHV glycoprotein; (B2) Nuclei of cells shown in B1 - dashed ring indicates nuclei of giant cell (C1-C5) KHV infected cells labelled with MAb 10A9 detecting virus envelope glycoprotein (C1) A clump of infected blebbing (*bc*) CCB cells exhibiting abundant glycoprotein antigen signals in the plasma membrane; (C2) Signals associated with the periphery of the nucleus (*pn*) and plasma membrane (*m*); (C3) nucleus stained by propidium iodide of same infected cell as C2 – curved arrow showing viral signal around periphery of nucleus; (C4) giant cell (*gc*) formation exhibiting glycoprotein staining around the plasma membrane. Note the concave distribution of fluorescence around the nucleus indicated by red star; (D) 3D image of contrasting nuclear staining by MAb 7C6 compared to 20F10. Cytoplasmic signals are also prominent. Blue = DAPI/propidium iodide stained nuclei (in overlay micrographs) apart from C3; Green = FITC staining of virus; Visual interpretation of D requires 3D spectacles as indicated by . Thin arrows = nuclear associated antigen; Thick arrows = membrane associated antigen; Arrow heads = cytoplasmic associated antigen.

5.4 – Discussion

5.4.1 KHV protein profiling and binding characteristics of MAbs

Initial screening of all MAbs by ELISA, using sucrose purified whole KHV virus as antigen, demonstrated the extent to which recognition of the whole virion differs depending on the epitope target of the MAb. The concentration of antibodies for 8 MAb hybridoma cell lines was standardised, thus differences in binding cannot be explained simply by variations in the number of antibodies present to recognise virus epitopes of the soluble antigen in the wells. Where MAbs were not concentrated and purified, very low absorbance values were obtained, e.g. with MAb 7C6, which has previously been shown to work very well in ELISA (Aoki *et al*., 2009), thus the hybridoma supernatant and ascites fluid were likely too dilute to give strong positive signals. Therefore the high level of antibody binding of MAb 10A9, for example, compared to MAbs 11A4, 12C4 and 20F10, may correspond to the fact that the envelope glycoprotein epitope is recognised by MAb 10A9 and an internal glycoprotein, primary envelope and capsid epitopes are recognised by MAbs 11A4, 12C4 and 20F10, respectively. It is therefore possible that the differences observed between antibody-antigen binding complexes were associated with envelope glycoprotein epitopes being more abundant and exposed in whole purified, concentrated KHV virions than epitopes associated with internal glycoproteins, primary envelope proteins and capsid proteins. Internal virus proteins, including capsid, and certain primary envelope proteins are not found in extracellular virions of alphaherpeviruses such as PrV and HSV-1, i.e. UL31, UL34 and gD (Klupp *et al*., 2000; Skepper *et al*., 2001; Mettenleiter, 2002; Loret *et al*., 2008). Thus, their presence in the purified KHV stock may have derived from co-purification of disrupted virions, albeit relatively few compared to intact virions, during ultracentrifugation, resulting in exposure to

and recognition by their respective MAbs. Co-purification of internal virion proteins in apparently isolated membranes, including those associated with capsids, has been reported from HSV-1 infected cells (Gibson and Roizman, 1972).

In the present study, 20-22 bands were observed after silver staining SDS-PAGE gels containing purified KHV virions. This is similar to the 21 bands found in Coomassie blue stained gels by Adkison *et al*. (2005), but is less than the 31 polypeptides reported by Gilad *et al*. (2002) and 25 bands by Michel *et al*. (2010b). Dong *et al*. (2011) recently reported at least 30 visible bands from the first isolated KHV virus from China. Although variations between geographically-associated virus genotypes has been reported for KHV isolates (Kurita *et al*., 2009; Han *et al*., 2013), homogeneity of the KHV proteome has been demonstrated (Gilad *et al*., 2002; 2003), thus the geographic origin of KHV isolates is unlikely to be a factor in protein profile variations. Instead, this perhaps highlights differences in the purification process and the subsequent SDS-PAGE. This also highlights limitations in using Western blotting to examine the immunogenic proteins of KHV, as important polypeptides may go undetected or may not be successfully transferred to the membrane for immunoblotting. The high proportion of KHV protein retained within the host cell, as demonstrated by both immunofluorescence approaches for determining antigen expression, i.e. the high levels of capsid antigen still associated with the nucleus after 7 dpi (Fig. 5.19 A and B), and the high copy numbers of viral DNA retained in cell pellets during infection (Fig. 5.10), may also help to explain the loss of some virus proteins, following purification of the virions by sucrose gradient ultracentrifugation, as the antigens remain associated with cellular compartments. Michel *et al*. (2010b) reported that there were a number of bands specific for KHV in infected cell lysates that were either absent or less intense following purification, thus indicating that a lot of the KHV antigen formed in cell culture is retained with cellular

membranous organelles, even after lysis. The dominant band was observed at 130 kDa, which has previously been identified as the dominant KHV polypeptide by several authors (Gilad *et al*., 2002; 2003; Adkison *et al*., 2005; Michel *et al*., 2010b; Dong *et al*., 2011). Interestingly, all the MAbs reacted with this 130 kDa band, as well as a high molecular weight protein at 250 kDa. Despite some conserved antigen-recognition by certain MAbs, differences were observed between the MAbs in the proteins they recognised, which was expected since they were raised to different KHV structural proteins, e.g. 10A9 recognising an envelope glycoprotein and 20F10 recognising a capsid antigen. A number of cellular proteins were still identified with the same MW as purified virion lysates indicating there could be some cellular proteins present either retained within the mature virion, or contaminants from lysed cells during harvest and purification. This is possible as extracellular virions were harvested after extensive cell lysis (full CPE, 10-14 dpi), whereby cell protein contamination was minimised in another study by harvesting virus at the peak of the virus growth curve (i.e. 4 dpi) (Michel *et al*., 2010b). It is known that host cell proteins are associated with KHV virus particles both *in vitro* (Michel *et al*., 2010b) and *in vivo* (Gotesman *et al*., 2013).

All concentrated MAbs detected KHV antigens in Western blotting and IFAT, while MAbs to rORF62 and rORF68 proteins (Aoki *et al*., 2011) were presumably too dilute to produce bands. Positive staining was, however, obtained using these MAbs in IFAT during a later stage of the infection, when copious viral antigen was present within infected cultured cells. The negative results in Western blotting may be associated with a low abundance of proteins encoded by ORF62 and 68 being transferred onto the membrane.

The multiple bands observed in immunoblots, were not expected as each MAb should be monospecific and recognise only a single epitope, however, previous characterisation of

MAb 7C6, detecting rORF68 resulted in 3 bands (Aoki *et al*., 2011). These findings were thought to be the result of ORF68 endcoding a polyprotein and thus the 3 proteins detected were either a result of cleavage products by proteases, or alternative splicing or glycosylation of the protein after cleavage (Aoki *et al*., 2011). Although no bands were observed using MAb 7C6 in the current study, due to the low concentration as mentioned earlier, all MAbs used at 20 µg mL^{-1} did show similar multiple banding by Western blot. In herpesviruses, protease precursors that undergo autoprocessing and cleavage in order to achieve cleavage of subsequent polyproteins during capsid maturation and shell assembly (Yu *et al*., 2005), can also exhibit multiple bands when screened by MAbs, e.g. the scaffold protein and protease noted for Marek's disease virus (MDV) (Laurent *et al*., 2007) and Lung-eye-trachea disease virus (LETV) (Coberley *et al*., 2002). Alternatively, MAbs detecting glycoproteins may also recognise the antigen at multiple molecular weights due to a larger molecule being synthesised following glycosylation and other post-translational modifications resulting in the addition of oligosaccharides, and/or glycosylation intermediates, which were shown previously for Feline herpesvirus (Mijnes *et al*., 1996) and Bovine herpesvirus-4 (Machiels *et al*., 2011), respectively. Protein processing and modifications during KHV infection could perhaps explain the bands observed for at least some of the MAbs that are known to detect KHV capsid antigens and glycoproteins. Following intensive blocking many of the multiple bands were eliminated, except for intense bands at 250 kDa, which may be associated with large glycosylated proteins with carbohydrate moieties that did not migrate quickly through the gel and may not have separated sufficiently. Part of the reason for reduced mobility can be the lack of binding of SDS to large carbohydrates reducing the charge to mass ratio of protein-SDS complexes resulting in aberrant migration and increased apparent molecular weights (Hames and Rickwood, 1990). However, an intensively stained band was still

evident at approximately 100 kDa, recognised only by MAb 20F10. MAb 20F10 was also the only MAb known to recognise a capsid-associated antigen, thus it could be hypothesised that this ~100 kDa protein is a non-glycosylated capsid antigen for which electrophoretic migration through the gel is not inhibited by large carbohydrate moieties. However, further analysis on detergent extracts of purified KHV virions, i.e. with Triton-X, similar to the methods used for envelope protein characterisation of other alloherpesviruses, e.g. CCV or AngHV-1 (Liu *et al*., 2011; Van Beurden *et al*., 2011b), using immunogold TEM or immunoprecipitation, should be undertaken to confirm this.

Due to the large panel of MAbs tested, high throughput analysis of their expression characteristics was deemed desirable to elucidate which of the recognised antigens play vital roles in KHV replication and virion formation, *i.e*. is abundantly expressed and/or associated with a specific stage during virion assembly.

5.4.2 Variation of KHV antigen expression detected by MAbs using microtitre plate immunofluorescence (IF)

The 96-well microtitre plate, semi-quantitative, IF approach developed for analysing KHV antigens in the present study provided a number of benefits over conventional methods that apply anti-viral MAbs for determining levels of infection in cells. These methods rely on manual counting of infected (fluorescently stained) and non-infected cells without any consideration to the amount of intracellular antigen (detected by the MAb) being produced within the cells during infection (Kao *et al*., 2001; Espinoza and Kuznar, 2002; Abaitua *et al*., 2012). Furthermore, as the approach designed in the current study employed a 96-well microtitre plate, high throughput analyses of various MAbs to the antigens of interest could also be undertaken on the same infection of the same batch population of sub-cultured cells.

The approach in this study followed a similar methodology to the microtitre plate assay developed for measuring the inhibitory effects of various reagents against Rotavirus (Xijier *et al*., 2011), except for the fact that the emphasis here was to determine KHV antigen expression characteristics during the course of infection, thus taking into consideration different stages of the virus replication and maturation. However, problems were encountered following attempts to screen the panel of anti-KHV MAbs against virus antigens expressed at different stages post infection due to background fluorescence, particularly in KF-1 cells, which was evident by the high level of non-specific binding of the MAb cocktail against mock infected cells. Despite this affecting the interpretation of relative antigen expression, there were still clear differences observed between the MAbs. Less background was observed with CCB cells, and the expression trends of antigens mirrored those seen with KF-1 cells at the later stages of the infection. Interestingly, elevated protein abundance was not detected by MAbs 10A9, 17A9 or 21D11, until the most advanced stages of virus infection, despite these producing the greatest recognition of whole purified KHV virions by ELISA (not shown). In contrast, progressively greater levels of protein were recognised by MAb 20F10 throughout the trial, despite this MAb producing much lower absorbance signals by whole virus ELISA. These results may support the previous hypothesis in Section 5.4.1 that the ELISA absorbance values were influenced by the virus structural protein detected by the MAb. Previous developments of quantitative assays for aquatic viral pathogens, i.e. IPNV, VHSV and IHNV, using microtitre plate immunofluorescence have also provided variable results (Falk *et al*., 1998), which may be due to low viral abundance of the target antigen in the infected cells. Thus, quantification of the protein abundance of different MAbs detecting various structural or non-structural proteins of the virus should perhaps be performed to reveal the most sensitive MAb for that application, especially for an assay designed for live

virus titration. This proposal is supported further, not only by the results of current investigation, but from studies relating to mammalian virus using immunofluorescence, *e.g.* the variation in sensitivity of the test developed for detecting the mammalian virus DV, where the MAb detecting the nucleoprotein, NS1, was, as expected, the most sensitive at an early stage of infection (Kao *et al*., 2001). Recently, a clinical shell vial immunofluorescence assay was used to determine the sensitivity of an improved ISAV culture technique, which was able to detect viral antigens after only 2 dpi in the absence of CPE, although quantitation relied on manual infected cell counts (Molloy *et al*., 2013). Better validation could perhaps be achieved by spectrophotometric quantitation of fluorescence.

Antigen expression of both the envelope glycoprotein and capsid protein was detected after only 24 hpi, but only capsid expression continued to increase considerably and at significantly greater levels than those of the envelope protein between 2-7 dpi. These differences could affect the sensitivity of MAb-based diagnostic assays such as those mentioned above. A recent study on IFN I responses to KHV *in vitro* using flow cytometry did not successfully detect KHV infected CCB cells until 4 dpi with a MAb detecting a KHV glycoprotein despite high viral virulence with 90% of cells positive by 6 dpi (Adamek *et al*., 2012). Thus spectrophotometric quantification of KHV infected cell immunofluorescence could be more, or at least as, sensitive as that determined by flow cytometry, especially if an internal virus capsid antigen is utilised. There was also only minimal detection of the envelope glycoprotein antigen investigated in the current study until later stages of infection.

There were a number of limitations to the microtitre method including the inability to (1) determine the percentage of cells infected, (2) assess virus induced cell abnormalities and (3) localise recognised antigen in infected cells. Non-specific binding of certain MAbs within

the control MAb cocktail, e.g. MAb 16A9 also made interpretation of the data difficult. An alternative approach was therefore sought to enable antigen quantitation by immunofluorescence while providing an opportunity for visual and qualitative analysis of virus induced cell deformation during the course of infection.

5.4.3 Kinetics of KHV morphogenesis and expression of capsid and envelope antigens

The morphogenesis of KHV has been described in cultured cyprinid cells, CCB, NGF-2 and NGF-3 (Miwa *et al*., 2007), however, analysis was only undertaken after 7 dpi. In the current study attempts were made to (1) determine the sequence of morphological changes of the KHV virion following initial infection within two of the most commonly used cell lines for KHV propagation, CCB and KF-1, and (2) relate this to the expression of antigens of different structural proteins recognised by MAbs.

One of the most notable findings of the current investigation was the presence of capsids within the cell nucleus after only 1 hpi. This finding was unexpected as DNA replication of other herpesviruses is not initiated until 3 hpi (Ben-Porat and Veach, 1980) and recently Ilouze *et al*. (2012b) demonstrated that KHV DNA synthesis occurs between 4-8 hpi in infected CCB cells. Capsid assembly would therefore not be expected until late mRNAs have been translated and the structural proteins incorporated into the nucleus, and furthermore, the finding is in contrast to previous reports of cultured cells infected with other alloherpesviruses and mammalian herpesviruses. Pseudorabies virus and channel catfish virus capsids for example were not detected in the nucleus of infected cells until 4 hpi (Wolf and Darlington, 1971; Granzow *et al*., 1997). This may therefore constitute an artefact caused by contaminating infected cells from the inoculum as a result of insufficient monolayer washing

following virus absorption. However, no evidence of later stages of virion maturation were observed at this stage of infection and, as expected, no discernible antigen (late structural proteins) expression was detected by either MAb 10A9 or 20F10 at this stage. Although high viral DNA loads were also detected within cell pellets and tissue culture supernatant at very early stages, viral DNA concentrations of KHV infected cells has previously been reported to be indistinguishable from the original inoculum after just 1 hpi (Dishon *et al*., 2007), which is not dissimilar to other herpesviruses (Ahlqvist *et al*., 2005). Thus there appears to be a rapid absorption of infectious virions to the cells following inoculation. Shifting of PrV infected cells from non-permissive to permissive temperatures results in virion attachment to the cell membrane within 1 minute and intracellular importation of virions after only 5 minutes (Granzow *et al*., 1997). Imported PrV nucleocapsids are found in close proximity to microtubules, sometimes already docked at the nuclear pore within 30 minutes (Granzow *et al*., 1997; Kaelin *et al*., 2000). In the current study, one particular cell was observed with a nucleocapsid-like particle in close proximity to a coated pit, e.g. possibly clathrin coated for receptor mediated endocytosis (Cross and Mercer, 1993), which extended intracellularly from the plasma membrane. Similar findings have been associated with viropexis (entry of virus to the cell via coated pits) in early infection stages of PrV (Granzow *et al*., 1997) and possibly HSV-1 (Nicola *et al*., 2003). Although no microtubules could be easily distinguished, this may have been a de-enveloped nucleocapsid migrating to the nucleus. In contrast, other cells contained electron dense and electron lucent capsid-like structures of approximately 100 nm that resembled early stage empty capsids of HSV-1 degraded particles and revertant mutants (Campadelli-Fiume *et al*., 1988; Abaitua *et al*., 2012). In the study by Abaitua *et al*., (2012) these capsids were situated around the nuclear envelope, unlike the cytoplasmic distribution in the current study. Nonetheless, one empty capsid was found less than 100 nm from a

potential nuclear pore potentiating its possible role in viral DNA release into the nucleus of that cell. Granzow *et al*. (1997) reported 'oddities' in the early infection stages of PrV where virions were found within coated pits, contained in coated vesicles and inside structures resembling secondary lysosomes within 20 minutes post infection, which did not affect virion structure, however, the large putative secondary lysosomes (distinguished by the lucent appearance and presence of degraded material in contrast to the homogenous electron density of primary lysosomes (Cross and Mercer, 1993)) that were also in close association with the early cytoplasmic capsids in the current study, may have released these unenveloped, empty capsids as disrupted virions. If these unusual virion formations are the result of receptor-mediated endocytosis, which is thought to result in a dead-end pathway of penetration in other herpesviruses (Campadelli-Fiume *et al*., 1988), they would not be expected to result in productive infection.

The capsids that were observed in the nucleus at this very early stage were always immature and devoid of DNA, and no later stages of capsid maturation were observed, thus they may be pre-formed capsids (procapsids) associated with 'early' late (γ-1) major structural proteins, which may be synthesised prior to DNA replication, e.g. VP5, the major capsid protein of HSV-1 (Ginsberg, 1988; Spencer *et al*., 1998). Structural protein transcripts of KHV have been detected in infected CCB cells as early as 2 hpi, prior to DNA replication (Ilouze *et al*., 2012b). Other structural proteins have also been found incorporated into herpesvirus infected nuclei during the early stages. 'Early' late tegument proteins derived from inoculum have previously been reported in the nucleus after 2 hpi, e.g. VP8 of BoHV-1 and pp65 of human cytomegalovirus (Grefte *et al*., 1992; Van Drunen Little-van den Hurk *et al*., 1995). However, further studies would have to be undertaken to determine the underlying factors associated with the empty cytoplasmic capsids and 'immediate' nuclear capsid

formations within KHV infected KF-1 and CCB cells observed after 1 hpi in the current study. Analysis of the processes involved in cell entry of herpesviruses, which are rapid, have been achieved previously by cold temperature manipulation after initial inoculation (Granzow *et al*., 1997; Klupp *et al*., 2000; Nicola *et al*., 2003; Abaitua *et al*., 2012).

Within the first day of infection cytopathic changes were observed including nuclear hypertrophy and margination of chromatin. This is similar, although not as rapid as the chromatin margination and initiation of syncytia after only 2 hpi reported in CCV infected cells (Wolf and Darlington, 1971). This is not surprising as infectious progeny virus can be isolated from CCV infected catfish after only 1 dpi (Kancharla and Hanson, 1996) compared to the lag time of KHV infected carp (Dishon *et al*., 2005; Matras *et al*., 2012; Dong *et al*., 2013), which may correspond to differences in viral replication kinetics. By 4 hpi capsids were found within the nucleus at all stages of maturation, which is similar to findings for other herpesviruses (Nii *et al*., 1968; Wolf and Darlington, 1971; Nii, 1991; Granzow *et al*., 1997). These exhibited 3 forms; the most abundant of which contained a circular sphere within the capsid composed of concentric circles with the inner containing heterogenous material, thought to be capsomers and scaffolding protein in PrV (Granzow *et al*., 1997). The other two types were either electron dense with varied morphology or empty with low electron density similar to those described previously for KHV and other herpesviruses (Granzow *et al*., 1997; Fuchs *et al*., 2007; Miwa *et al*., 2007; Miyazaki *et al*., 2008). Primary envelopment of nucleocapsids was also observed at this stage, but not in the cytoplasm, unlike the nucleocapsids reported for CCV (Wolf and Darlington, 1971), which indicates an eclipse stage of the infection was still ensuing (i.e. no production of infectious particles (Flint *et al*., 2009)) as mature infectious enveloped virions were absent until analysis after 1 dpi.

The envelope glycoprotein antigen was detected by IF after just 4 and 8 hpi in KF-1 and CCB cells, respectively, using the more sensitive confocal laser microscope image analysis technique, compared to the spectrophotometric microtitre plate technique where the antigen was not detected until 24 hpi. The KHV antigen recognised by the envelope glycoprotein MAb 10A9 was recently identified as the product of ORF56 (Gotesman *et al*., 2013) and transcripts of this ORF have previously been detected as early as 2 hpi in KHV infected CCB cells (Ilouze *et al*., 2012b). Herpesvirus envelope glycoproteins, such gB of HSV-1, are synthesised prior to DNA replication, albeit in small amounts (Ramachandran *et al*., 2010), thus the early detection of the KHV envelope glycoprotein antigen may be prior to DNA synthesis, glycosylation, and other post translational modifications. Furthermore, as this early expression is also associated with the nucleus, the protein may be present in the nuclear envelope as well as being abundant in extracellular virions, similar to the major glycoprotein, gB as well as gD, which are found in both primary and secondary enveloped virions (Gilbert and Ghosh, 1993; Gilbert *et al*., 1994; Campadelli-Fiume and Roizman, 2006). This would correspond to the nucleocapsids first found fused with the inner nuclear lamella and within the peri-nuclear cisterna after 4 hpi by TEM. The capsid antigen was also first detected 4-8 hpi by confocal microscopy and image analysis, the cytoplasmic and nuclear associated protein abundance of which was similar, but slightly more to that of the envelope antigen at this stage. Not until after 24 hpi was there a pronounced difference in the expression and abundance of capsid antigen to envelope glycoprotein, which may be associated with the subsequent infection of neighbouring cells and the high proportion of non-infectious immature virions (i.e. with capsid protein) compared to mature infectious enveloped virions containing envelope glycoproteins.

Some of the intranuclear capsids had formed paracrystalline-like arrays after just 4 hpi, which have previously been reported within infected carp gill epithelial cell nuclei (Hedrick *et al*., 2000), and in the cytoplasm of a newly developed koi caudal fin cell line (KCF-1) (Dong *et al*., 2011), however this formation, which is considered a rare sighting in KHV infected cell cultures (Dong *et al*., 2011), is typical of other herpesviruses (Nii *et al*., 1968; Granzow *et al*., 1997). The inclusions were clearly seen by light microscopy, and may be associated with the intra-nuclear inclusion bodies (IIB) presented as one of the more characteristic histopathological signs of fish tissues with KHVD (Hedrick *et al*., 2005; Miyazaki *et al*., 2008; El-Din, 2011). In the current study many cells exhibited these capsid formations, which may be associated with the virulence of the isolate. These have been described as pseudocrystals in PrV infected cells, which are hypothesised to dissolve during replication and release individual capsids as they are not found in necrotic cells following replication (Granzow *et al*., 1997). The current study supports this as these capsid formations were no longer observed after 3 dpi, despite being found in numerous cells prior to this.

Although fluorescence signals of MAb 10A9 detecting the envelope glycoprotein was localised to the nucleus following densitometric measurements by image analysis, this was never seen by confocal microscopy and synthesis of this protein *de novo* would not be expected in the nucleus. Further examination of the envelope glycoprotein localisation by stereo imaging at later stages of infection revealed compartmentalised signals at the periphery of the nucleus and around the plasma membrane, but not within the nucleus. This may be explained by protein synthesis of the antigen being recognised before post-translational modification, i.e. synthesis at the endoplasmic reticulum (peripheral nuclear staining) prior to its translocation to the golgi apparatus for glycosylation. The concurrent plasma membrane signals could represent the integration of viral glycoprotein into the viral envelope for later

budding events of the infectious virions at the cell membrane. Similar distributions of the highly conserved and immuno-dominant gB in HSV-1 (Pereira, 1994; Mocarski *et al*., 2007) have been demonstrated (Gilbert and Ghosh, 1993; Gilbert *et al*., 1994). This supports the earlier hypotheses for the multiple protein bands observed by Western blot (Section 5.4.1) as the MAb may recognise the same protein epitope prior to glycosylation, which is similar to the radio-immunoprecipitation and immunofluorescence studies undertaken on gE and gI of Feline herpesvirus (Mijnes *et al*., 1996). The antigen recognised by MAb 10A9 has been detected in peripheral blood leukocytes of potential reservoir fish for KHV such as sturgeon and goldfish (Kempter *et al*., 2009; Bergmann *et al*., 2010c), thus the antigen is not easily degraded *in vivo*. Furthermore, MAb 10A9 was recently utilised for affinity purification of KHV viral proteins in infected carp tissues, which successfully yielded 5 KHV proteins and a large number of host proteins (Gotesman *et al*., 2013). The antigen was subsequently identified as a glycoprotein encoded by ORF56 by electron ionisation coupled to mass spectrometry (ESI-MS) (Gotesman *et al*., 2013), although the expected molecular mass differed from that observed by Western blot analysis in the current study, this could be a result of modifications *in vitro* compared to *in vivo*.

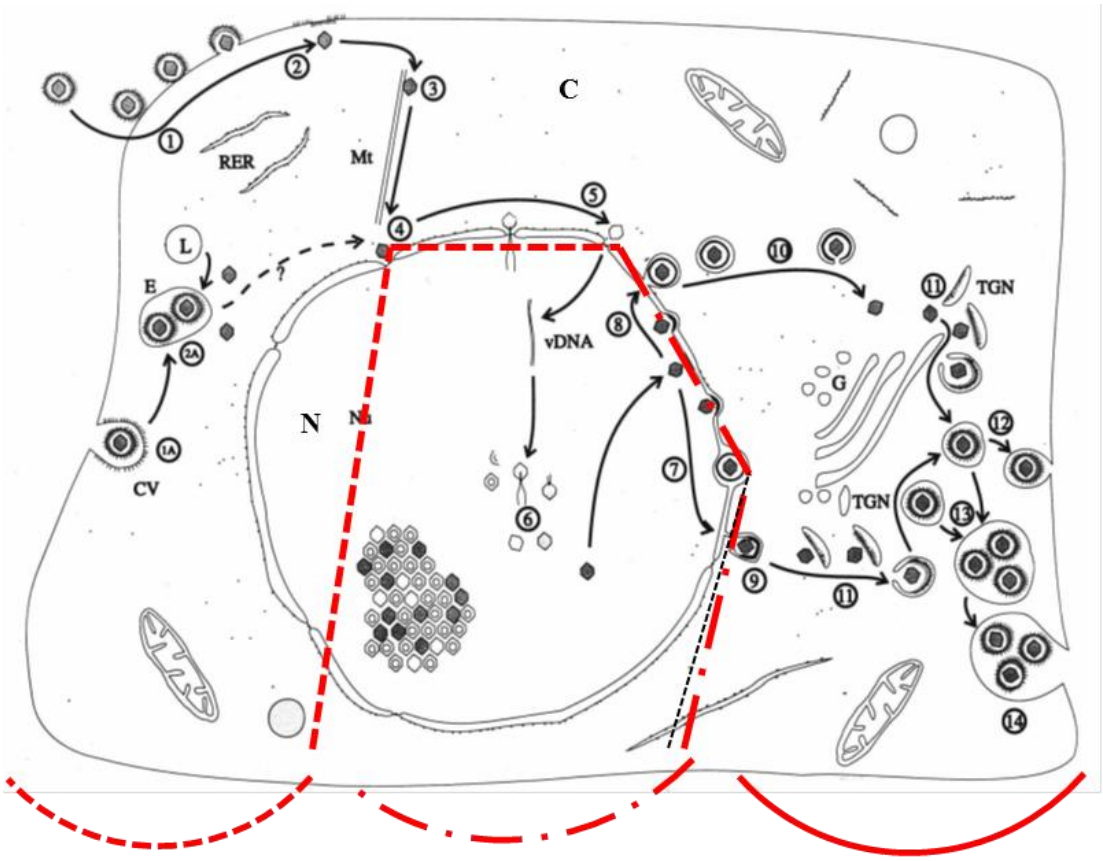
In contrast to the rapid production of progeny virus within 10-12 hours of the alloherpesvirus, CCV (Wolf and Darlington, 1971), extracellular infectious virion release appears much slower for KHV and other herpesviruses (i.e. 3-5 dpi) identified by growth curves (Ahlqvist *et al*., 2005; Dishon *et al*., 2007; Costes *et al*., 2008; 2009; Dong *et al*., 2011). This was similar in the current study and corresponding real-time qPCR indicated elevated viral DNA concentrations from 3 dpi in cell pellets and 4 dpi in the media. Previous studies have indicated an increase in KHV viral DNA from 2 hpi – 4 dpi (Dishon *et al*., 2007; Ilouze *et al*., 2012b), which was not observed in the current study, but an increased

production of infectious secondary enveloped virions and glycoprotein antigen expression was seen after 3 dpi, in accordance with increased intracellular viral DNA. At later stages of infection all stages of virus morphogenesis could be observed and the sizes of capsids, nucleocapsids, primary enveloped and secondary enveloped virions were in agreement with other studies on KHV and other allopherpesviruses (Wolf and Darlington, 1971; Hedrick *et al*., 2000; 2005; Miwa *et al*., 2007; Miyazaki *et al*., 2008). There was a notable increase in expression and abundance of capsid protein after 1 dpi compared to envelope glycoprotein, which corresponds with the much fewer enveloped mature virions, i.e. containing envelope glycoproteins, until the later stages of infection, compared to the masses of non-enveloped capsids and nucleocapsids of various stages of maturation observed throughout the infection. Even these aberrant particles are able to leave the cell through exocytosis in other herpesviruses (Granzow *et al*., 1997), thus they may increase the production of non-infectious particles. Some non-infectious herpesvirus particles may be formed by the absence of a 'primary envelopment – secondary envelopment' step pathway, e.g. in HSV-1 mutants where gD is retained to the ER, the production of extracellular virions with rare gD positive particles is still evident, which are derived directly from the cytoplasmic compartments during viral egress, either by possible cell lysis or a secretory pathway that bypasses the golgi apparatus (Skepper *et al*., 2001). Production of infectious KHV particles in cell cultures appears to be similar to other herpesviruses for which only ~100 virus particles may be infectious from ~10^4-10^5 total particles (Ginsberg, 1988).

A high competition between nucleocapsids for budding, via the perinuclear envelope and intracytoplasmic vesicles of the TGN, may have contributed not only to the irregular formations found within the nuclear envelope, but also to the formation of syncytia. Re-duplication of the nuclear envelope, intranuclear folds and incorporated vesicles may occur in

herpesvirus infected cells through the accumulation of virus-derived antigens within the cisternae, which corresponds to the intense perinuclear signals obtained with MAb 10A9 at later stages of KHV infection. Similar formations are found in alphaherpesviruses, where primary enveloped virions accumulate in the perinuclear region in the absence of proteins that are required for successful egress and further maturation (Granzow *et al*., 2004). This can result in thickening (Ghadially, 1997) leading to nuclear envelope proliferations, fusions and subsequent abnormal concentric lamellar structures (Nii, *et al*., 1968). These are characteristic cytopathologies observed in KHV and other herpesviruses (Nii *et al*., 1968; Wolf and Darlington, 1971; Nii, 1991; Ghadially, 1997; Miwa *et al*., 2007). Formation of syncytia on the other hand, is thought to result from mutations in glycoprotein genes (Pereira, 1994), with an extensive production of intracellular mature and immature virus particles, which occurred more often in CCB cells than KF-1 cells, the latter of which were more prone to lysis. Syncytial formation has previously been described in KHV infected CCB cells (Adamek *et al*., 2012), which also occurred in the current study. Where giant cells had formed, concave formations around the nucleus were apparent with abundant glycoprotein staining (MAb 10A9) as well as number of multinucleated cells. This may explain not only the reduction in antigen abundance towards the end of the trial in KF-1 cells, but also the progressive increase in CCB cells, as viral particles may have been released gradually through budding instead of cell lysis. As a result of cell lysis there appeared to be a greater loss of virus from KF-1 cells. This may explain the higher copy numbers of viral DNA in the KF-1 cell supernatant, but greater copy numbers in CCB cell pellets at the latest stages of infection. The overall high viral DNA concentrations and expressed antigen, do not, however, directly correlate with the number of virus particles as, noted from HSV-1, as only about 25% of viral DNA and protein is considered to be assembled into virions (Ginsberg, 1988).

The increased number of cytopathic vacuoles at later stages, often containing infectious virus particles, may also be associated with vacuolation (i.e. CPE) at this time. which have also been associated with competitive budding processes in high infection experiments. This is due to either fusion of a large number of secretory vesicles, or many virions budding through limited golgi-derived vesicles (Granzow *et al*., 1997), and also corresponded with the greater expression of glycoprotein antigen at the later stages of infection. Elevated expression of the capsid antigen was localised to the nucleus, but was also highly abundant in the cytoplasm, which is comparable to nucleocytoplasmic shuttling proteins such as VP19C of HSV-1, which has been suggested to function for anchoring viral DNA to the capsid and facilitating transport of competent proteins to the site of capsid assembly (Zhao and Zheng, 2012). Thus the capsid antigen in the current study may play a similar role. The associations between stages of KHV morphogenesis and the abundance and expression of capsid and glycoprotein antigens are illustrated schematically in Fig. 5.21.



En (RAE)	0	≤ 0.03	≤ 0.56
Ca (RAE)	0	≤ 0.07	≤ 1.27

After Granzow et al. (1997) for illustration of Pseudorabies virus infection cycle

Figure 5.21 Schematic diagram representing putative relationships between koi herpesvirus morphogenic development and capsid and envelope glycoprotein antigen expression. Different time divisions for the period when the respective virus particle are observed in infected cell cultures (CCB and KF-1) are represented by broken and stable red lines. The antigen expression determined by image analysis of confocal microscope derived *z* stacks are indicated below the illustrated cell. N = nucleus; C = cytoplasm; CV = coated pit; RER = Rough endoplasmic reticulum; E = endosome; Mt = Microtubule; TGN = Trans-golgi network; V = vesicle; Pa = paracrystalline array; vDNA = Viral DNA; En = enveloped glycoprotein; Ca = Capsid; RAE = Relative antigen expression

Although image analysis was only undertaken for MAb 10A9 and 20F10, focal nuclear signals, unlike those observed for the capsid antigen, were seen for the protein encoded by ORF68, which was also previously reported in KHV infected cells (Aoki *et al*., 2011). These signals may be associated with tegumentation of the capsid prior to primary envelopment, similar to early stage intranuclear tegumentation in BoHV-1 reported by Van Drunen Little-van den Hurk *et al*., (1995). However, although the role of the protein encoded by ORF68 is unknown from proteomic analysis (Michel *et al*., 2010b), the recombinant antigen to which this MAb was raised is an N-terminal region of a myosin-like protein with a trans-membrane region (Aoki *et al*., 2009; 2011), thus is exposed through the viral envelope and is more likely to represent an uncharacterised envelope glycoprotein, which may be similar to gB of HSV-1, which is also found at the nuclear membrane and cell plasma membrane (Gilbert *et al*., 1994). Transcripts of KHV ORF68 were, however, not previously detected in infected CCB cells until 8 hpi, categorising the product of ORF68 as a γ (late) protein (Ilouze *et al*., 2012b), whereas gB is synthesised prior to DNA replication in HSV-1 (Ramachandran *et al*., 2010). Further characterisation of the antigen detected by anti-ORF68 MAbs would be necessary to elucidate its role in KHV maturation and pathogenesis.

5.4.4 Sensitivity limits, advantages and disadvantages of immunofluorescence for antigen quantitation

Image analysis appeared marginally more sensitive than the microtitre procedure, as relative antigen expression of the envelope and capsid proteins (compared to mock infected cells) could be determined much earlier and in greater abundance, especially when compared to a corresponding mock-infected control at each time point, to help eliminate non-specific background labelling.

Although cell counts were not undertaken, this is possible using the designed macro, which could then be used to validate the results compared to other highly sensitive and specific methods like flow cytometry. Finally this approach provides an opportunity to analyse cytopathology of infected cells in detail. The expression of viral proteins involved in pathogenesis often revolves around the use of gene expression studies, *i.e*. by measurements of the levels of transcripts over the course of infection (Øster and Höllsberg, 2002; Dishon *et al*., 2007; Ilouze *et al*., 2012a; b). However, such measurements do not take into account post-transcriptional processing such as translation initiation, elongation and termination (Plotkin, 2010) and up to 60% of the variation in protein concentration may be unexplained by measurement of mRNAs alone (Vogel and Marcotte, 2012). By utilising MAbs for antigen quantification in the current study, it was possible to elucidate characteristics of protein abundance associated solely with the final folded protein epitope. The image analysis approach is limited by minimal cell coverage, the time lag of processing of *z* stacks and the requirement of specialist technical input, and as a result it is difficult to analyse a large panel of MAbs through a time course of viral infection. The 96 well plate procedure, adapted from previous studies on ISAV (Falk *et al*., 1998) and RV (Xijier *et al*., 2011), provides a very convenient, simple approach with high throughput, and having an infection procedure that can be accompanied by typical virus titration experiments. The microtitre IF technique could provide an alternative diagnostic virus titration method, not only for KHV, but for other viruses, *e.g*. ISAV, where the development of CPE can be slow, especially in low titre samples, and by utilising an anti-capsid MAb the sensitivity may be improved enabling antigen detection after only 1 dpi. The immunofluorescent tests should be validated with different MOI of KHV to gauge the sensitivity of these assays. A greater number of analysed fields under confocal microscopy would also be required to accurately assess the expression

of antigens over the whole monolayer, which is an advantage of the microtitre plate technique that can screen the whole monolayer in each well.

5.4.5 Concluding remarks

Much greater capsid antigen expression and abundance were observed over envelope glycoprotein production using the novel semi-quantitative 96-well microtitre plate and confocal microscopy-image analysis immunofluorescence approaches: In contrast, greater antibody binding to the envelope glycoprotein by MAb 10A9 in a whole virus KHV ELISA, indicated that a higher abundance of this protein is present on released whole virions, whereas a greater proportion of capsid antigen is produced and retained within the cell. A large number of unenveloped capsids were also found within infected cells at an ultrastructural level compared to secondary enveloped infectious virions. Following cell lysis, the release of these abundant capsid antigens may therefore be exposed to carp B cells and thus the production of specific antibodies against them. Further characterisation of these, and the other MAbs from the panel, should be undertaken. The application of the immunofluorescence approaches developed in the current study may contribute to this, as knowledge of the biologic and antigenic role of KHV proteins is still limited. Determining the antigenicity of the epitopes recognised by the MAbs in carp could provide invaluable information of their potential application in diagnostics and/or vaccination. Such information is vital for development of DIVA strategies.

Chapter 6

Applications of serological diagnostics for KHV using a DIVA strategy

6.1 Introduction

6.1.1 Problems encountered with diagnosis of 'latently' Koi herpesvirus (KHV) infected fish

The rapid spread of KHV worldwide has partly been attributed to the limited sensitivity of molecular diagnostics for detecting KHV DNA in subclinically infected, and infected fish with a possible latent infection (Bergmann *et al*., 2010a; Pokorova *et al*., 2010; Matras *et al*., 2012). This may result in false negative results following screening of carp and subsequent transmission of the virus. Not only have cultured carp, i.e. for both food and the live ornamental carp trade, been affected (Haenen *et al*., 2004; Pokorova *et al*., 2005; Ilouze *et al*., 2006a), but several reports of KHV detection and outbreaks in fisheries and wild stocks (Grimmett *et al*., 2006; Taylor *et al*., 2010, 2011; Uchii *et al*., 2009; 2011; Garver *et al*., 2010) have raised concerns over the spread of KHV beyond the confines of fish farms. The presence of KHV in natural waters (Takashima *et al*., 2005; Shimizu *et al*., 2006; Matsui *et al*., 2008; Murwantoko, 2009; Minamoto *et al*., 2011; 2012) and wild non-KHVD susceptible fish species may also have accelerated the spread of KHV among cultured stocks (Fabian *et al*., 2012).

Following recovery from an outbreak, decreased virus expression occurs while the virus resides within the host. A number of studies have demonstrated latent-like infection characteristics of KHV both *in vitro* (Dishon *et al*., 2007; Ilouze *et al*., 2012a) and *in vivo* (Gilad *et al*., 2003; 2004; St-Hilaire *et al*., 2005; 2009; Eide *et al*., 2011a) that occur especially at non-permissive water temperatures. Other aquatic herpesviruses such as carp pox, Cyprinid herpesvirus-1 (CyHV-1) (Sano *et al*., 1993), channel catfish virus (*Ictalurid herpesvirus-1*) (Gray *et al*., 1999) and eel herpesvirus (*Anguillid herpesvirus*) (Rijsewijk *et al*., 2005) also establish apparent latent infections, which can be reactivated. This has made

detection of apparently latently infected, clinically healthy fish, by molecular diagnostic methods a challenge, as noted for KHV in experimentally challenged carp (Gilad, *et al*., 2003; St-Hilaire *et al.*, 2005). Pooling organs from as little as two fish for real-time PCR has compromised the detection of such carp or koi (Bergmann *et al*. 2010a). Little is known about where the virus resides in the fish when latent, although Yuasa *et al*. (2007) showed that KHV DNA could only be detected in the brain of common carp 3 months to 1 year following an experimental challenge, but not in other organs. More recently, Eide *et al*. (2011a) demonstrated that KHV may become latent in leukocytes as well as other organs. Clinically normal carrier koi were also shown to shed KHV for a longer duration and take longer to express clinical signs of KHV disease (KHVD) at lower water temperatures (Yuasa *et al*., 2008).

Exposure of carrier koi to permissive water temperature or certain stressors causes reactivation of KHV (Gilad *et al*. 2004; St-Hilaire *et al*., 2005; Eide *et al*. 2011a; Bergmann and Kempter, 2011), and koi could be held at these temperatures before testing to increase virus expression and improve detection rates (Gilad *et al*. 2003; Yuasa *et al*. 2008; Eide *et al*., 2011a). In temperate countries, on-farm collection of fish for KHV screening is seasonal and timed to coincide with rising water temperatures in spring (Yuasa *et al*. 2008; Taylor *et al*., 2010), which are optimal for virus expression. In tropical climates where water temperatures remain around 28^{o}C all year round, clinical signs of KHVD usually manifest in carrier fish following transport stress or during periods of heavy rain when water temperatures are lower. Holding koi at lower water temperatures before testing them by real-time PCR would be very costly as it involves chilling the water and the quarantine area (Diana Chee, pers. comm.). A more sensitive and precise method of identifying these carrier fish is required. This can be achieved by indirect detection of serum antibodies with specific affinity for the virus.

6.1.2 Serological approaches for KHV: successes and challenges

The use of serological tests for disease monitoring in aquatic animals is rare compared to terrestrial animals (LaPatra 1996; Denzin and Staak, 2000; Kibenge *et al*., 2002), and requirements for health certification and screening for disease freedom of source farms based on OIE guidelines for KHV (OIE, 2012) currently place more emphasis on molecular tests due to difficulties in validation of serological methods. Serological methods are indirect tests as they detect the host response to the pathogen rather than direct detection of the pathogen. Serology methods (e.g. ELISA) have, however, been developed (Ronen *et al*., 2003; Adkison *et al*., 2005; St-Hilaire *et al.*, 2005; 2009) and some are described in the OIE manual (OIE, 2012). Serology is a useful tool for screening koi as the presence of specific anti-KHV antibodies indicates that koi have been exposed to the virus and could be carriers. Current tests however, are not completely specific for KHV, especially at serum dilutions of 1/400 and below (Adkison *et al*., 2005; St-Hilaire *et al*., 2009). This limits the usefulness of this test in detecting previously infected populations of koi where antibody titres might have dropped to below a 1/400 dilution. The use of real time PCR to detect KHV DNA together with a specific, sensitive serological test for detection of koi antibodies to KHV, could improve identification of persistently or latently infected batches of koi. The common bottlenecks in the development of a serological test is (1) the process of culturing, purifying and quantifying the virus to coat the ELISA plates (Dixon *et al*., 1994) and, in the case of pathogens like KHV that harbour cross-reactive epitopes (Adkison *et al*., 2005), is (2) the specificity of the assay. However, another problem encountered with serological diagnostics for this virus, due to its notifiable status, is that fish antibodies induced to infection cannot be distinguished from those induced by vaccination. This complicates the potential of vaccination programmes as it cannot be determined whether fish have been vaccinated or infected, and whether those vaccinated fish are subsequent carriers of infectious virus. Consequently, vaccinated carp

may transmit fatal KHVD to naïve unvaccinated carp. This may have contributed to the spread of wild type virus in vaccinated carp where apparently 'protected' carp have been imported (Peeler *et al*., 2009). An ELISA test that can detect antibodies to the virus specifically and enable differentiation between antibodies induced to the infectious virus and to a protective vaccine strain would be highly desirable for KHV.

6.1.3 DIVA approaches based on various antigens of the pathogenic agent

Although ELISAs have been developed for KHV based on whole virus antigen (Ronen *et al*., 2003; Adkison *et al*., 2005; St-Hilaire *et al*., 2009), the application of recombinant proteins in assays can provide many advantages with respect to their safety, stability and cost-effectiveness as highlighted in previous developments for mammalian and avian viruses (Mohan *et al*., 2006; Pérez-Filgueira *et al*., 2006; Muller *et al*., 2010; van der Wal *et al*., 2012). Recombinant ELISAs utilising various structural (Makkay *et al*., 1999; Suarez, 2005; Mohan *et al*., 2006; van der Wal *et al*., 2012) and non-structural (Birch-Machin *et al*., 1997; Tumpey *et al*., 2005; Barros *et al*., 2009) proteins of viruses have been used in the development of serological diagnostic tests for mammals and avians, especially to enable the differentiation of antibodies induced to the vaccine and to infection (Suarez, 2005; Barros *et al*., 2009). Serological tests, and especially DIVA diagnostic assays for herpesviruses, are usually targeted against envelope glycoproteins of the virus (van Zijl *et al*., 1991; Van Oirschot *et al*., 1996; Gómez-Sebastián *et al*., 2008), although the internal capsid antigens have been found to provide a useful target for detection of viral induced antibodies (Coberley *et al*., 2002). Recombinant protein ELISAs have also been applied, with varying success, for detecting anti-virus antibodies in fish, e.g. against nodavirus (Huang *et al*., 2001) and rhabdoviruses, e.g. VHS (Encinas *et al*., 2011a; b).

The antigenicity of KHV proteins is largely unknown apart from a characterised glycoprotein, considered to be the major envelope protein encoded by ORF81 (Rosenkranz *et al*., 2008). A membrane protein encoded by ORF2 was also reported to possess putative B and T cell epitopes using bioinformatics (Murwantoko, 2009). Two antigenic peptides of KHV encoded by ORF62 and ORF68 were recently identified using a phage display library and expressed as recombinant proteins to generate anti-sera, and ultimately monoclonal antibodies (MAbs), specific for KHV (CyHV-3) preventing cross-reactions with the closely related CyHV-1 (Aoki *et al*., 2011). These MAbs subsequently provided a foundation for developing a diagnostic test to detect KHV antigen specifically, i.e. through lateral flow technology, as suggested by the authors (Aoki *et al*., 2011), although such a test has yet to be developed for KHV using these MAbs. However, the specificity of these antigens for KHV also makes them ideal candidates for developing recombinant protein ELISAs to detect anti-KHV antibodies in the serum of carp and koi. The proteins were previously characterised (Table 6.1) as a cysteine protease (recombinant protein of ORF62; rORF62) and myosin like protein or lipoprotein (recombinant protein of ORF68; rORF68) and are expressed as γ (late) proteins (Aoki *et al*., 2009; 2011; Ilouze *et al*., 2012a; b). Preliminary data has previously been reported indicating that rORF68 was recognised by infected carp IgM (Aoki *et al*., 2009), which supports its application in a serological test. Since ORF62 and ORF68 express different structural proteins of KHV, ORF62 expresses an internal tegument protein (Michel *et al*., 2010b), while ORF68 expresses an unknown protein with a transmembrane domain (Aoki *et al*., 2009; 2011), the recombinant antigens derived from these ORFs may also constitute properties for developing a DIVA strategy for a KHV vaccine. The expression kinetics of these antigens, among others, was previously investigated *in vitro* in Chapter 5, and determining their antigenicity with infected carp sera could provide further information on their role in host-pathogen interactions.

6.1.4 Aims

The aim of this chapter was to improve the sensitivity and specificity of KHV serological diagnostics and enable the implementation of DIVA strategies using recombinant proteins of KHV.

The antibody response of carp and koi to KHV proteins was compared using ELISAs in which the plates had been coated with either whole virus or recombinant proteins of ORF62 and ORF68, with the aim of improving the specificity and sensitivity of KHV serological diagnostics. Western blot analysis was subsequently carried out on select sera samples in order to determine the most immunogenic antigens of KHV for application in diagnostics and vaccine development. Assessing the recognition of antibodies against rORF62 and rORF68 was also intended to highlight the feasibility of exploiting carp and koi antibody responses for implementing a DIVA strategy. Since rORF62 constitutes an internal structural protein of KHV and rORF68 an N terminal extracellular region of a protein with a transmembrane domain, they may induce differential antibody responses in infected fish. If immunogenic in infected fish, but absent or not presented to the host immune system to the same extent in vaccinated fish, such recombinant proteins could potentially permit discriminatory diagnosis between infected and vaccinated carp.

6.2. Materials and Methods

6.2.1 Control sera

High titre (1/1600) anti-KHV anti-sera pooled from experimentally infected Koi, kindly provided by Dr. Keith Way (Centre for Environment, Fisheries and Aquaculture Science (CEFAS), UK), was used as a positive control for ELISA screening. Mirror carp, originally obtained from a farm with no previous history of KHVD (Hampshire Carp Hatcheries, UK),

were maintained in a recirculation system at the ARF, Institute of Aquaculture, Stirling, and sera from these fish used as negative control sera.

Table 6.1 Koi herpesvirus (KHV) antigenic recombinant protein characteristics

KHV ORF*	Protein family*	Primers used to amplify antigen gene nucleic acid sequences *	Amino acid (aa) length*	Trans-membrane domain*	Localisation of protein in KHV virion**
62	OUT-like cysteine protease domain	ORF62-F: AAGGATCCCATATGGATCA GATTCCCCCCGTCCCAT, ORF62-R: TTGAATTCTCACATCGCGG TGGCGTCAAACTT	570 aa	No	Tegument
68	Similar to myosin	ORF68-F: AAGGATCCCATATGGATCA GTTCAAGCAGACCACGG, ORF68-R: TTGAATTCTCACTGCGACT CGAGCCTGGAGTT	501 aa	Yes	Unknown

***Characterisation and information of KHV recombinant antigens according to Aoki *et al.* (2009; 2011)**

**** Putative localisation of proteins following mass spectrometric analysis according to Michel *et al*. (2010b)**

6.2.2 Virus and cell propagation

CCB cells were cultured at 20ºC for propagation and purification of KHV, as described in Sections 2.2.2 and 2.3.2, respectively.

6.2.3 Carp serum samples

A total of 224 serum samples were analysed during the study. This included 162 serum samples collected from individual koi submitted as part of the national KHV surveillance programme in Singapore, 11 serum samples from a KHV infection trial conducted in Singapore, 25 serum samples from a KHV vaccination and challenge trial using a live attenuated vaccine conducted in Israel, 25 serum samples from a KHV vaccination trial using

an inactivated vaccine conducted in Scotland (Section 3.2.5) and the 1 positive control sample pooled from fish with high antibody titres to KHV (Section 6.2.1).

Blood was sampled from the caudal vein of fish and allowed to clot overnight at 4ºC before collecting serum as described in Section 3.2.4.2(a).

6.2.3.1 Field case serum samples from Asia

Serum was taken from live Koi that had been submitted to the Animal and Plant Health Laboratories, Agri-Food and Veterinary Authority of Singapore, as part of a KHV surveillance programme on imported Koi between December 2008 and December 2010, kindly provided by Ms. Yahui Wang (Agri-Food and Veterinary Authority of Singapore (AVFA), Lorong Chencharu, Singapore). A minimum of 30 fish were randomly collected from 1 import consignment or 1 premise in China, Japan, Malaysia and Singapore from each of the cases reported (Table A1, Appendix 1). Six organ pools from each submitted case, consisting of brain, gill, kidney, intestine, liver and spleen from 5 fish, were tested for KHV using the PCR described by Yuasa *et al*., (2005) and Bercovier *et al*, (2005). The cases were divided into 2 groups depending on their KHV status. Group 1 consisted of samples taken from fish where KHV DNA had been detected in tissues by either conventional PCR (Yuasa *et al*., 2005; Bercovier *et al*., 2005) or real-time PCR (Gilad *et al*., 2004) or where the sampled fish had been associated with KHV infected fish. Group 2 consisted of fish sampled from regions with no association with KHV and where no KHV DNA had been detected in tissues by PCR. The source of country, clinical signs of koi and PCR results from all the cases were recorded (Table A1, Appendix 1).

6.2.3.2 Singapore KHV experimental challenge

An experimental KHV challenge was carried out in Singapore by the Animal and Plant Health Laboratories, Agri-Food and Veterinary Authority of Singapore as part of a

collaboration with Mrs. Diana Chee (AVFA). Koi sourced from a farm where KHV had never been detected, were prophylactically treated to remove external parasites, moved to a containment facility for aquatic animals and acclimatised in 25ºC ozone-treated water in 2 x 1 tonne circular tanks on a recirculation system with daily 50% water changes. All the discarded water was ozone-treated and all biological waste was autoclaved prior to disposal. Post mortem, parasitological and bacteriological examination was carried out on 5 fish, while gill biopsies were taken from 130 anaesthetised fish from the population to pre-screen for KHV DNA by PCR (Yuasa *et al*., 2005; Bercovier *et al*., 2005). After acclimatising fish for 1 week, 3 groups of 12 carp were exposed to KHV that had been propagated in KF-1 cells with minimum essential medium (MEM), similar to that described in Section 2.3.2., with 2% foetal calf serum at different infectious doses by bath immersion for 1 h at 20ºC. The 3 positive groups received doses of 1.2 (high dose), 0.12 (moderate dose) and 0.012 $TCID_{50}$ mL^{-1} (low dose) by the titration method according to Reed and Muench (1938) with a fourth group exposed to MEM media alone, which were used as a control group. The differential virus doses were used in order to enable comparisons to be made on antibody responses of carp to KHV following different onsets of clinical and subclinical disease. The fish were held in 4 separate 120 L glass tanks for 4 weeks and monitored daily for any clinical signs associated with KHVD. Fish expressing signs of distress or dyspnoea, lethargy or excessive skin ulceration were euthanized and individually screened for KHV by real-time TaqMan PCR (Gilad *et al*., 2004). Serum samples were collected from the first 2 groups 16 and 18 days post infection (dpi), and from the third group and control group 29 dpi (Table 6.2). The serum samples from this trial were sent to IoA, University of Stirling for analysis.

6.2.3.3 Israeli KHV vaccination (live attenuated) and experimental challenge

A vaccination/challenge trial was conducted in Israel by Dr. Ofer Ashoulin at the commercial Koi farm Magnoy, Maadan, Ma'agan Michael, Israel using 14 month old koi with an average

bodyweight of 135 g. The fish were fed a diet of commercial koi pellets at 1% body weight per day and were held in brackish well water with an average temperature of 23 to 24ºC. Fourteen Koi were vaccinated by immersion with KV3 vaccine (KoVax Ltd, Israel), which is a live attenuated KHV vaccine (Ronen *et al*., 2003; Perelberg *at al*., 2005), by immersion for 45 min at 22ºC with 10 plaque forming units (PFU) per mL^{-1} water. Fourteen other koi were vaccinated with 0.1 mL ip of the same vaccine following anaesthesia in phenoxyethanol. Control groups were transferred to similar 20 L tanks as the treated groups and held at 22ºC water for 45 min without vaccination. The serum samples from this trial were sent to IoA, University of Stirling for analysis.

Table 6.2 Challenge dose, clinical signs, DNA and serum sampling of koi experimentally infected with Koi herpesvirus (KHV) in Singapore

Experimental group	KHV inoculation dose by immersion ($TCID_{50}\ mL^{-1}$)	Clinical signs	PCR results and primers used	Time of serum collection
High dose	1.2	Diseased, moribund with acute mortality seen.	+ve real-time TaqMan PCR.	16 dpi
Moderate dose	0.12	Diseased	+ve real-time TaqMan PCR.	18 dpi
Low dose	0.012	Healthy	-ve real-time TaqMan PCR.	29 dpi
Control	0	Healthy	-ve real-time TaqMan PCR.	29 dpi

Fish were subsequently held in separate 100 L flow-through tanks each for the immersion vaccination, ip vaccination or control groups. The 2 vaccinated groups were challenged by cohabitation with 8 infected koi per tank 26 days following immunisation. Sera from 5 fish

were sampled 25 days post vaccination (dpv) and from the challenged groups at 14 days post challenge (dpc) (Table 6.3).

6.2.3.4 United Kingdom KHV vaccination (inactivated)

Mirror carp were vaccinated with formalin inactivated KHV vaccine containing an aluminium hydroxide adjuvant (Henderson Morley Ltd., 2009) as described in Section 3.2.5.1(b).

Table 6.3 Collection points of koi serum from KV3 vaccinated and experimentally challenged koi with Koi herpesvirus in Israel

Experimental Group ID	Description	Sampling for serum
IP	Intraperitoneal vaccinated fish	Day 25
IPC	Intraperitoneal Vaccinated and Challenged fish	Day 40
IM	Immersion Vaccinated fish	Day 25
IMC	Immersion Vaccinated and Challenged fish	Day 40
C	Control fish	Day 25

IP = Intraperitoneal vaccinated; IPC = Intraperitoneal vaccinated then challenged; IM = Immersion vaccinated; IMC = Immersion vaccinated then challenged; C = Control

6.2.4 Whole KHV ELISA

A sucrose gradient purified KHV ELISA, developed as described in Section 3.2.5.3(c) was used for detection of serum antibodies to whole virus antigens.

6.2.5 KHV MAbs and ascites fluid

Hybridoma supernatant, from MAb 10D10 and MAb 7C6 recognising recombinant protein antigens of KHV ORF62 and ORF68, respectively, ascitic fluid from mice immunised with

either rORF62 or rORF68 (Asc10D10 and Asc7C6) were obtained, cultured and maintained as described in Sections 2.1.1 and 5.2.1.1.

6.2.6 KHV recombinant protein antigens

Transformed *Escherichia coli* BL21 cells containing plasmids of rORF62 and rORF8 (Aoki *et al*., 2011) were a kind gift from Dr. Tae Sung Jung, Aquatic Biotechnology Center, College of Veterinary Medicine, Gyeongsang National University, Korea and Professor Takashi Aoki, Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Tokyo, Japan.

6.2.6.1 Recombinant protein expression

(a) Pre-trial expression and characterisation

Antigenic recombinant proteins of KHV ORF62 (rORF62) and ORF68 (rORF68) were produced and characterised as described by Aoki *et al*. (2009; 2011) information for which is shown in Table 6.1. Initially a preliminary culture was grown and products were characterised to ensure that the proteins could be successfully expressed from the plasmids. The transformed BL21 *E.coli* cells (DE3) from the pET-28a system (pET-28a KHV-ORF62/68) were cultured on LB agar (1.5% w/v agar, 1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7) containing 50µg mL^{-1} kanamycin at 37°C for 18 h. Ten colonies were picked from 2 strains of bacteria, each one containing a plasmid expressing either rORF62 or rORF68, and inoculated into 20 mL LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH, 7) broth containing 50µg mL^{-1} kanamycin in a 50 mL centrifuge tube and incubated at 37°C on a shaking platform. The cultures were incubated for 14-18 h before adding 50 µL of culture to 5 mL of fresh LB medium and shaken for 3 h, after which the absorbance was measured at OD_{450nm} on a portable spectrophotometer, using LB medium as a zero reference. The bacterial culture was adjusted to an absorbance of 0.4-0.6 and 3 mL of culture was split

between two 15 mL polypropylene tubes (1.5 mL each). Six microliters of 0.1M isopropyl-D-thiogalactopyranoside (IPTG) (Quigen) was added to induce one of the tubes (at a final concentration of 0.4 – 0.8mM), before culturing both tubes for 3-6 h at 37°C with shaking. A 300-500 µL aliquot of each bacterial culture (in suspension) was harvested after 1-3 h and mixed 1:1 v/v with 2 x SDS sample buffer for soluble protein analysis. After 6 h the cultures were harvested by centrifuging at 3000 x *g*. The pellets were re-suspended in PBS and mixed with 2 x SDS sample buffer, as described above, as was the supernatant, for insoluble and soluble protein analysis, respectively.

The samples were subsequently tested by SDS-PAGE to determine protein expression and Western Blot to determine epitope conservation using MAbs 10D10 and 7C6 to recognise their respective recombinant proteins.

(b) Transformed E.coli glycerol stocks

Once recombinant proteins had been successfully produced, glycerol stocks of transformed bacteria were made by mixing broth cultures of transformed *E.coli* bacterial suspensions in 15% v/v glycerol to provide bacterial stocks (8.5 mL bacterial suspension, 1.5 mL glycerol), which were stored at -70°C until use. The stocks were always thawed quickly on dry ice with ethanol prior to growing new cultures.

(c) Large scale recombinant protein production

The protocol for large scale production of KHV recombinant proteins, were similar to that described above for the pre-trial expression and characterisation (Section 6.2.6.1(a)). An aliquot of transformed BL21 *E.coli* was thawed as described in section 6.2.6.1(b) cultured at 37°C in LB broth for 18 h and 5 mL was inoculated into 2 x 500 mL of 2 x YT broth (1.6% bacto tryptone, 1% yeast extract, 0.5% NaCl) containing 50 µg mL^{-1} kanamycin. The bacteria broths were then induced with 1M IPTG 3 h after inoculation when an OD_{600nm} 0.8 was

obtained. The induced bacteria were cultured for a further 4 h at 37°C with shaking after which the broths were pooled and centrifuged at 3000 x *g* for 15 min at RT. The supernatant was discarded and the pellets (containing the insoluble recombinant proteins) were stored at -20°C until used for protein extraction.

6.2.6.2 Bacterial lysis and protein extraction

Recombinant proteins of KHV were extracted from inclusion bodies of lysed, transformed *E.coli* BL21 (pET 28a) cells using the CelLytic B II bacterial cell lysis extraction reagent kit (Sigma-Aldrich) according to the manufacturer's instructions with modifications. Briefly, bacterial pellets were freeze thawed 3 times prior to initiating the extraction procedure in a warm water bath. The bacterial pellet was then weighed by comparing to a zeroed centrifuge tube on a balance. The wet cell paste was reconstituted by adding 5 mL working lysis solution (CelLytic B II) to 1 g of wet cell paste. Benzonase (Sigma-Aldrich) was added to a final concentration of 5µg mL^{-1} in order to reduce the viscosity of the solution and yield a solid pellet. Lysozyme (Sigma-Aldrich), required to break down the bacterial cell wall, was then added at a final concentration of 0.4 mg mL^{-1}. The extraction suspension was incubated for 10 - 15 min at RT to fully extract soluble proteins from cells and then centrifuged at 25,000 x *g* for 15 min to pellet the insoluble material. The majority of the soluble protein (approximately 90-95%) was now contained within the suspension fraction. The cell pellet was then resuspended in the required volume of CelLytic B II, vortexed for 1 – 2 min to completely resuspend the cell pellet and was incubated with 0.4 mg mL^{-1} lysozyme at RT for 5 – 10 min with shaking to allow the lysozyme to fragment the cell bacterial wall. A 1:20 solution of CelLytic B 2 was prepared in dH_2O (Lysis wash buffer) and 30 mL was added to the sample mixture and mixed well before centrifuging the sample again at 25,000 x *g* for 15 min to pellet the cell debris. The pellet was again reconstituted in 40 mL Lysis wash buffer and vortexed for 1 – 2 min to completely remove the remaining cell debris after

centrifugation again for 15 min at 25,000 x *g*. These steps were repeated 2 more times to completely remove any soluble proteins and cell wall from the inclusion bodies. The pellets were finally resuspended in 40 mL phosphate buffer (0.5M NaCl, 20mM NaPO4, pH 7.4) by vortexing again for 1-2 min and centrifuged at 25,000 x *g* for 10 min. The washed inclusion bodies (pellets) were then solubilised in urea buffer (8M urea, 20mM $NaPO_4$, 0.5M NaCl, 10mM imidazole, pH 8), vortexed and shook at 37°C for 1 h in order to maximise solubilisation of proteins. The samples were left to incubate at 4°C overnight prior to purification. Samples from fractions were taken at each stage of the procedure and placed 1:1 v/v in 2 x SDS sample buffer, which were subsequently tested by SDS-PAGE following protein purification described in Section 6.2.6.4.

6.2.6.3 Recombinant protein purification and quantitation

The ionised metal affinity chromatography (IMAC) system was used for purification of solubilised recombinant proteins using the same ÄKTA prime liquid affinity chromatography system (Amersham Biosciences) used for purifying MAbs (Section 2.1.1.3).

The solubilised inclusion bodies were brought to RT then centrifuged at 10,000 x *g* for 10 min and the pellet retained. The samples were prepared through filtration via 0.45 µm then 0.2 µm filters (GE Healthcare, UK) using a vacuum. A Hi-Trap chelating HP 1 mL column, previously charged with Nickel (Ni^{2+}) by loading 0.5 mL of 0.1M $NiSO_4$, was used for binding His-tagged proteins. All buffers contained 8M urea and were filtered through 0.45 µm filters and the column was washed thoroughly with binding buffer (8M urea, 20mM imidazole, 50mM Na_2PO_4, 300mM NaCl, pH 8) to elute non-specifically bound metal ions that could otherwise be eluted during the desorption process.

After washing and equilibration of the ÄKTA prime affinity chromatography system, the sample in binding buffer was applied to the Hi-Trap chelating HP 1 mL column. The

sample was passed through the system at a rate of 1 mL min^{-1} and the bound (HIS)6-tagged proteins of interest were eluted with the addition of elution buffer (8M urea, 250mM imidazole, 50mM Na_2PO4, 300mM NaCl, pH 8.5). The absorbance of collected fractions were analysed spectrophotometrically after zeroing the system with buffer containing 500mM imidazole. The purified proteins were subsequently concentrated through a Millipore 10 kDa centrifuge tube (Millipore, Cork, Ireland) with PBS buffer, and dialysed against PBS through a 12 kDa cut off dialysis membrane similar to that described for MAbs in Section 2.1.1.3.

6.2.6.4 Recombinant protein validation

(a) SDS-PAGE

Bacterial lysates of IPTG induced and non-induced *E. coli*, as well as extracted and purified recombinant proteins were subjected to SDS-PAGE (stained with both Coomassie blue or Silver stain) as described Section 5.2.1.3.

(b) Western Blot with anti-rORF62 and anti-rORF68 MAbs

Western Blot screening of recombinant proteins of ORF62 and ORF68 with specific MAbs developed against the proteins (Aoki *et al*., 2011) was undertaken as described in Section 5.2.1.4.

6.2.7 Recombinant protein ELISA development

6.2.7.1 Recombinant KHV ELISA optimisation

The recombinant ELISAs for screening carp serum antibodies were developed through a series of optimisation steps involving the use of various types of 96-well microtitre ELISA plates including Immulon-4 HBX (Dynax Technologies Inc., Fisher Scientific, UK), Polysorb (Nunc) and Multisorb (Nunc). Different reagent blockers were also compared for blocking non-specific binding including 10 % and 5% casein (Marvel, Ireland), 1 % BSA (Fisher

Scientific, UK) and 1x Rotiblock (Roth, Germany). Addition of blockers to the diluent was also attempted, including *E. coli* lysate and casein, and different concentrations of mouse anti-carp IgM MAbs (Aquatic Diagnostics Ltd, Stirling, Scotland), goat anti-mouse HRP-conjugated IgG MAbs (Sigma-Aldrich) and different concentrations of positive and negative control carp sera. The final assay was determined by end-point checkerboard assays.

6.2.7.2 Final established recombinant KHV ELISA protocol

Following numerous optimisation assays, a final protocol was established that resulted in the greatest positive to negative ratio from control test sera. The developed assay was similar to that used for screening carp anti-sera to KHV whole virus antigens described in Section 3.2.5.3(c), but with modifications. The differences of these assays compared to whole KHV ELISA were as follows: 100 µL of antigen, either 20 µg mL^{-1} recombinant protein (rORF62 or rORF68) or BSA diluted in 0.05M carbonate-bicarbonate buffer, were coated to the plate. Non-specific binding sites were blocked with 250 µL of 5 % casein in dH_2O for 5 h at RT. One hundred microliters of Mouse anti-carp IgM (Aquatic Diagnostics Ltd, Stirling, Scotland) diluted 1:55 in 0.1 % BSA in PBS (100 µl $well^{-1}$) was added to each well and incubated for 1 h.

From the surveillance programme and Singapore experimental trial, strong responders were re-tested at doubling dilutions from 1/100 to 1/400, moderate responders at doubling dilutions from 1/50 to 1/200, and weak responders at doubling dilutions from 1/20 to 1/80. Strong responders from the Israel experimental trial were re-tested at doubling dilutions from 1/100 and 1/200, moderate responders at 1/50 to 1/100 and weak responders at 1/20 to 1/40. All serum samples from carp immunised with the inactivated KHV vaccine (K and GK) and the control group from the GFP marker vaccine trial (Section 3.2.5) were re-tested at doubling dilutions from 1/200 – 1/3200. Serum screening was also repeated with 5 % casein in PBS as a serum diluent instead of PBS only.

A total of 71 serum samples were re-screened using the recombinant ELISAs and 32 of these samples were selected for screening by Western Blot, performed as described below in Section 6.2.8. Screening of some samples was repeated on different blots.

6.2.8 Western Blot screening of serum

SDS-PAGE was undertaken as described in Section 5.2.1.3, with rORF62, rORF68, bovine serum albumin (BSA) and whole KHV polypeptides electrophoresed in a polyacrylamide gel. The gels were then transferred to nitrocellulose membranes as described in Section 5.2.1.4. For screening sera against the various proteins, the membranes were divided into strips. A strip from each membrane was stained with 0.1% amido black solution (0.1% amido black v/v in 40% methanol, 10% acetic acid) and incubated for 1 h RT in order to visualise separated protein bands. Unstained membrane strips were blocked overnight with 5% casein in Tris buffered saline (TBS: 0.02M Trisma base, 0.5 M NaCl, pH 7.5). The membranes were then washed 3 x TBS-Tween (TBST: TBS with 0.1% v/v Tween-20) with 5 min incubation per wash, and then cut into strips for which each strip was incubated with test sera diluted 1/50 in 2% casein in TBST for 3 h. Control strips were incubated with TBS only (negative control), KHV negative Mirror carp sera, KHV positive Koi sera (CEFAS) and anti-KHV MAbs 10D10 and 7C6. Anti-carp IgM MAbs (ADL, Stirling, Scotland) were then added to membranes incubated with carp anti-sera diluted 1/50 in TBST for 1 h, followed by 1 h incubation with goat anti-mouse IgG biotin-conjugated MAbs (Sigma-Aldrich, UK) diluted 1/250 in TBST. After a final incubation with streptavidin-conjugated HRP (Vector Laboratories Ltd, UK) diluted 1/250 in TBST, the membranes were washed 3 x 5 min with TBST followed by a 1 min wash with TBS without tween. The reactions were developed with the 4 CN peroxidase substrate system (2-C: KPL, US) according to the manufacturer's instructions. All incubations were carried out at RT, with the TBST wash steps described above.

6.2.9 MALDI-TOF/TOF MS - Mass spectrometry

After Western blotting, selected protein bands that were recognised by infected and vaccinated fish were excised from stained (Bio-Safe Coomassie G-250 stain, Bio-Rad, US) SDS-PAGE gels and in gel digestion was performed with trypsin as previously described for 2-D gel electrophoresis of *Streptococcus iniae* (Shin *et al*., 2006). As part of a collaboration, MALDI TOF/TOF MS analysis was carried out at the Aquatic Biotechnology Center, College of Veterinary Medicine, Gyeongsang National University, Korea, by Dr. Taesung Jung as described previously for identification and characterisation of ranavirus-1 antigens (Kim *et al*., 2011) using a Voyager-DE-STR mass spectrophotometer (Applied Biosystems, Framingham, MA). Mass spectra were subsequently obtained in the reflection/delayed extraction mode. Accumulated data was analysed by using SequazymeTM Peptide Mass Standard Kit (Calibration 1, Applied Biosystems). Screening of Monoisotopic peptide masses was achieved using MoverZ (http://www.proteomics.com) and searching of the NCBInr database was undertaken using MASCOT software (http://www.matrixscience.com). The parameters used in the database searches were as follows: mass tolerance of 50 ppm, one missed cleavage, oxidation of methionine permitted, and cysteine modification by iodoacetamide allowed.

Identification of proteins by MS and MS/MS analyses was undertaken on an ABI 4800 Plus TOF-TOF MS Spectrophotometer (Applied Biosystems) using a 200 Hz ND: YAG laser operating at 355 nm. Subsequent MS/MS analysis in the 1 kV mode using 800-1000 consecutive laser shots was undertaken for the 10 most and 10 least intense ions per MALDI spot, with signal/noise ratios >25. Air served as the collision gas. The output data were then subject to analysis using Mass Standard Kit for the 4700 Proteomics Analyser (Calibration Mixture 1, catalogue number 4333604, Applied Biosystems). Searches were then performed to find matches to the MS/MS spectra on the NCBInr database using ProteinPilot v 3.0, and

MASCOT was applied as the database search engine, with peptide and fragment ion mass tolerances of 50 ppm. During the peptide searches allowances were made for carbamidomethylation of cysteines and oxidation of methionines.

6.2.10 Statistical analysis

Antibody values (absorbance $OD_{450nm)}$ were tested for statistically significant differences in responses to different antigens. Data was tested for normality and homogeneity of variance as described in Section 3.2.6. Non-parametric testing was subsequently carried out using Kruskal-Wallis one-way ANOVA by ranks and median test. Pairwise comparisons of medians were made between carp antibody responses using the Mann Whitney-U test (CI = 95%). Minitab (Minitab 16) was employed for performing all statistical analyses.

6.3 Results

6.3.1 Detection and assessment of anti-KHV antibody responses of carp from case studies in Asia

Of all the serum samples screened from field cases using the whole KHV ELISA, 54.9% (89/162) of the samples were seronegative, while 45.1% (74/162) were seropositive at ≥1/200 dilution. Of the seropositive samples, 6.2% were categorised as strong (≥1/800) responders, 22.2% were moderate (1/400) responders and 16.7% were weak (1/200) responding fish. After dividing responders into groups based on PCR results and associations with KHV-positive sites, 35.8% (58/162) were allocated to Group 1 (KHV +ve by PCR and associated with infected farms/sites), and 64.2% (104/162) were allocated to Group 2 (KHV –ve by PCR and no association with infected farms/sites). Only 31% of carp from Group 1 were seropositive (Fig. 6.1 A). In contrast, as many as 52.9% of Group 2 carp were seropositive for

KHV antigen, although these had previously been reported negative for the presence of KHV DNA by PCR (Fig. 6.1 B).

Of the seropositive fish from Group 1, 12.1% were strong responders, 8.6% were moderate responders and 10.3% were weak responders (Fig. 6.1.A). The majority of seropositive fish from Group 2 were moderate (29.8%) and weak responders (20.1%) (Fig. 6.1 B), although a number moderate responders from this group had much higher absorbance values (OD_{450nm}) than the cut-off when initially tested at a 1/400 dilution, which were found to be strong seropositive responders when rescreened up to a dilution of 1/1600 (fish 47, 48, 51, 61, 63, 66, 68, 72, 73). Furthermore, a number of fish had absorbance values greater than 2x the cut-off at a dilution of 1/400 (fish 78, 106, 140, 143, 163 and 167), that were not titrated further, thus may also have constituted strong responders (results not shown).

The presence of anti-KHV antibodies was found in a large proportion of the cases (69%) by ELISA where PCR to detect KHV DNA was negative (Table 6.4). Very few cases (9%), and even fewer fish within these cases, were positive by both PCR and ELISA (Table 6.4).

6.3.2 Anti-KHV antibody responses in koi vaccinated with a live attenuated KHV vaccine and experimentally challenged with KHV

All four fish from the high dose and moderate dose virus challenges in Singapore, two fish from each group, were strongly seropositive, whereas there were no antibody responses induced in koi challenged with the low dose of virus (Fig. 6.2 A; Table 6.5). All five fish from both the IP and IM vaccinated groups from the Israel experimental trial were seropositive.

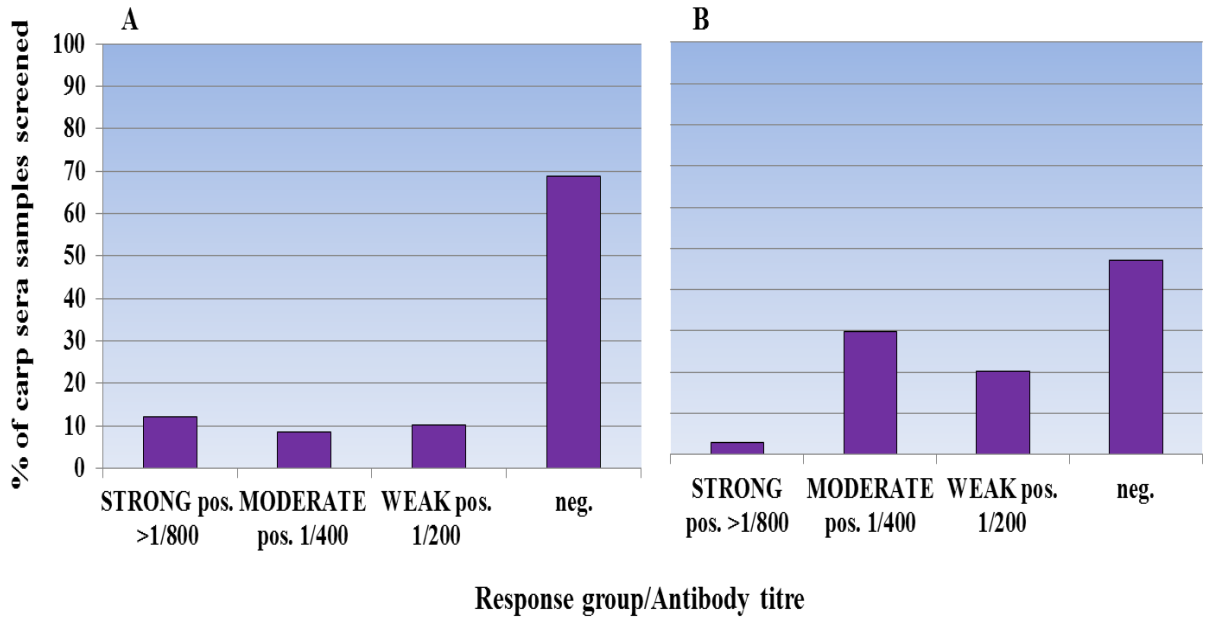


Figure 6.1 Variation of koi antibody responses to koi herpesvirus (KHV) from field cases of a KHV surveillance programme in Asia (2008-2010). (A) Antibody responses of koi in group 1 (KHV +ve by PCR and associated with infected farms/sites) (*n*=58); (B) Antibody responses of koi in group 2 (KHV –ve by PCR and no association with infected farms/sites) (*n*=103). Bars represent different categories depending on antibody titre of responders designated as: strong (≥1/800), moderate (1/400), weak (1/200), or negative at a 1/200 dilution.

Table 6.4 Results of PCR and ELISA screening of carp from a koi herpesvirus surveillance programme in Asia

	PCR+ ELISA +	PCR+ ELISA -	PCR- ELISA+	PCR-ELISA-
Cases (*n*=35)	**3**	**0**	**24**	**8**
Fish (*n*=162)	**6***	**18***	**62**	**76**

***Cases that were positive by both ELISA and PCR, although some individual fish were only positive by PCR and negative by ELISA**

These were all strong responders to the virus, however, only 4/5 ip vaccinated and challenged (IPC) fish and 3/5 immersion vaccinated and challenged (IMC) fish were strongly

seropositive (Fig. 6.2 B; Table 6.5). One fish from the IPC group (IPC1) was seronegative resulting in a lower mean antibody response compared to the IMC group fish (Fig. 6.2 B). There were 2/5 fish from the control group that were seropositive, one weak and 1 moderate responder (Table 6.5).

The majority of experimentally challenged and/or vaccinated fish were strong antibody responders (Fig. 6.3). Of these responders, the antibody responses to the high and moderate doses of virus used for challenging fish with KHV in Singapore (which were sufficient to induce disease in these fish) were greater than the responses seen in fish from Israel, which had been vaccinated with the attenuated vaccine and subsequently challenged with KHV (Fig. 6.2).

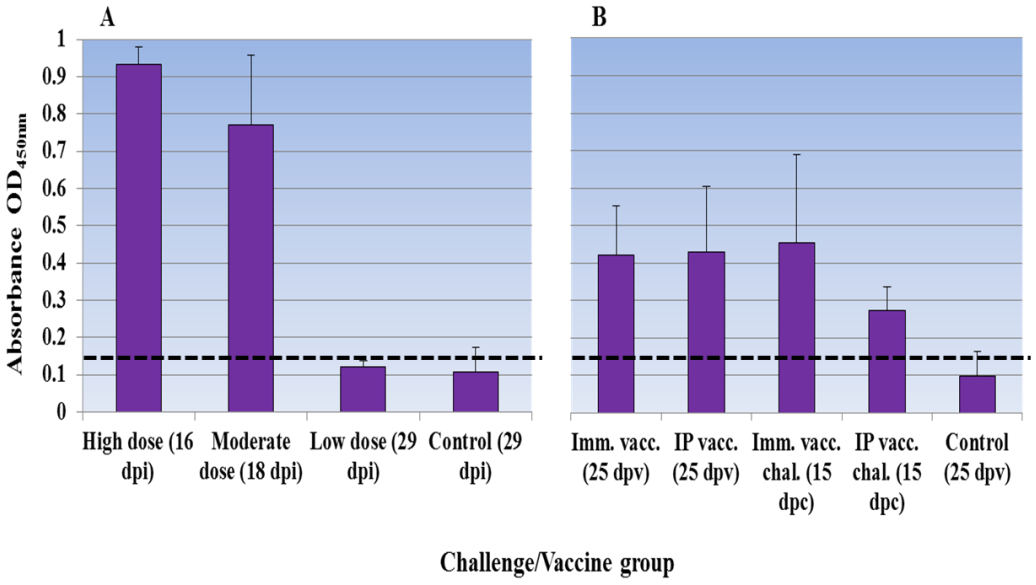


Figure 6.2 Mean antibody responses in serum of koi to koi herpesvirus following an experimental challenge and vaccination challenge trial in Singapore and Israel. (A) Experimentally challenged koi in Singapore with different doses of virus. (B) Koi vaccinated by ip (IP vacc.) and immersion (Imm. vac.) with an attenuated live vaccine (KV3) then subsequently challenged by cohabitation (IP vac. chal; Imm. vac. chal., respectively). Sera screened at 1/200 from individual fish. Data are mean ± SD (for A: *n*=2: high dose 16 dpi and moderate dose 18 dpi; *n*=4: low dose 29 dpi; *n* = 3: control 29 dpi; for B *n*=5 for all groups). Dashed black line = positive threshold for ELISA

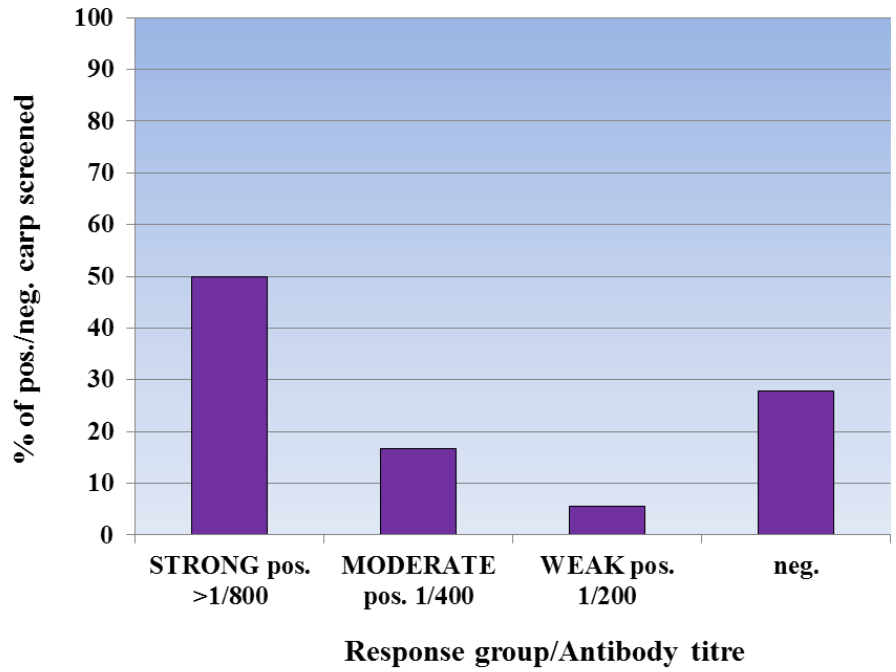


Figure 6.3 Variation of antibody responses to koi herpesvirus in serum from experimentally challenged and vaccinated koi. Categorised antibody responders of koi immunised by attenuated vaccine and experimental infection are shown from the Singapore and Israeli trials (*n*=36). Bars represent different categories depending on antibody titre of responders designated as: strong (≥1/800), moderate (1/400), weak (1/200), or negative at a 1/200 dilution.

The results obtained in the current study were validated at the 1/1600 cutoff serum dilution employed by CEFAS Weymouth laboratory (St-Hilaire *et al*., 2009) according to their criteria. Thus, for this validation a fish was considered positive when the OD at 1/1600 was greater than the average for the negative control, at all dilutions tested from 1/400-1/3200, plus 3x the standard deviation with all other lower dilutions exhibiting higher OD readings than the previous one (St-Hilaire *et al*., 2009). This enabled verification that the ELISA developed in the current study provided results that would be comparable if utilising previously published protocols. The results obtained were not dissimilar when the experimental fish sera from the Singapore and Israeli trials were assessed using the St-Hilaire *et al*. (2009) method in parallel to the current protocol (Table 6.5). Therefore the results in the current study were considered to be reliable and in accordance with published methodology.

However, the 1/200 serum dilution was applied here, rather than the 1/1600 dilution of CEFAS, in order to improve assay sensitivity and avoid false negative screening of fish with low antibody titres. Furthermore, the specificity at this dilution could be compared with that of a KHV-specific recombinant protein ELISA in Section 6.3.3.

Table 6.5 Comparison of positive threshold cutoff applied in the current study to that employed by the CEFAS Weymouth Laboratory for developing a specific KHV ELISA (St-Hilaire *et al*., 2009)

KHV Trial	No. pos. >1/200 by current ELISA	No. pos. at 1/1600 by current ELISA	No. pos. by St-Hilaire *et al*. (2009) ELISA
Sing. High dose chal.	2/2	2/2	2/2
Sing. Mod dose chal.	2/2	2/2	2/2
Sing. Low dose chal.	0/4	0/4	0/4
Sing. Control	1/3	0/3	0/3
Israel control	2/5	0/5	0/5
Israel IP vacc.	5/5	3/5	3/5
Israel IM vacc.	5/5	4/5	4/5
Israel IP vacc/chal.	5/5	3/5	3/5
Israel IM vacc/chal.	5/5	3/5	4/5

Sing. = Singapore; chal. = challenge; IP = intraperitoneal; IM = Immersion; vac. = vaccination
CEFAS ELISA cutoff: A fish is considered positive if OD reading at 1/1600 dilution was greater than the average for the negative control (at all dilutions tested from 1/400-1/3200 for duplicate negative controls) plus 3x standard deviation, and if all dilution have a higher OD than the previous dilution. (St-Hilaire *et al*., 2009)

6.3.3 Recombinant ELISA development for detection of specific anti-KHV antibodies

A number of selected fish serum samples (*n*=45) from those designated as strong, moderate, weak and negative responders to KHV were screened further by the recombinant protein ELISAs to assess if improved serological assay sensitivity (i.e. <1/1600) could be achieved with KHV (CyHV-3) specific antigens, rather than using whole virus to coat the ELISA plates.

6.3.3.1 Expression, extraction and purification of KHV recombinant proteins rORF62 and rORF68 through transformed E.coli BL21 cells (pETLys system)

Both rORF62 and rORF68 were successfully over-expressed in transformed *E. coli* producing high yields of protein, which increased over time post-induction and both proteins were identified at expected molecular weights in Coomassie blue stained SDS-PAGE gels (not shown).

The recombinant proteins were both highly expressed within inclusion bodies of *E. coli* and during protein extraction no discernible recombinant protein was observed in soluble fractions by SDS-PAGE (Fig. 6.4 A-B). The majority of both rORF62 and rORF68 were retained after IMAC purification, but rORF62 had a number of breakdown products, which may have been associated with the protein precipitating after dialysis into PBS (Fig. 6.4 A). Analysis of the final proteins revealed a high degree of purity (Fig. 6.4 C), and these were of the correct approximate molecular weights (MW) of 60 kDa for rORF62 and 56 kDa for rORF68 (Aoki *et al*., 2011).

6.3.3.2 Antigenicity of purified and concentrated rORF62 and rORF68

Both recombinant proteins were recognised by KHV positive anti-sera (fish 12) on Western blot, which also recognised purified KHV polypeptides of 100 kDa and 250 kDa (Fig. 6.5 A-C). Negative sera also produced a faint band to the highest molecular weight (70 kDa) polypeptide from the breakdown product of rORF62. MAb 10D10 produced intense bands against rORF62 and all its breakdown products, but no band was observed against rORF62 by MAb 7C6, while there were some faint background bands observed with PBS (Fig. 6.5 B). Recombinant protein rORF68 was recognised by MAb 7C6 and positive anti-sera, but not negative anti-sera and only a faint band was observed with MAb 10D10 (Fig. 6.5. C). A very

faint non-specific band was observed against BSA when incubated with serum and MAb 10D10, but not 7C6 or PBS (Fig. 6.5 D).

6.3.3.3 Recombinant protein ELISA optimisation

Sera from fish 6 and fish 12 were selected as negative and positive control anti-sera, respectively, for optimising the recombinant ELISA, as well as the pooled positive koi sera supplied by CEFAS and the naïve mirror carp sera from stock fish of the previous marker vaccine trial (Section 3.2.5).

The optimal positive:negative signal ratio for the recombinant KHV protein ELISAs was obtained when no diluent blocking was applied to serum and when the concentration of casein for post-coat blocking was reduced to 5% from the original 10% applied for whole virus ELISA plates (Section 3.2.5.3(c)). It was only possible to screen a selected number (n=71) of carp serum samples with the recombinant proteins. The serum samples screened in the recombinant ELISA included fish of the Asian group 1 (n=8), Asian group 2 (n=10), the Israeli vaccination/challenge (n=22), the Singapore experimental challenge (n=5) and the marker vaccine trial using an inactivated KHV vaccine (Chapter 3, Section 3.2.5 (n=25)) as well as pooled high titre anti-KHV koi serum from CEFAS (n=1).

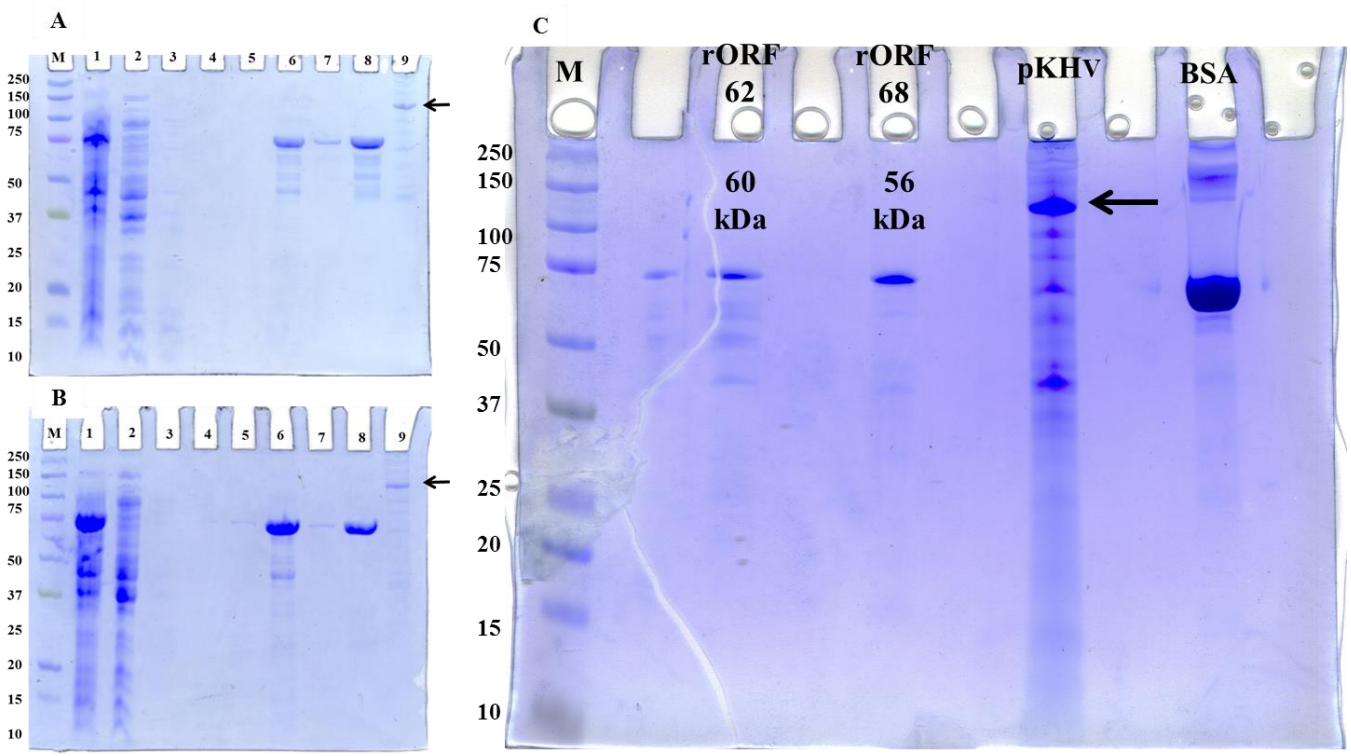


Figure 6.4 Coomassie blue stained polyacrylamide gels showing insoluble and soluble protein products of koi herpesvirus (KHV) recombinant proteins, rORF62 and rORF68 during protein extraction and purification compared to polypeptides of KHV. Extraction process of (A) rORF62 and (B) rORF68 from lysed BL21 bacterial cells; (C) Purified rORF 62 and 68, pKHV and BSA . M = molecular weight markers (kDa) ; Lanes 1-9 in (A) and (B) : (1) Thawed *Escherichia coli* cell in lysis buffer, (2) Soluble fraction of protein, (3) Second centrifugation with lysis buffer, (4) third and fourth centrifugation without lysozyme (1/20 LB), (5) fifth and sixth centrifugation without lysozyme (1/20 LB), (6) Solubilised pellet after incubation in urea binding buffer, (7) Purified protein before concentrating, (8) Purified protein after concentrating, (9) purified KHV polypeptides for comparison. rORF62 = purified rORF62; rORF68 = purified rORF68; pKHV = purified KHV; BSA = bovine serum albumin. Note the more efficient purification of rORF68 compared to rORF62. Arrow indicates putative major capsid protein.

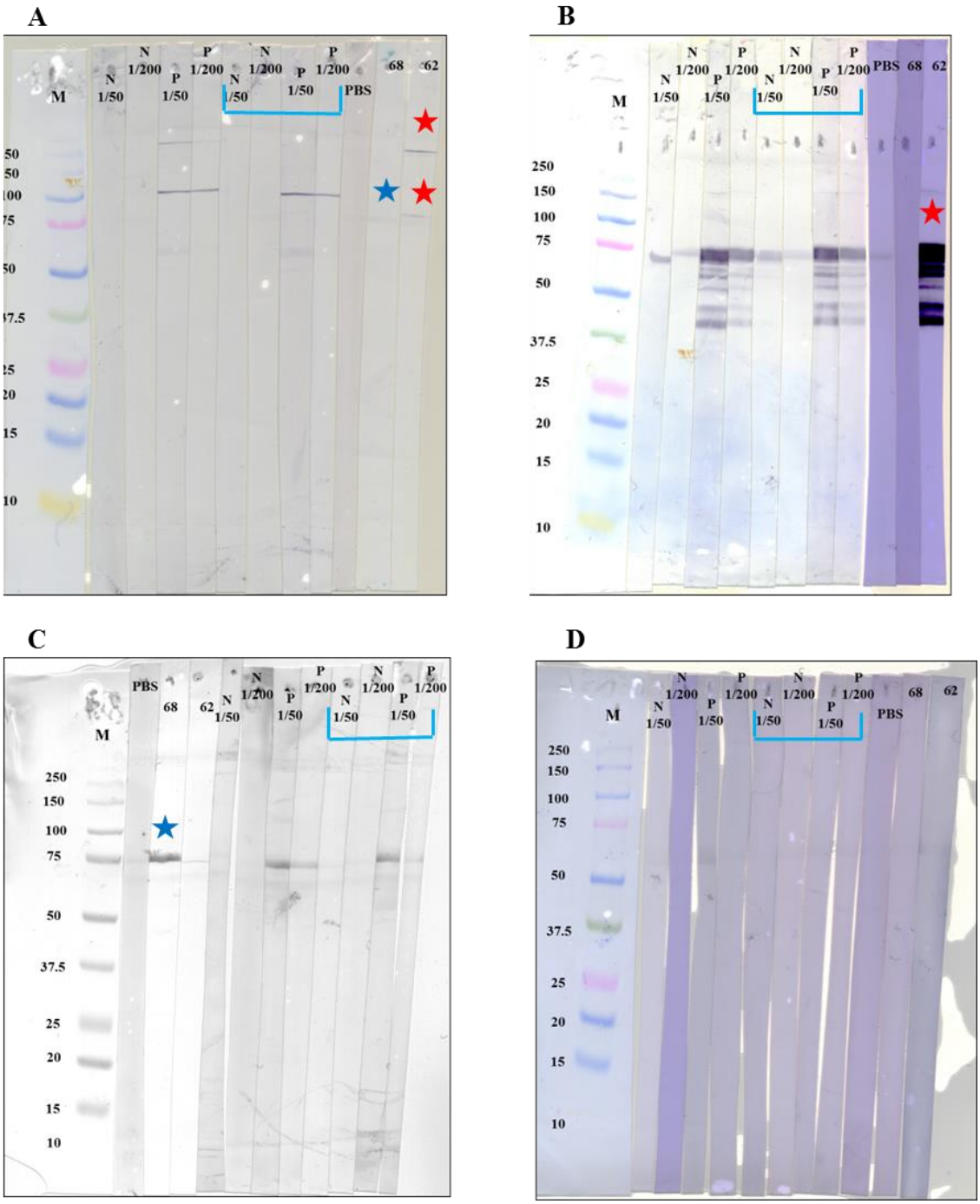


Figure 6.5 Validation of expressed koi herpesvirus (KHV) recombinant proteins by Western blot. (A) Separated polypeptides of purified KHV; (B) rORF62; (C) rORF68; (D) BSA. Strips of membrane were incubated independently with MAbs or sera as follows: N 1/50 = neg. sera (fish 34) 1/50; N 1/200 = neg. sera 1/200; P 1/50 = pos. sera (fish 12) 1/50; P 1/200 = pos sera 1/200; PBS = Phosphate buffered saline control; 68 = ASc. 68; 62 = ASc. 62. Red stars indicate protein bands detected by ASc. 62 (MAb 10D10) and blue stars indicate protein bands detected by ASc. 68 (MAb 7C6). Note faint bands were observed to BSA and rORF68 by MAb 10D10. Light blue underline indicates sera with diluent blocking.

6.3.3.4 Antibody recognition of recombinant KHV antigens rORF62 and rORF68 by infected/exposed fish

Many of the carp that had produced moderate or strong antibody responses to whole KHV antigen also produced high antibody values to both rORF62 and rORF68 when rescreened using a 1/200 serum dilution (Fig. 6.6). However, there were large variations between individual fish to the two recombinant antigens. This was reflected in the number of positive fish to rORF62 and rORF68 when screened at serum dilutions from 1/50–1/200. All moderate responders were positive for rORF62 (11/11), while 73 % (8/11) were positive for rORF68 at dilutions ≤1/200. Of the fish categorised as strong responders, 71 % (5/7) were positive for both recombinant antigens. There were no significant differences between antibody values produced to rORF62 and rORF68 by strong (p=0.16) and moderate (p=0.13) responders of KHV infected and/or exposed carp at a serum dilution of 1/200 (Fig. 6.6).

Two of the five serum samples of fish from field cases that had tested positive by PCR were seropositive on both recombinant ELISAs, while 3/5 were seronegative on both (results not shown). Eight of the 13 serum samples of fish from field cases that had previously tested negative by PCR were seropositive on both recombinant protein ELISAs, four were seropositive on the rORF62 ELISA alone, while one was seronegative on both ELISAs (results not shown).

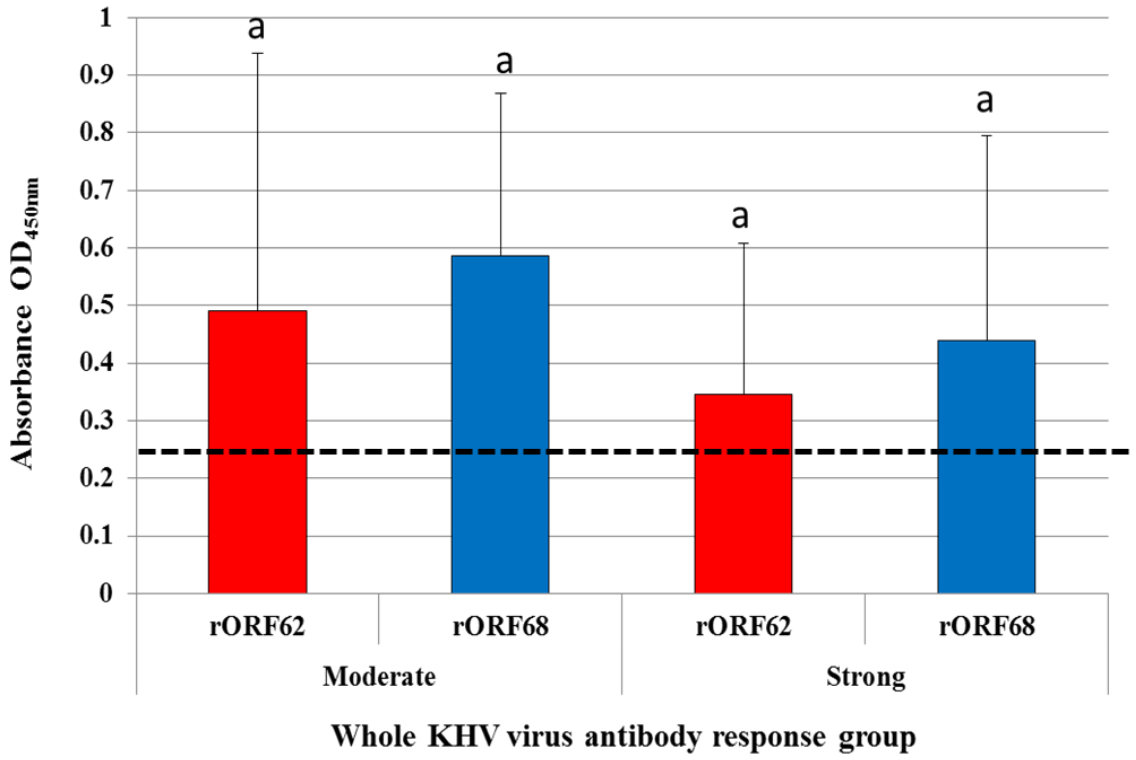


Figure 6.6 Mean antibody response of koi herpesvirus infected/exposed carp. Antibody responses to rORF62 and rORF68 ELISA. Sera screened at 1/200 from individual fish. Data are mean ± SD (*n*=11 moderate responders; *n*=7 strong responders). Different letters above error bars indicate significant differences between medians (Mann Whitney-U pairwise comparison test; $p<0.05$ [95% CI]). Broken black line = cut-off OD

6.3.3.5 Antibody recognition of recombinant antigens rORF62 and rORF68 in vaccinated fish

Mean antibody values to KHV recombinant antigens at a serum dilution 1/200 differed considerably between fish groups vaccinated with an inactivated vaccine and those vaccinated with a live attenuated vaccine (Fig. 6.7). The mean antibody response of fish vaccinated with the attenuated vaccine against rORF62 were comparable to their response with whole KHV antigen, whereas their response to rORF68 was lower (Fig. 6.7), however no significant differences in responses to the two recombinant antigens were found ($p>0.05$). In contrast, fish vaccinated with an inactivated vaccine had weak mean antibody responses to rORF62, but interestingly, had much greater mean antibody values to rORF68. The mean

responses to rORF68 in fish vaccinated with the inactivated vaccine were comparable to responses to whole KHV antigen (Fig. 6.7).

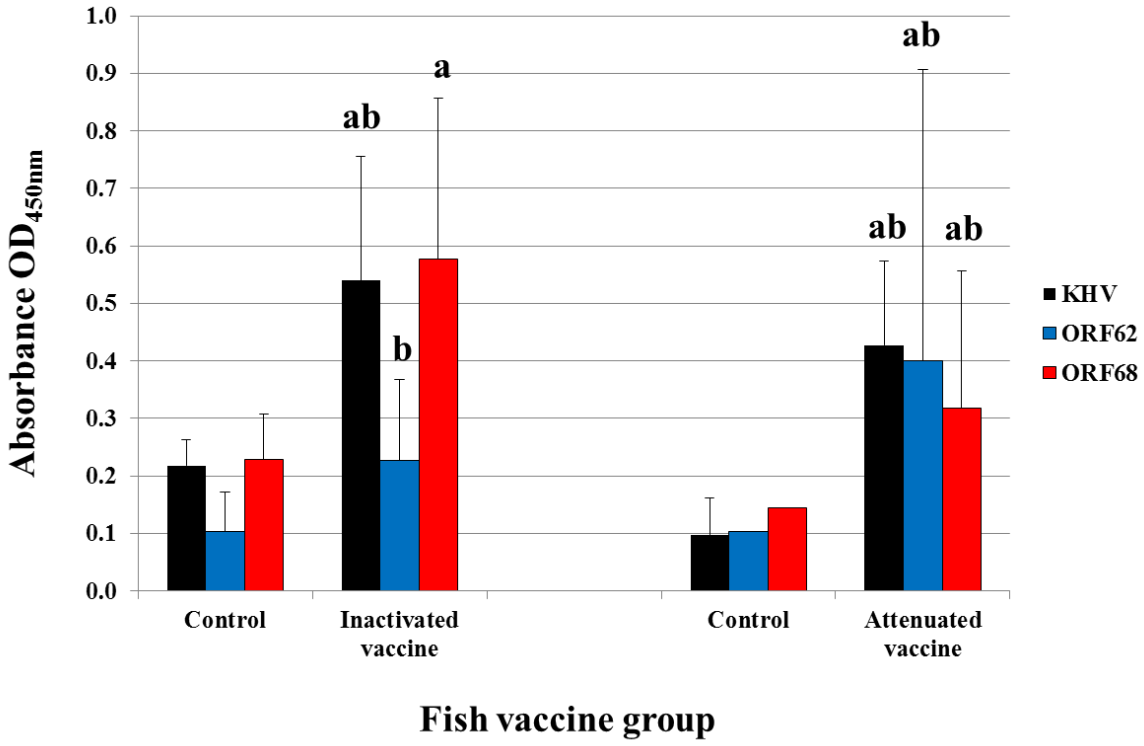


Figure 6.7 Mean antibody responses to recombinant proteins of koi herpesvirus (KHV) in uninfected carp vaccinated with an inactivated and live attenuated KHV vaccine. Antibody responses to rORF62 and rORF68 ELISA from the same serum samples of individual fish. Sera diluted 1/200. Data are mean ± SD: Inactivated vaccinated control fish (PBS injected) n = 8; inactivated vaccine immunised fish n = 15; attenuated vaccine control fish n = 1; attenuated vaccine immunised fish n =7. Different letters above error bars indicate significant differences between medians (Mann Whitney-U pairwise comparison test; p<0.05 [95% CI])

6.3.3.6 Differential antibody recognition of recombinant antigens rORF62 and rORF68 by fish vaccinated with an inactivated vaccine

Mean antibody responses of inactivated vaccinated fish were ~2.5 times greater to rORF68 than to rORF62 at a 1/200 dilution. The greater antibody response to rORF68 than rORF62 was highly significant (p<0.001). Mean antibody values of inactivated vaccinated fish

recognising rORF62 were low (<OD 0.25) at all dilutions, whereas, even at a dilution of 1/800, antibody values to rORF68 were higher than to rORF62 at a 1/200 dilution (Fig. 6.8). However, antibody values to both recombinant antigens at a 1/200 dilution were significantly higher than antibody values measured from control fish (i.e. injected with PBS and montanide adjuvant) screened against rORF68 ($p<0.01$) and rORF62 ($p=0.02$) (Fig. 6.8).

Although antibody values were much lower to rORF62 than rORF68, a number of inactivated vaccinated fish were above the cut-off value for positive antibody detection at low serum dilutions. However, at a serum dilution of 1/800, when background was eliminated and all negative fish were below the cut-off, only 1/15 vaccinated fish were positive for rORF62, which was a particularly strong responder (fish GK5, 1/1600 titre to whole virus, Table 6.6), whereas 7/15 were positive for rORF68 and 12/15 were positive by whole KHV ELISA (Fig. 6.9).

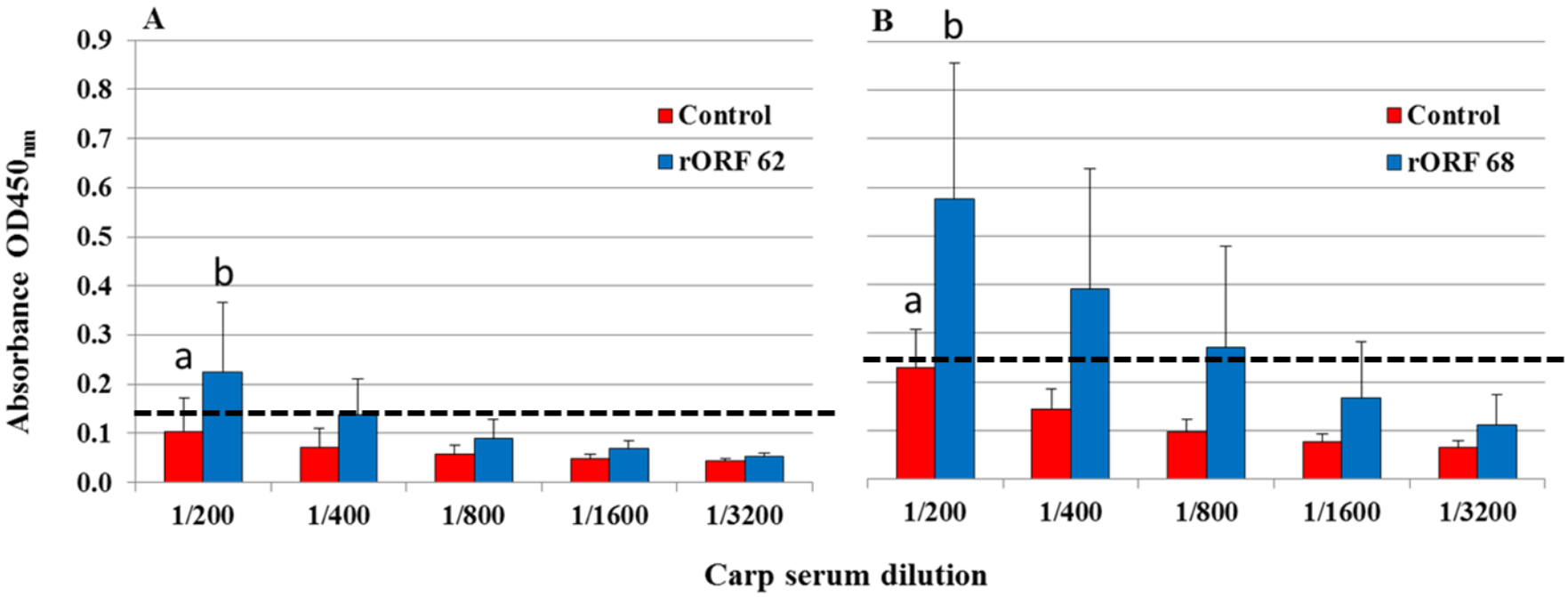


Figure 6.8 Mean antibody responses at 2-fold serum dilutions to different recombinant proteins of koi herpesvirus (KHV) in uninfected carp vaccinated with an inactivated KHV vaccine. Antibody responses to rORF62 and rORF68 ELISA from the same serum samples of individual fish. Data are mean ± SD (*n*=8 Control fish (PBS injected); *n*=15 Vaccinated fish). Different letters above error bars indicate significant differences between medians (Mann Whitney-U pairwise comparison test; $p<0.05$ [95% CI]). Dashed black line = cut-off OD

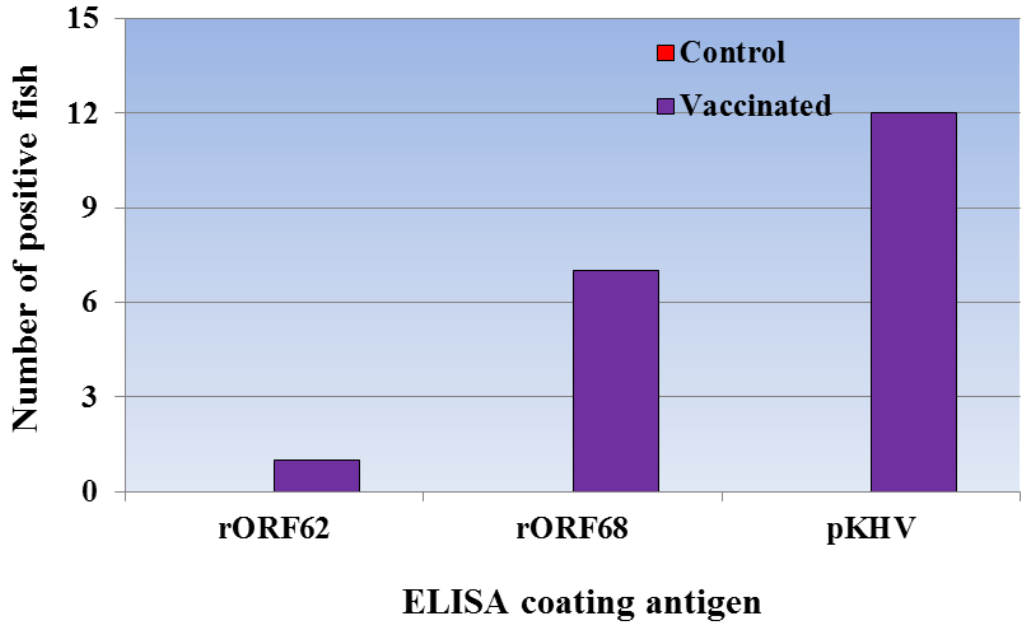


Figure 6.9 Number of fish vaccinated with an inactivated koi herpesvirus (KHV) vaccine positive by whole virus or recombinant KHV antigen ELISA at 1/800 serum dilution. Bars indicate number of fish positive after screening serum from vaccinated (*n*=15) and control (*n*=8) fish.

6.3.3.7 Variable antibody recognition of infected and/or exposed fish to antigens rORF62 and rORF68

Some carp appeared to produce much stronger antibody responses against rORF62 than rORF68, (e.g. fish 32) whereas others had much stronger responses to rORF68 than rORF62 (e.g. fish 12, 68 and 78) (Fig. 6.10 A-B), regardless of whether fish were strong or moderate antibody responders to whole KHV antigen. Non-responders to both antigens were also evident, despite being categorised as strong responders to whole KHV antigen (e.g. fish 21 and 23) (Fig. 6.10 A).

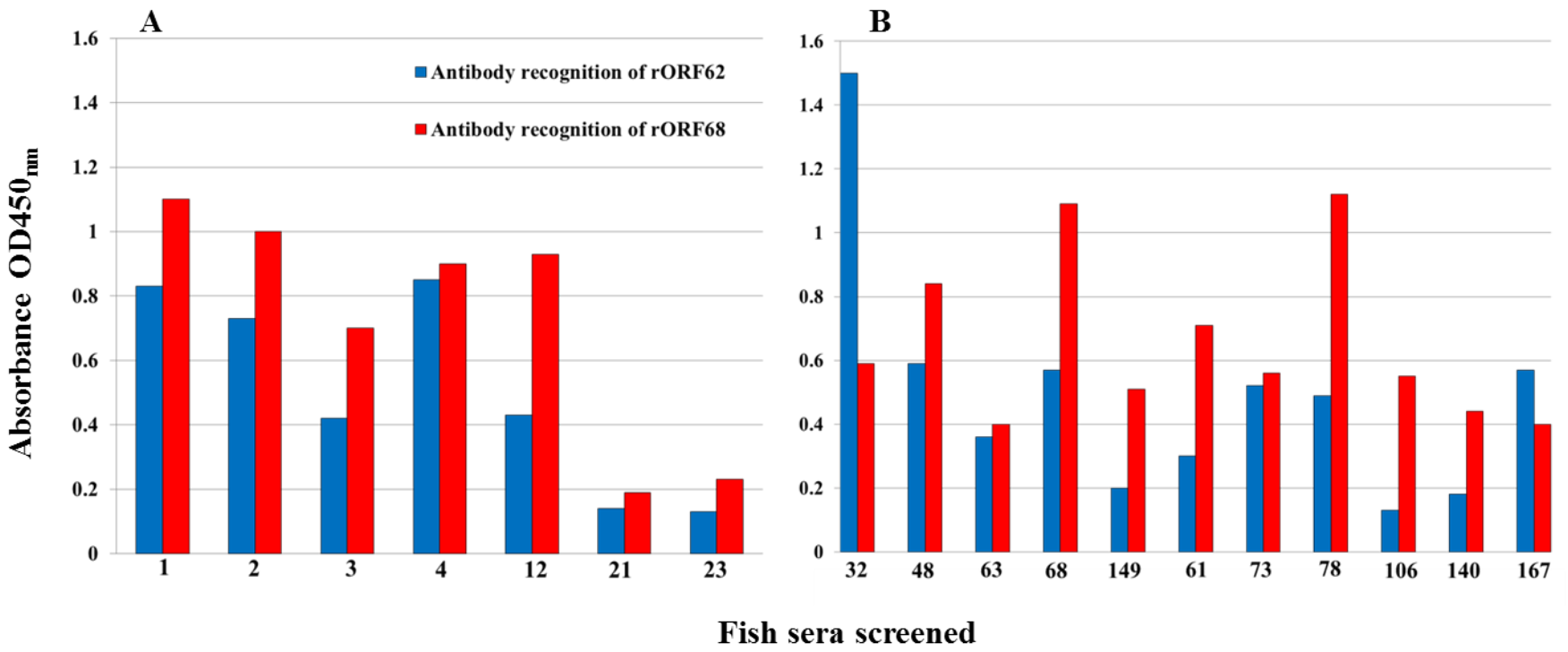


Figure 6.10 Strong and moderate antibody responses of koi herpesvirus (KHV) infected and/or exposed carp from field cases in Asia positive by recombinant KHV antigen ELISA at 1/200 serum dilution. Bars indicate antibody response (absorbance value OD_{450nm}) of individual vaccinated (*n*=15) and control (*n*=8) fish to the two recombinant ELISAs. (A) Strong antibody responders to whole KHV antigen; (B) Moderate responders to whole KHV antigens. Numbers on the *x* axis indicate the identity of serum samples from individual carp.

6.3.4 KHV antigen characterisation

6.3.4.1 Western blot analysis of sera from KHV infected, vaccinated and exposed carp

Despite only a maximum of 18 bands being detected by amido black staining, a total of 19 different molecular weight protein bands were detected following screening by carp sera in Western blot. Individual fish recognised as few as 1 protein band to as many as 12 from the sera of strong responders (e.g. fish 61 which had a titre of 1/1600; Fig. 6.11; Table 6.6). However, the number of protein bands recognised by individual serum samples was not directly correlated with the antibody titre to whole virus antigen by ELISA, where titres of 1/6400 recognised between 2-4 protein bands and 1/800 recognised between 1-6 bands (Table 6.6).

A high molecular weight protein band of approximately 250 kDa was the most frequently recognised protein by all of the fish that were screened (*n*=21). Responders to this protein included infected and vaccinated carp with faint staining also obtained with negative sera (Table 6.7). The next most recognised protein band (53 %) was of 130 kDa. Interestingly, this band was not recognised by fish vaccinated with an inactivated vaccine, but was recognised by infected fish, vaccinated fish with a live attenuated vaccine and 2/4 apparently negative fish (Fig. 6.11; Table 6.7). A band of 170 kDa was recognised by half of all serum samples screened including infected/exposed fish and both groups of vaccinated fish, but not negative fish (Table 6.7).

A band of approximately 100 kDa was recognised by 28% of all screened fish serum including infected/exposed carp and carp vaccinated with an attenuated vaccine, but similar to the 130 kDa band, this protein was not recognised by carp immunised with an inactivated

vaccine. Negative carp sera did not produce any bands of this molecular weight (Fig. 6.11; Table. 6.7). A 40 kDa band was recognised by 16% of screened immunised carp sera, the majority of which were fish that had been vaccinated with an inactivated vaccine (Table 6.7).

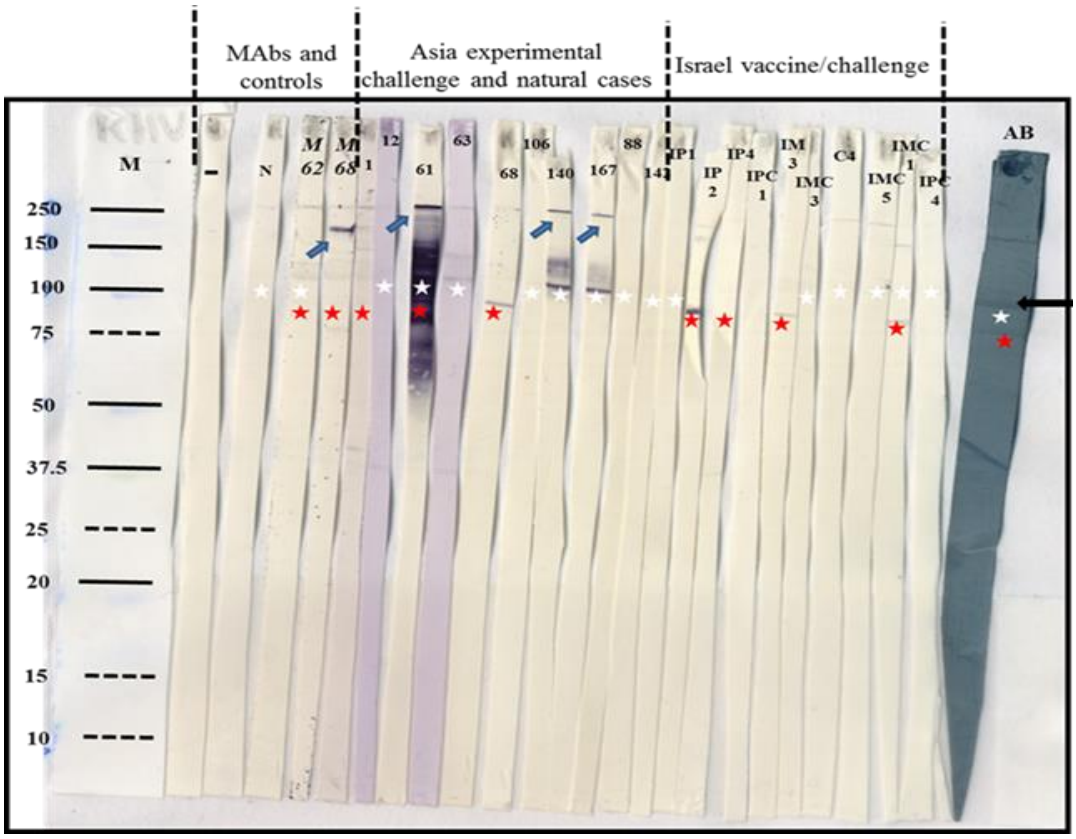


Figure 6.11 Immunoblots of purified koi herpesvirus screened with specific monoclonal antibodies and carp sera. Each membrane strip (lane) was incubated individually with MAbs and carp sera Lanes: *Mabs and controls:* - = TBS; N= neg. mirror carp sera; M62 = MAb 10D10; M68 = MAb 7C6; *Asia experimental Challenge and natural cases;* infected fish: 1, 12, 61, 63, 68, 106, 140, 167, 88, 142; *Israel Vaccination/challenge trials;* vaccinated fish: IP1, IP2, IP4, IM3; challenged vaccinated fish: IPC1, IMC3, IMC5, IMC1, IPC4; Control C4; AB = Amido black. Red stars = 100 kDa protein recognised by fish; white stars = 130 kDa recognised by fish; arrows indicate other immunodominant bands.

A number of other low molecular weight protein bands of 50, 37, 30 and 25 kDa, were also only observed on immunoblots screened with anti-sera from carp vaccinated with the

inactivated vaccine (Table 6.7). Other protein bands that were commonly recognised by carp anti-sera were of 230, 150, 140 and 60 kDa (Table 6.6; Table 6.7).

Table 6.6 Koi herpesvirus (KHV) polypeptides recognised by the sera from individual carp and their antibody titre to whole KHV antigen

Fish ID (case origin)	Whole KHV ELISA titre	Polypeptides recognised by Western blot (kDa)
1 (St)	1/12800	250, 230, 170, 150, 140, 130, 100, 60, 40
12 (M)	1/12800	250, 100, 130
61 (M)	1/1600	250, 230, 170, 150, 140, 130, 110, 100, 80, 75, 60, 55
63 (M)	1/800	250, 170, 130
IP1 (I t)	1/6400	130, 60
IP2 (I t)	1/1600	250, 230, 170, 100, 45
IP4 (I t)	1/6400	250, 170, 100, 40
IPC1 (I t)	1/3200	170, 140
IM3 (I t)	1/6400	250, 100, 75
IMC3 (I t)	1/800	130
68 (S)	1/400	250, 100
106 (S)	1/400	130
140 (S)	1/400	250, 170, 130, 120
167 (J)	1/400	250, 130
C4 (I t)	1/400	250, 130
IMC5 (I t)	1/400	250, 130
88 (J)	1/200	130
142 (M)	1/200	130
IMC1 (I t)	1/200	250, 230, 130, 100
IPC4 (I t)	-	130
NCMC1 (U t)	-	250, 130
C10 (U t)	1/200	-
K1 (U t)	1/3200	170
K3 (U t)	1/1600	250, 170, 140, 40
K7 (U t)	1/3200	250, 170, 140, 50, 40
GK3 (U t)	1/800	250, 170, 40, 37, 30, 25
GK4 (U t)	1/800	250, 170
GK5 (U t)	1/1600	250, 170, 60
32 (M)	1/400	170, 150, 100
CEFAS (A)	1/1600	250, 130
GK6 (U t)	1/1600	250, 170, 40
34 (M)	-	-

Case origin is indicated in brackets (more details are given in Appendix 1, Table A1)
A = Archival material; I t = Israel trial; J = Japan; M = Malaysia; S = Singapore; S t = Singapore trial; U t = UK trial

Table 6.7 Consistency of koi herpesvirus polypeptide recognition by carp antisera

Protein approx. MW (kDa)	No. fish pos.	% of pos. fish	*Inf. (17)*	*At. V (4)*	*In. V (7)*	*Neg (4)*
250	21	66	10	3	6	2
230	4	13	4	0	0	0
170	16	50	6	2	7	0
150	3	9	3	0	0	0
140	6	19	3	1	2	0
130	17	53	14	1	0	2
120	1	3	1	0	0	0
110	1	3	1	0	0	0
100	9	28	6	3	0	0
80	1	3	1	0	0	0
75	2	6	1	1	0	0
60	4	13	2	1	1	0
55	1	3	1	0	0	0
50	1	3	0	0	1	0
45	1	3	0	1	0	0
40	5	16	1	0	4	0
37	1	3	0	0	1	0
30	1	3	0	0	1	0
25	1	3	0	0	1	0

Abbreviations of source of immunisation are denoted as: Inf. = infected; At. V = attenuated vaccine; In. V = inactivated vaccine; Neg. = negative fish. Brackets under these are no. of fish screened

6.3.4.2 Characterisation of antigenic proteins by mass spectrometry

As the protein bands of 130 and 100 kDa were recognised by a relatively large proportion of the screened sera of infected fish and fish vaccinated with a live attenuated vaccine, for which 3/4 fish detected the 100 kDa protein (Fig. 6.11; Table 6.7), but not an inactivated vaccine, these were further characterised by mass spectrometry. Furthermore, a 120 kDa protein was characterised as it was difficult to distinguish the precise molecular weight of the 130 kDa band. The lower molecular weight protein of 40 kDa was included in the mass spectrometric analysis as it was recognised by 4/7 inactivated vaccinated fish (Table 6.7).

MALDI-TOF/TOF analysis confirmed that the antigenic protein bands of 130, 120 and 100 kDa were KHV specific, but identification of the 40 kDa band was unsuccessful (Fig. 6.12). The 120 and 130 kDa bands were identified as both constituting the major capsid protein encoded by ORF92 with an estimated molecular weight of 140 kDa (Fig. 6.12; see Fig. A1 appendix 1). The 100 kDa protein band was identified as an uncharacterised KHV-specific protein encoded by ORF84 with an estimated molecular weight of 86 kDa (Fig. 6.12; Fig. A2 see appendix 1). Both KHV proteins were identified from the full KHV genomic sequence (Aoki *et al*., 2007).

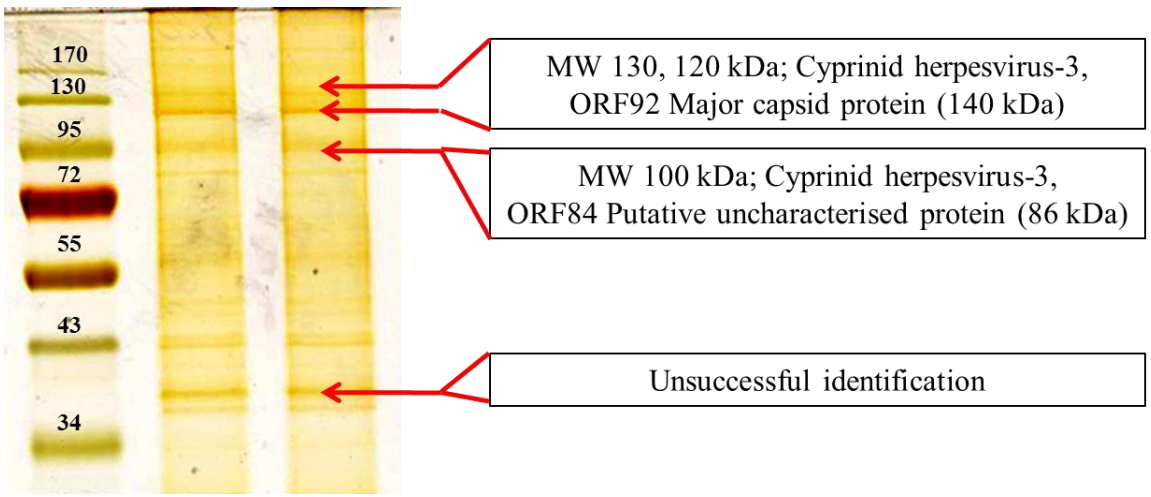


Figure 6.12 SDS-PAGE gel containing separated koi herpesvirus polypeptides for extraction and characterisation by mass spectrometry and the subsequent identification of virion proteins. Red arrows indicate the protein bands eluted from the gel and the attached text box indicates the identity of the proteins following MALDI-TOF/TOF analysis.

6.4 Discussion

6.4.1 Challenges of sensitive and specific detection of antibodies to KHV

Avoidance of false positive antibody detection during sero-surveillance is important for notifiable diseases (Pérez-Filgueira *et al*., 2006), which has been a barrier for development of sensitive serology testing for KHV. Until serology can be fully validated, confirmation of KHV by the OIE in the absence of clinical disease or mortalities is still only accepted by virus detection methods using a combination of ISH, PCR and IFAT on carp tissues (OIE, 2012). However, 45% of all koi sera screened by whole KHV ELISA, from cases in Asia, were seropositive in the current study at a 1/200 dilution, despite a lack of clinical signs in the majority of these cases (Table A1, Appendix 1). Many of these fish would otherwise have been diagnosed as sero-negative by the published ELISA protocols available, which employ high cut-off dilutions in order to prevent detection of cross-reacting antibodies with closely related aquatic herpesviruses such as CyHV-1 (carp pox) (Adkison *et al*., 2005; St-Hilaire *et al*., 2005; 2009; Taylor *et al*., 2010). This represents a high level of risk regarding misdiagnosis of carrier, possibly latently infected fish, with low antibody titres, although the dilemma of false positives was also apparent in the current study. Negative control fish were seropositive at a 1/400 dilution highlighting the difficulties in detecting only antibodies specific for KHV at low serum dilutions. Such cross-reactions from naïve fish may also be a result of natural antibodies that are particularly abundant in cyprinids (Dixon *et al*., 1994; Kachamakova *et al*., 2006; Sinyakov and Avtalion, 2009), which is why stringent blocking steps were included in the assay. Nonetheless, serological detection of KHV antigen can be highly specific as Perelberg *et al*. (2008) reported increased antibody titre and affinity to KHV following inoculation with attenuated virus and repeated exposure to infectious virus.

Differences between putative anti-KHV specific antibody responses of Group 1 and 2 fish from the Asia cases, based on previous association with KHV or presence of KHV DNA in carp tissues, highlighted the extent to which carp are insufficiently screened for KHV using molecular methods in isolation. As much as 69% of cases from different regions/farms in Asia were PCR negative, but seropositive for KHV by ELISA at a dilution of 1/200, whereas only 9% were positive by both PCR and ELISA. This emphasises the necessity to accompany molecular diagnostics with serology for control and surveillance of KHV. Negative cases by real-time PCR may have resulted from dilution of viral DNA after pooling of tissue samples from infected koi, as some tissues will harbour lower concentrations of virus DNA, especially during potential latent infections (Gilad *et al*., 2004; Bergmann *et al*., 2010a; Eide *et al*., 2011a). However, variable detection sensitivities of PCR may also depend on water temperatures during clinical KHVD (Matras *et al*., 2012). Nonetheless, serology appeared more sensitive than molecular methods for detecting KHV positive cases in the current study (i.e. not just individual fish) and similarly has also proved useful for epidemiological screening of carp populations in fisheries and farms (Taylor *et al*.; 2010; 2011; Azila *et al*., 2012). Previous studies of naturally exposed fish to KHV have also demonstrated greater numbers of KHV seropositive fish compared to PCR positive fish (Uchii *et al*., 2009), which indicates that persistent infections (with low viral loads) can be present that are undetected by PCR. Alternatively, periodic reactivation of latent virus may continuously boost antibody responses. This may explain the high antibody titres > 1 year following transfer of infected fish to virus-free water, as reported by Adkison *et al*. (2005), and the variation in antibody titres in the current study between fish from various cases. Variations in virus dose, exposure periods, temperature and natural antibody abundance may have contributed considerably to long term antibodies detected in previously exposed fish.

Such long term detectable antibody titres to KHV have also been reported in experimentally infected (Ronen *et al*., 2003; Adkison *et al*., 2005; St-Hilaire *et al*., 2009; Taylor *et al*. 2010) and naturally infected (Uchii *et al*., 2009) fish. The high titre antibody responses of a number of healthy fish from Group 2 cases are therefore likely to be long term responders that have recovered from KHVD.

Fish from cases associated with areas where KHVD outbreaks were present and/or molecular methods were positive, were often seronegative, thus these fish may have been experiencing an acute KHV outbreak prior to sero-conversion, similar to findings from experimental bath inoculation challenges (Matras *et al*., 2012). However, following recovery, detectable antibodies are usually evident, i.e. 3-6 weeks post infection/vaccination (Ronen *et al*., 2003; Adkison *et al*., 2005; Perelberg *et al*., 2008; Matras *et al*., 2012). Matras *et al*., (2012) showed that a rise in detectable KHV-specific antibody titres occurs following cessation of clinical KHVD in experimentally infected fish using SNT. This suggests that neutralisation and clearance of the virus occurs, thus reducing viral loads resulting in negative PCR results (Matras *et al*., 2012). This also corroborates earlier findings by Perelberg *et al*. (2008) where mortality of recently challenged and/or vaccinated carp is inversely correlated with rising antibody titres, and where KHV DNA could not be detected in carp surviving experimental challenge (Gilad *et al*., 2004). However, other studies have demonstrated that antibodies are only partially protective (Adkison *et al*., 2005) and cellular immunity plays an important role in protection (Adkison *et al*., 2005; Perelberg *et al*., 2008; Rakus *et al*., 2012).

Strongly seropositive Group 2 fish that originated from cases in Japan (C15), China (C16), and Singapore (C20) were largely asymptomatic (Table A1, Appendix 1). Therefore, regular screening of carp, preferably by non-lethal methods, such as blood sampling for both

molecular detection in blood leukocytes (Eide *et al*., 2011a, b; Bergmann and Kempter, 2011), gill swabs (Eide *et al*., 2011a; Bergmann *et al*., 2010a) and antibody detection in serum (Adkison *et al*., 2005; St-Hilaire *et al*., 2005; 2009; Taylor *et al*., 2010; Azila *et al*., 2012; Matras *et al*., 2012) should be carried out regardless of reported disease outbreaks.

A dose-dependent induction of antibodies to KHV was observed with higher challenge doses in experimentally infected fish in Singapore. Vaccination with a live attenuated vaccine also generally induced strong responses with antibody kinetics comparable to previous studies (Ronen *et al*., 2003; Perelberg *et al*., 2008; Matras *et al*., 2012), but slow or absent sero-conversion was apparent in carp receiving a low dose infection, i.e. after 29 dpi. This presents a major problem for KHV serological diagnostics, and DIVA strategies, and emphasises the need for improved sensitivity of current whole KHV ELISA protocols that require high serum dilutions (Adkison *et al*., 2005; St-Hilaire *et al*., 2009).

6.4.2 Potential for improved sensitivity and specificity of KHV serology utilising recombinant proteins

The expressed and purified recombinant proteins from transformed *E. coli* were validated according to molecular weight (Aoki *et al*., 2009; 2011) (60 and 56 kDa for rORF62 and rORF68, respectively), purity and antigenicity by screening with their respective MAbs, or carp sera on immunoblots. Apart from breakdown products observed for rORF62, serum antibodies still recognised these, which suggested that the proteins were correctly folded and that the positive recombinant ELISA results represented the presence of anti-KHV carp antibodies specific for rORF62. Breakdown products of recombinant proteins are commonly observed following harvesting and purification procedures of the product, e.g., precipitation of insoluble products at physiological pH as reported after purification of recombinant protein

G of VHSV (Encinas *et al*., 2011b). This may be associated particularly with proteolysis (Gómez-Sebastián *et al*., 2008), possibly explaining the greater presence of rORF62 breakdown products, which is itself a protease (Aoki *et al*., 2009; 2011). The degraded products did not affect the epitopes, which were still recognised by MAb10D10 and carp anti-sera. Recombinant protein, rORF68 was also recognised by infected carp sera. Recombinant antigens of aquatic viruses produced through prokaryotic expression systems such as *E. coli* have previously been successfully utilised for fish serology, e.g. for detection of nodavirus (Huang *et al*., 2001), iridovirus (Kim *et al*., 2007b) and VHSV (Encinas *et al*., 2011b) and appears to be an effective and simple expression system for recombinant ELISA development for KHV.

Some strong responders to whole KHV antigen were negative for both rORF62 and rORF68 highlighting that alternative epitopes are involved in humoral immunity to KHV and expressed products of ORF62 and ORF68 may not generate a high proportion of specific antibodies by all infected individuals, which may be associated with the restricted antibody repertoire of fish (Du Pasquier, 1982), especially to a complex virus like KHV. Therefore, alternative antigens may be needed to enhance the reliability of these recombinant anti-KHV antibody ELISAs. A 1/200 single serum dilution was employed to provide greater sensitivity than the ELISA screening methods developed to date (Adkison *et al*., 2005; St-Hilaire *et al*., 2009) while aiming to retain assay specificity. Furthermore, this screening approach is highly cost-effective and provides high sample throughput with reduced plate-plate variations among fish (Alcorn and Pascho, 2000). Although no cross-reactivity of MAbs raised against the recombinant antigens (MAb 10D10 and MAb 7C6) was previously observed to CyHV-1 and CyHV-2 (Aoki *et al*., 2009; 2011), anti-CyHV-1 serum that is free of anti-CyHV-3 (KHV) antibodies would need to be screened on the recombinant ELISAs developed in the

current study to validate the specificity of these assays for KHV. This would also enable the determination of minimum working dilutions of carp serum required for these assays to detect anti-KHV antibodies specifically, while remaining negative for antibodies to CyHV-1.

A number of known negative fish were positive by recombinant ELISAs, despite these recombinant proteins being antigenically specific for KHV (CyHV-3) and have been shown not be recognised by cross reacting antibodies, i.e. to closely related herpesviruses such as CyHV-1 (Aoki *et al*., 2009; 2011). These negative fish gave false positive results especially at dilutions of ≤1/200. At these lower dilutions false positives are likely to be associated with natural antibodies (Kachamakova *et al*., 2006; Sinyakov and Avtalion, 2009). However, the inclusion of high titre anti-sera to CyHV-1, that is negative for antibodies to CyHV-3, as a heterologous antibody control on the plate, would confirm this and could enable a distinction between natural antibody, cross-reactivity and anti-CyHV-3 antibodies. Cyprinids expressing high levels of natural antibody are thought to be less capable of expressing specific IgM following immunisation, whereas fish with low natural antibody levels tend to produce higher specific antibody titres (Sinyakov and Avtalion, 2009). This presents another challenge for improving the sensitivity of KHV diagnostic serology. Fish that do not produce high titres of specific antibody may alternatively synthesise high levels of innate, non-specific natural antibodies, which are characteristically cross-reactive. Thus determining the specificity of antibodies to KHV is problematic. High backgrounds have been previously reported in ELISAs using recombinant proteins, for which Encinas *et al*., (2011a; b) used solid-phase antibody captured virus to enable a better distinction between specific and non-specific antibody binding. Alternatively, antibody responses directed to bacterial derived antigen, i.e. *E. coli* lysate, may have contributed to background signals. This is a common problem encountered with recombinant ELISA assays that are coated with *E.*

coli-derived expression products (Villinger *et al*., 1989; Hemmatzadeh *et al*., 2013), which can be reduced by blocking with *E. coli* lysate in the serum diluent (De Diego *et al*., 1997). However, during optimisation of the assay in the current study, these approaches were attempted, but no discernible differences in ELISA signals were noticed between sera with and without *E. coli* lysate.

Interestingly, variations in response to rORF62 and rORF68 were evident between carp vaccinated with a live attenuated vaccine (rORF62 < rORF68) and those vaccinated with an inactivated vaccine (rORF62 > rORF68). It could be that B cell clonal expansion against rORF68 occurs more in inactivated vaccinated fish compared to attenuated vaccinated fish.

Stronger antibody responses to rORF62 in attenuated vaccinated fish than inactivated vaccinated fish, suggests that this antigen is exposed to fish more abundantly within a replicating virus. Therefore because of the higher diversity of antibodies induced by the live attenuated vaccine, i.e. from a larger number of expressed proteins, there may be a limited repository of specific anti- rORF68 antibodies compared to the inactivated vaccine, which was evident in the current study, as this vaccine lacks active expression. Different antibody responses between fish to the same viral protein, but to different fragments of that protein, have been demonstrated for VHSV (Encinas *et al*., 2011b). This may be associated with disulphide-dependent conformational epitopes (Einer-Jensen *et al*., 1998). Thus, such antigenic epitopes of the protein, i.e. of ORF62, may be masked by cross-linking during formalin inactivation of the virus when developing the inactivated vaccine and the native form is subsequently not recognised by these immunised fish. However, altered conformation of virus epitopes or incorrect protein folding following expression through *E.coli*, can also affect antibody detection on the diagnostic test. This is particularly true when the proteins

form aggregated inclusion bodies within the bacterial cell, as is the case for rORF62 and rORF68 (Aoki *et al*., 2011). Proteins expressed as inclusion bodies are generally mis-folded and often biologically inactive (Sørensen and Mortensen, 2005). However, this can be reduced by culturing the transformed *E. coli* at lower temperatures (Sørensen and Mortensen, 2005), e.g. as with VHSV recombinant proteins ELISA (Encinas *et al*., 2011b). Utilising eukaryotic expression pathways have been shown to have a number of advantages over prokaryotic systems like *E. coli* including a lower permissible temperature for culture (Macauley-Patrick *et al*., 2005).

Vast variations in immunoreactivity to rORF62 and rORF68 were also noted in experimentally infected or naturally exposed carp. As KHV encodes a vast number of proteins, including at least 13 envelope proteins (Michel *et al*., 2010b), many may induce strong antibody responses to various epitopes, which may complicate the development of highly specific, convenient and cost-effective recombinant protein ELISAs (Gómez-Sebastián *et al*., 2008). Variation in antibody response to the recombinant proteins in the ELISA can be related to antigenic drift of virus strains alternating their epitope binding site (Jacobs and Kimman, 1994; Pérez-Filgueira *et al*., 2006). Although little variation between protein profiles is noted between KHV strains (Gilad *et al*., 2002; 2003), differences may be present in antigenic determinants of certain proteins in field strains, which may result in the variation in antibody responses seen in the current study. Similar antibody responses have been observed with field strains of African Swine Fever Virus (ASFV) p30 (Pérez-Filgueira *et al*., 2006) or PRV glycoprotein E (gE) (Gómez-Sebastián *et al*., 2008) in pigs. The ELISA may require a cocktail of KHV-specific antigens to overcome this.

The 19 KHV protein bands detected by various fish in western blot analysis is greater than the predicted number of envelope proteins of KHV, considered to be the most immunogenic proteins (Rosenkranz *et al*., 2008; Michel *et al*., 2010b). Therefore, alternative KHV structural (and non-structural) proteins must also induce antibody responses. Large variations in immunoreactivity between individual animals have been reported for mammalian herpesviruses, e.g. bovine herpesvirus, equine herpesvirus and asinine herpesvirus (Van Drunen Little-van den Hurk and Babiuk, 1986; Crabb and Studdert, 1990). Variable neutralising and non-neutralising antibody responses can arise from exogenous antigens recognised by B cells or antigen presenting cells (APCs) that are presented to $CD4^+$ T helper cells via the MHCII pathway (Hangartner *et al*., 2006). However, endogenous antigens can also be processed via the MHCII pathway, and stimulate the production of IFN γ, and the subsequent production of non-neutralising antibodies (Hedge *et al*., 2003). Non-neutralising antibodies may be highly abundant and long-lived in fish surviving infection, e.g. to rhabdoviruses (Olesen *et al*., 1991; Enzmann and Konrad, 1993; Lorenzo *et al*., 1995). Thus, in the interest of diagnostics, antigens other than the neutralising epitopes of envelope glycoproteins should be investigated.

The most commonly recognised KHV antigens in western blot analysis had molecular weights of 250, 170, 130 and 100 kDa. These were recognised by more than a quarter of all the sera analysed, while a protein band of approximately 40 kDa was recognised by 16 % of all the sera. Adkison *et al*. (2005) also reported a band of 97 kDa to be recognised by the majority of KHV infected carp, suggesting it too is potentially immunodominant. The authors also suggested that protein bands of 170, 130 and 56 kDa are potentially cross-reactive with CyHV-1 polypeptides. The protein band of ~100kDa in the current study, which is likely to represent the same 97 kDa protein reported by Adkison *et al*. (2005), was identified as an

unknown CyHV-3 specific protein product of ORF84 by mass spectrometry. This protein was recognised by 6/17 naturally exposed or infected carp and 3/4 fish vaccinated with the attenuated vaccine, but not by uninfected or inactivated vaccinated fish. Thus, this protein could represent a useful alternative recombinant antigen for screening for anti-KHV antibodies. Fish 68 and IMMC1, the only fish screened on the recombinant protein ELISAs that were classified as moderate-weak responders in whole virus ELISA, possessed antibodies recognising the KHV specific rORF62 and the 100 kDa protein, indicating that it may be a useful antigen for improving the sensitivity and specificity of KHV serology.

In the current study, KHV protein bands of 250 kDa and 130 kDa were detected from healthy, uninfected carp sera. Thus, the 130 kDa band does appear to constitute a cross-reactive protein. This band was consistently detected as a dominant band on SDS-PAGE gels and an immuno-dominant band by Western blot, and was confirmed as the major capsid protein (MCP) of KHV encoded by ORF92 by mass spectrometry, in accordance with other studies (Gilad *et al*., 2002; 2003; Michel *et al*., 2010b; Dong *et al*., 2011). Fish 88, IP1 and 142 recognised the 130 kDa protein, but neither of the recombinant proteins possibly suggesting that their antibody titres detected on whole KHV ELISA were associated with cross-reactivity to the MCP, which appears to be highly conserved among the cyprinivirus genus. Indeed the MCP encoded by KHV (CyHV-3) ORF92 is homologous to the MCP of AngHV-1, IcHV-1, RaHV-1 and RaHV-2 (Aoki *et al*., 2007; Michel *et al*., 2010b; Van Beurden *et al*., 2011b). This supports previous suggestions that low dilution antibody responses of carp to whole KHV antigen is often due to this cross reactivity (Adkison *et al*., 2005; St-Hilaire *et al*., 2009), for which this antigen may play a major role. This finding is useful as the apparently healthy status of fish 88 and 142 can be confirmed as KHV seronegative. Fish 142 was associated with a case including a fish with a high anti-KHV

antibody titre (Fish 143), thus is an example of the risks involved in pooling of serum samples for antibody detection. This has been proposed for studies on KHV prevalence in carp populations (St-Hilaire *et al*., 2009; Taylor *et al*., 2010; 2011), but pooling of sera from each case in this study would have resulted in Fish 142 diluting the high titre antibodies of Fish 143 leading to potentially false negative diagnosis of that carp population. However, this finding also demonstrates complications in detecting antibodies in vaccinated fish, such as IP1, which produced strong antibody responses to the 130 kDa MCP, but not rORF62 or rORF68, making it difficult to determine if that response is induced to the KHV vaccine specifically. This is even more problematic with non-responding fish such as IPC4, which had been vaccinated with an attenuated live vaccine in the trial in Israel, and subsequently challenged with a virulent KHV isolate, but was negative by all ELISAs. This fish was only positive by Western blot to the 130 kDa MCP band. Thus low level KHV-specific antibodies may be induced to this highly conserved antigen, which could only be used reliably for diagnostics if the KHV-specific epitopes were determined, i.e. by epitope mapping, similar to that undertaken for the G protein of rhabdoviruses, IHNV and VHSV (Encinas *et al*., 2011b). Virus protein bands of 250 and 170 kDa were detected by 67% and 52% of screened fish. Further investigation into the role of these polypeptides would be worthwhile as the former represented another major cross-reactive antigen and the latter was detected by infected and vaccinated fish with both vaccines.

Some fish that were positive by PCR, were negative by both recombinant protein ELISAs at 1/200 serum dilution, while others were negative by PCR, but seropositive on both recombinant protein ELISAs. A capsid antigen-based recombinant protein ELISA was previously shown to successfully detect antibodies to nodavirus in barramundi after only 6 dpi, while brain and eye tissues were positive by RT-PCR after only 3 dpi (Huang *et al*.,

2001). The majority of anti-KHV antibodies are not detected until ~3 wpi (Ronen *et al*., 2003; Perelberg *et al*., 2008). Thus even with improved sensitivity and specificity, the application of such tests for the control of KHVD need to be undertaken in conjunction with molecular methods both for early and late stage diagnostics. However, the advantages of utilising KHV (CyHV-3)-specific antigens was made clear with 12/13 fish positive for antibodies to rORF62 and 8/13 positive for rORF68 at a low (1/200) serum dilution from cases that had previously tested negative by PCR.

As there were a number of moderate and weak responders from the whole KHV ELISA that were positive for the recombinant protein ELISAs, these fish would have been considered seronegative using the current conventional ELISA protocols (Adkison *et al*., 2005; St-Hilaire *et al*., 2009). Thus retesting serum with antibody titres of 1/400 or lower from the KHV whole virus ELISA on KHV recombinant protein ELISAs may lead to the detection of more seropositive fish and decrease the number of false negatives. However, the characteristics of carp immunoglobulins must be taken into account when enhancing the sensitivity of KHV antibody ELISAs, due to the problems with non-specific binding and natural antibody activity (Sinyakov and Avtalion, 2009) particularly for DIVA diagnostic test development.

6.4.3 DIVA vaccination strategy using an inactivated KHV vaccine

The differential antibody responses to rORF62 and rORF68 may pave the way for potential DIVA strategies, i.e. where vaccinated fish produce antibodies to rORF68 and not rORF62, whereas infected/exposed fish recognise both recombinant proteins. The principle behind such a DIVA strategy is explained in Section 1.6.6 and illustrated in Figure 1.6 C-D. Differential antibody responses have been reported previously in fish vaccinated with

formalin inactivated Lymphocytivirus compared to infected fish (Jang *et al*., 2011), where fewer antigens were recognised by the former. Such differences are considered to be related to viral replication kinetics. Viral protein is produced and presented to the immune system in abundance during replication with subsequent induction of specific antibodies to each antigenic viral protein (Flint *et al*., 2004), including non-neutralising antibodies (Olsen *et al*., 1991; Adkison *et al*., 2005; Encinas *et al*., 2011a) e.g. to KHV. In contrast, formalin inactivated virus does not induce the production of new antibody responses to all the viral proteins. This can be related to cross-linking of viral proteins and nucleic acids, which eliminates viral infectivity (Jang *et al*., 2011). However, KHV is a herpesvirus and the virion possesses a glycoprotein envelope (Hedrick *et al*., 2000; 2005; Miwa *et al*., 2007; Miyazaki *et al*., 2008). Therefore, the cysteine protease, which is a late protein, encoded by ORF62 (Aoki *et al*., 2007; 2009; 2011; Ilouze *et al*., 2012b), characterised as a tegument protein of the virion (Michel *et al*., 2010b) would not be expected to be exposed on the surface of the virion as it is an internal viral structural protein. The fact that the protein encoded by ORF68 possesses a transmembrane domain, but not ORF62 (Aoki *et al*., 2009; 2011), may support the hypothesis that the protein encoded by ORF62 is not exposed to the immune system, at least not to the same extent, as that encoded by ORF68. Thus no/or limited specific antibodies are produced to this protein in fish vaccinated with whole, formalin inactivated KHV. However, even external viral structural proteins can induce differential antibody responses following formalin inactivation during vaccine development. This was observed by Kwon *et al*. (2009) when developing a marker vaccine and DIVA system for avian influenza. In that study, birds did not respond to the N2 (neuramindase) envelope protein, despite being vaccinated with an inactivated H9N2 vaccine (Kwon *et al*., 2009), until they were challenged

with wild-type H9N2 AIV. This was suggested to be a result of formalin-induced conformational changes of the recognised NA epitopes.

Previous recombinant protein ELISA developments for diagnostics against VHSV, another notifiable fish pathogen, highlighted that lower absorbance values observed from hyper immunised trout to recombinant G protein epitopes were molecular size dependent (Encinas *et al*., 2011a). The VHSV G protein constitutes a major immunogenic structural protein of rhabdoviruses (Gaudin *et al*., 1999). Due to the larger size of the recombinant protein G21-465 (445 aa) compared to small peptide fragments of the same protein (55 - 199 aa) on coated ELISA plates, a greater number of molecules of smaller fragments per surface unit, increases epitopic density and exposure (Encinas *et al*., 2011a) resulting in a greater number of antibody-antigen complexes. The recombinant protein of ORF62 is also larger (570 aa) than that of ORF68 (501 aa) (Aoki *et al*., 2009; 2011). Despite this, significantly greater antibody responses to rORF68 compared to rORF62 were only observed in sera from inactivated vaccinated fish, but not the live attenuated or infected/exposed fish. This suggests that poorer antibody responses to rORF62 appear to be specific to uninfected fish vaccinated with this inactivated vaccine. There were however, significantly higher antibody responses to rORF62, compared to control fish, detected in fish vaccinated with the inactivated vaccine. This would suggest that there is some, albeit minimal, recognition of this protein in these vaccinated fish prior to infection. Thus, this would limit the effectiveness of this antigen for DIVA strategies. However, differences in the adjuvant administered to vaccinates and control fish should also be considered. Vaccinated fish were immunised with aluminium hydroxide adjuvant, which stimulates a potent Th2 response (Cox and Coulter, 1997; Kool *et al*., 2008), and this may have influenced the greater production of antibodies to rORF62 compared to the controls, which received only montanide. This may therefore also have resulted in greater

antibody levels in general and perhaps non-specific reactivity with rORF62. Control fish would need to be immunised with the same adjuvant to determine if this had an influence on the DIVA serology test.

Complications with fish IgM cross-reactivity can occur even when small antigens are coated onto solid phase ELISA plates, such as the ELISA plates coated with rORF62 and rORF68 in the current study. This is due to the solid phase resembling the surface of complex antigen when it is densely coated (Denzin and Staak, 2000). The coated antigen may subsequently be displayed to IgM as repetitive epitopes, to which the 'starshaped' multivalent fish IgM binds by adopting a 'crab-like' structure (Roitt, 1997). This may have resulted in non-specific binding on the recombinant ELISAs, possibly affecting the DIVA compatibility of rORF62 to distinguish inactivated vaccinated fish as uninfected. Competitive ELISA using suspended antigen can prevent artefactual binding like this, as demonstrated previously to whole KHV antigen (Perelberg *et al*., 2008). It is possible to improve the specificity, and thus sensitivity, of recombinant protein based ELISAs, which is critical for DIVA diagnostics, by employing competitive assays. Such assays have also proved effective for carp serology with other viruses, e.g. Spring Viraemia of Carp Virus (SVCV), by including high affinity mammalian IgG (Dixon *et al*., 1994; Denzin and Staak, 2000). Competitive recombinant ELISAs have been utilised for a number of DIVA diagnostic tests (van Oirschot *et al*., 1988; 1996; Bosch *et al*., 1997; Floegel-Niesmann, 2001; Kirkland and Delbridge, 2011) and could be developed for the current DIVA diagnostic tests utilising specific MAbs for rORF62 and rORF68, (i.e. 10D10 and 7C6, respectively). However, the epitopes recognised by the MAbs and antigenic determinants recognised by carp IgM are as yet unknown. A competitive ELISA using these MAbs may lack the ability to inhibit carp serum antibodies if carp antibodies recognise alternative epitopes of the recombinant proteins, thus limiting the

validity of the assay. Furthermore, this impairs the possibility to engineer the ELISA into a rapid diagnostic test using lateral flow technology that would subsequently be used for in-field testing.

Recombinant ELISAs against different viral structural proteins have been utilised in a number of studies to differentiate infected animals, i.e. producing antibodies to both 'external' membrane glycoproteins and 'internal' nucleoproteins, from vaccinated uninfected animals, which produce antibodies only to the external membrane glycoproteins (Makkay *et al*., 1999; van der Wal *et al*., 2012). Such DIVA strategies usually comprise the administration of subunit and recombinant vaccines lacking the internal, usually non-protective, viral proteins in conjunction with recombinant ELISAs. These DIVA assays employ plates coated with (1) the vaccine antigen and (2) a viral antigen absent in the vaccine. However, whole virus inactivated vaccines have also been utilised for DIVA strategies, which are usually based on accompanying ELISA testing of serum antibodies to non-structural virus proteins, i.e. only exposed to the host when the virus is replicating (Birch-Machin *et al*., 1997; Tumpey *et al*., 2005; Barros *et al*., 2009; Avellaneda *et al*., 2010). In spite of promising results, this strategy has proved difficult and often unreliable for some viruses, such as AIV, as a lack of sero-converting birds results in false negative diagnosis of infected birds (Avellaneda *et al*., 2010). Structural viral proteins, as utilised for KHV in the current study, may prove more reliable by inducing stronger antibody responses. A structural protein of AI, the M2e membrane protein, has proved an effective antigen for DIVA strategies using inactivated AI vaccines (Lambrecht *et al*., 2007; Kim *et al*., 2010; Hemmatzadeh *et al*., 2013). As this protein is not abundant in the virion, but is highly abundant in infected cells, it induces strong antibody responses in infected, but not vaccinated birds. Therefore rORF62, an internal tegument protein of KHV, which is also not vastly

abundant in whole virus particles (Michel *et al*., 2010b), was utilised for a similar purpose in the current study, and although promising results were obtained with regards to a DIVA potential, the protein was still recognised by 1 fish at 1/800 serum dilution. Problems were also encountered with the number of false negatives (5/12) to rORF68 compared to whole virus. Nonetheless, differential responses were observed, but the problems encountered, including non-responsiveness and lack of sero-conversion, typical of DIVA vaccination (Uttenthal *et al*., 2010) make it difficult to incorporate this strategy for individual fish, even if the serological tests were optimised.

The highly immunogenic envelope glycoproteins have proved to be the most effective target antigens for DIVA vaccination development for mammalian and avian herpesviruses (Heffner *et al*., 1993; Jacobs and Kimman, 1994; Bosch *et al*., 1997; Shil *et al*., 2012). However, after screening the KHV sero-positive carp by Western blot, only 1 fish appeared to possess antibodies to a low molecular weight antigen of approximately 26kDa, which would correspond to the size of the putative immunodominant envelope protein, the product of ORF81 (Aoki *et al*., 2007; Rosenkranz *et al*., 2008, Michel *et al*., 2010a; b). This may have been due to low concentrations of this protein transferred to the membrane. However, other highly antigenic structural proteins could be utilised for serological diagnostics and DIVA strategies. Recognition of the internal MCP of KHV by the sera screened in the current study indicates that it is exposed to the immune system during a lytic infection. However, as the 130 kDa protein may represent a cross-reactive antigen with closely related herpesviruses, alternative capsid antigens could provide a more specific alternative for detection of antibodies induced by infection, i.e. to internal KHV antigens, compared to a non-replicating inactivated vaccine virus strain. The other antigenic protein of 100 kDa (that may be analogous to the immunodominant 97 kDa protein described by Adkison *et al*. (2005)) is

encoded by ORF84 with no known role in KHV assembly or antigenicity (Michel *et al*., 2010b), but may be a suitable candidate for this purpose.

6.4.4 Concluding remarks

Results from the KHV whole virus ELISA showed that negative PCR results from pooled organs of a population of clinically healthy fish does not indicate KHV-free status alone and should be accompanied by serological testing from individual koi. The recombinant proteins of ORF62 and ORF68 can be useful for this purpose as they encode for KHV-specific antigens and the sequences of these are nearly identical among KHV isolates from USA, Europe and Japan (Aoki *et al*., 2007; 2011), which may alleviate problems of serological cross-reactivity with closely related aquatic herpesviruses. Both recombinant protein ELISAs have shown promising results for detection of anti-KHV antibodies in potentially latent infected koi with low antibody titres, which would otherwise be considered negative using a whole KHV ELISA employing a high dilution cut-off and PCR testing. Differential antibody responses to the two recombinant proteins, which are antigens derived from internal and exposed viral structural proteins, appeared to occur between uninfected fish vaccinated with an inactivated vaccine and fish that are infected, suggesting the feasibility of DIVA vaccination strategies in carp to KHV. A DIVA approach could be implemented by determining positive detection of only anti-rORF68 responses in uninfected vaccinated fish but responses to both antigens in infected fish. However, further optimisation and testing of these ELISAs would be required before they could be reliably used as serological assays for KHV screening of carp and koi, and/or DIVA diagnostic tests for an inactivated KHV vaccine, especially as differential responses were mostly limited to a serum dilution of 1/800. The variation in reactivity of carp antibodies to KHV needs to be taken into account for

optimised reproducibility of recombinant protein ELISA testing, and perhaps alternative KHV antigens may be useful for sensitive diagnostics and DIVA strategies, such as that encoded by ORF84.

Chapter 7

General Discussion

7.1 A series of investigations to find a suitable marker/DIVA vaccine approach for an aquatic virus

Although aquatic animal production differs from that of terrestrial animals, lessons can be learned from the design and implementation of disease control and eradication programmes used in the meat and poultry industries, for use in the aquaculture industry (Moennig, 2005). The successful approach of DIVA vaccination, discussed in detail in Chapter 1, has facilitated eradication programmes for Aujeszky's Disease (AD), bovine herpesvirus type-1 (BoHV-1) and rinderpest (RP), and has improved control of foot-and-mouth disease (FMD), avian influenza (AI) and classical swine fever (CSF) (Van Oirschot *et al*., 1996; Van Oirschot, 1999; Suarez, 2005; 2012; Beer *et al*., 2007; Meeusen *et al*., 2007; Uttenthal *et al*., 2010). These successes highlight the potential for control programmes for notifiable diseases affecting aquaculture to be more ethical and cost-effective. The vast array of vaccines and companion diagnostic tests designed to make DIVA vaccination strategies feasible for mammals and birds (see Table 1.1 and 1.2) have varied considerably depending on the disease agent. Assessing the feasibility of different approaches for DIVA vaccination in fish has been the focus of the current thesis. In this regard, two notifiable diseases, infectious salmon anaemia (ISA) and koi herpesvirus disease (KHVD) were used as they represent two of the most important threats to the global Atlantic salmon and common carp/koi industries, respectively (Kibenge *et al*., 2004; Ilouze *et al*., 2011; Michel *et al*., 2010a).

Inactivated ISA and KHV vaccines were used for marker and DIVA vaccination in the current study. Inactivated vaccines have been effective for a number of fish viral diseases, and they are commercially available for use in the field (Dhar and Allnutt, 2011; Gomez-Casado *et al*., 2011). The initial approach, using an exogenous marker vaccine (Chapter 3), was intended to elucidate (1) whether the inclusion of foreign antigens to the vaccine could

induce an additional antibody response in vaccinated fish and might thus be utilised as a 'positive marker'; (2) whether the vaccine could induce a reliable and detectable antibody response, which is a prerequisite for DIVA vaccination (Van Oirschot *et al*., 1996; Van Oirschot, 1999). A number of challenges facing exogenous marker and DIVA vaccination strategies for fish were highlighted by these initial studies.

The conditions within the aquatic environment of poikilothermic animals may affect the induction of detectable marker-specific antibodies and thus the potential success of marker vaccination. The cold temperatures that were experienced during the majority of the experimental period for vaccinated Atlantic salmon (<6ºC; Fig. 3.9) are known to inhibit antibody responses to the thymus dependent (TD) antigen, but not to the thymus independent (TI) antigen, which could be attributed to the temperature sensitivity of T cells (Bly and Clem, 1992; Secombes *et al*., 1996; Le Morvan *et al*., 1998). Where antibody responses were induced to one of the marker antigens (KLH) the greatest number of responders was <50% at any one time point, despite the very low serum dilutions used (Table 3.2; Fig. 3.6 C). Thus, none of these immunogens (TT, KLH, or FITC) are suitable for use as markers for ISAV vaccines in salmon. Therefore, alternative DIVA strategies were considered such as by exploiting structural proteins of ISAV that are absent in ISA vaccines.

Vaccines developed so far for ISA have included a recombinant subunit protein vaccine (Pharmaq, Norway; Dhar and Allnut, 2011), a DNA vaccine (Mikalsen *et al*., 2005) and a salmon alphavirus replicon vaccine (Wolf *et al*., 2013), which have all been developed specifically to express the protective surface haemagglutinin esterase (HE) protein, but not other ISAV proteins. Since internal structural proteins of ISAV, i.e. the nucleoproteins (NP), has been reported to be highly antigenic for anti-ISAV Atlantic salmon sera (Dr. Kim Thompson pers. comm.; Falk pers. comm. cited in Wolf *et al*., 2013) this protein holds potential for use in a DIVA strategy. The application of ISAV NP and HE protein on a

companion serology test could enable differentiation between infection and vaccination if detectable antibodies from vaccinated fish could be detected by a HE-specific ELISA and infected fish detected by an NP-specific ELISA. Similar approaches have been applied for DIVA strategies for other orthomyxoviruses, such as equine influenza virus (EIV) (Minke *et al*., 2004; Kirkland and Delbridge, 2011) and have been considered for avian influenza virus (AIV) (Suarez, 2005). However, poor antibody responses were also observed to the inactivated ISA vaccine used in Chapter 3. This is commonly reported in ISA vaccination studies prior to challenge (Brown *et al*., 2000; Mikalsen *et al*., 2005; Lauscher *et al*., 2011; Wolf *et al*., 2013), although following challenge protection and antibody production are usually reported (Jones *et al*., 1999b; Brown *et al*., 2000; Mikalsen *et al*., 2005; Lauscher *et al*., 2011; Wolf *et al*., 2013). Regardless of protection, however, a specific antibody response must be detectable prior to infection for the DIVA strategy to be feasible, which clearly does not appear to be possible in the field for Atlantic salmon, at least with regards to ISA. Thus, despite some of the antigens used in this study having previously been established as immunogenic in salmonids, temperature may be a major influential factor as these studies were carried out in warmer water (>10ºC) (Jones *et al*., 1999a; Cain *et al*., 2002; Swan *et al*., 2008; Valdenegro-Vega *et al*., 2013). The vaccination regime used in the current study followed that used for vaccination of commercially farmed stock fish. This study therefore highlighted the fact that inevitable temperature fluctuation in the field would be detrimental for DIVA vaccination programmes for ISA.

Despite the effectiveness of exogenous marker vaccination being demonstrated in mammals and birds (Walsh *et al*., 2000a; b; James *et al*., 2007; 2008; Fang *et al*., 2008), the same approach has not so far been successful for fish using the same antigens, i.e. TT, in Atlantic salmon, or GFP in carp. The lack of response to TT and GFP was not considered to be associated with antigenic competition as has been reported in teleost fish (Taussig, 1977;

Killie and Jørgensen, 1994; 1995), thus, the lack of recognition may be associated with immunological differences between fish and higher vertebrates. The immunogenicity of molecules in mammals often leads to inconsistent results when administered to fish (Alcorn and Pascho, 2002) and the high titre and high affinity of IgG and IgY noted in horses and chickens, respectively, is lacking in fish, as antibody isotype switching does not occur for circulating antibodies (Kaattari, 1994; Cain *et al*., 2002). Furthermore, the ISA vaccination studies undertaken previously were performed in pre-smolt Atlantic salmon (Mikalsen *et al*., 2005; Lauscher *et al*., 2011; Wolf *et al*., 2013). Therefore, the possible immunosuppressive effects during smoltification, i.e. reduced total immunoglobulin levels (Melingen *et al*., 1995b) should perhaps also be noted with regards to ISA vaccination strategies, especially where sensitive and specific sero-diagnostics is vital. These immunological differences between higher vertebrates and teleosts must be taken into consideration for DIVA vaccination and diagnostics. The difficulties in obtaining protective responses and high antibody titres with inactivated ISA vaccines have been previously demonstrated (Lauscher *et al*., 2011). It appears to be very much dose-dependent, and culturing of high yields of virus in ISAV-susceptible cell lines was also found to be very challenging during this thesis (Chapter 2). This is not to say DIVA vaccination is unfeasible in all teleost fish, as the antibody titres detected from carp to an inactivated KHV vaccine were very encouraging 6 wpv following the successful optimisation of a whole-KHV ELISA (Fig. 3.12). Subsequently, the majority of the project focused on approaches for establishing a DIVA strategy, and its potential application, for *Cyprinus carpio* against KHVD.

Despite the high antibody titres obtained to the inactivated KHV vaccine, anti-KHV antibodies induced after infection have not been not reliably detected until 3-6 weeks post infection (Ronen *et al*., 2003; Adkison *et al*., 2005; Perelberg *et al*., 2008; Matras *et al*., 2012), which leaves a broad window for misdiagnosing fish as uninfected/exposed, *e.g.*

during a sero-surveillance programme. An experimental challenge was therefore designed (Chapter 4) to induce an acute KHVD propagation using a virulent isolate to investigate KHV pathogenesis and determine the most sensitive diagnostic approach to detect KHV directly in fish immediately post infection. Various conclusions have been drawn regarding the portal of entry of KHV (Gilad *et al*., 2004; Pikarsky *et al*., 2004; Miyazaki *et al*., 2008; Costes *et al*., 2009; El-Din, 2011; Raj *et al*., 2011; Fournier and Vanderplasschen, 2011; Fournier *et al*., 2012; Lee *et al*. 2012; Adamek *et al*., 2013), and strong evidence has recently demonstrated the skin to be an important tissue during the early infection stages, and a possible point of entry (Costes *et al*., 2009; Fournier and Vanderplasschen, 2011; Fournier *et al*., 2012; Adamek *et al*., 2013). However, the findings in the current study by ISH and real-time qPCR indicated that the gills and gut may still represent points of virus entry (Fig.4.7 & Fig. 4.12). Variations in experimental design and analytical approaches often lead to contrasting results regarding the portal of entry of pathogens, which has also been observed with other intensively studied notifiable aquatic viruses such as the rhabdoviruses IHNV and VHSV (Yamamoto and Clermont, 1990; Yamamoto *et al*., 1990; Harmache *et al*., 2006; Montero *et al*., 2011). Indeed, the initial infection site of an important human pathogen, poliovirus, was still unconfirmed for 50 years following contrasting proposals for the initial infection site (Bergelson, 2003). Nonetheless, such studies on viral infection and pathogenesis provide an amalgamation of important information for developing effective diagnostic approaches critical for surveillance programmes.

The results obtained from studies on VHSV pathogenesis have contributed markedly to optimising diagnostic methodology for its detection (Cornwell *et al*., 2013). This is particularly important for different pathogens that have different courses of infection as non-lethal sampling methods for VHSV diagnostics using gill and skin biopsies were shown to be less sensitive compared to pooled internal organ samples (Cornwell *et al*., 2013). This is in

contrast to KHV diagnostics where use of pooled internal organs can be detrimental to detection of the virus, resulting in false negative reporting for some fish (Bergmann *et al*., 2010a), thus the same diagnostic approaches applied for VHSV could not be used for KHV. It was clear from the study carried out in Chapter 4 that pooling of internal tissues during an early stage of infection would be particularly unfavourable for KHV detection. An apparent eclipse period of infection, *i.e.* < 5 dpi, noted in this study corroborates previous findings (Costes *et al*., 2009; Fournier *et al*., 2012) characterised by low and undetectable viral loads within internal organs. This resulted in many false negative results using molecular methods, but highlighted the most sensitive PCR assays and suitable sampling tissue, the gill (Table 4.4; Fig. 4.20). Although the skin was not deemed to be a tissue of KHV entry based on results of the current study, the significance of this tissue in KHV pathogenesis highlighted in previous studies (Costes *et al*., 2009; Fournier *et al*., 2012; Adamek *et al*., 2013) and in the current study, perhaps makes it a useful biopsy for early KHV detection. As suggested by Adamek *et al*. (2011), skin biopsies could be obtained non-lethally, i.e. by fin clipping. However, such invasive approaches are undesirable for high value koi, and damage to the skin may actually facilitate an enhanced KHV infection following any encounter with the virus as demonstrated previously (Raj *et al*., 2011). Non-lethal skin mucus swabs proved to be the most effective method to detect KHV (Table 4.5; Fig. 4.23) as the virus appears to be shed via the skin at very early stages of infection, similar to other alloherpesviruses such as CCV (Kancharla and Hanson, 1996). Such a sampling approach could complement blood sampling for DIVA sero-surveillance strategies enabling both a direct and indirect approach to detecting KHV infected carp.

Although numerous *in situ* diagnostic methods were assessed in the study including IHC, TEM, IFAT and ISH, only DNA detection by ISH proved to be sensitive and reliable during early KHV infection stages. Information regarding the expression kinetics of various

KHV proteins could be very useful for devising DIVA diagnostic tests with specific viral structural proteins. DIVA approaches for inactivated AIV vaccines have recently demonstrated the effective application of structural proteins that are expressed in abundance in infected cells inducing strong antibody responses, but not in uninfected vaccinated birds (Lambrecht *et al*., 2007; Kim *et al*., 2010; Hemmatzadeh *et al*., 2013). Attempts were therefore made to analyse the expression of proteins on fixed tissues from experimentally challenged carp using IHC and IFAT. However, this was often inconclusive using MAbs against a tegument protein (ORF62) (Aoki *et al*., 2009; 2011), a lipoprotein with a transmembrane domain (ORF68) (Aoki *et al*., 2011; Ilouze *et al*., 2012a; b) and a capsid associated-protein (ORF84; Chapter 6). The majority of signals obtained were over-abundant and did not correspond with localisation of KHV DNA by ISH (Fig. 4.18). An exception was the MAb of the tegument protein, which produced focal signals in liver sections, but further validation is required to confirm these were specific (Fig. 4.16-4.18). Furthermore, no antibody responses were detected in any fish from this trial (during the first 10 dpi) except for a surviving fish, which had a high specific antibody titre of 1/10,000, but a neutralising antibody titre of only 1/45 (Sven Bergmann pers. comm.).

Subsequent studies therefore investigated differential expression kinetics of KHV proteins *in vitro* using MAbs (Chapter 5) and the application of recombinant proteins coated onto ELISA plates for improved sensitivity and specificity of KHV serology (Chapter 6). Ultimately the four approaches from the various studies of this thesis could complement each other to (1) allow detection of antibodies to exogenous 'positive' markers (Chapter 3); (2) enable direct virus detection by non-lethal sampling at early stages post infection with a highly sensitive PCR-based assay (Chapter 4); (3) provide further information of KHV antigens during replication (Chapter 5) and (4) enable detection of specific KHV antibodies

at low serum dilutions with the possibility of differentiating between infected and vaccinated carp (Chapter 6).

The development of two novel immunofluorescence (IF) techniques in Chapter 5 facilitated antigen expression studies *in vitro*. These techniques enabled comparisons of the relative quantification of KHV antigen expressed in infected cells. This was determined through antibody-antigen complexing of MAbs to their epitopes from various virus structural proteins during a time course of KHV infection in cultured cells. Two MAbs from a panel of 10 were screened using both a microtitre plate IF procedure and confocal microscopy followed by image analysis. These MAbs were of particular interest as they indicated significantly different expression kinetics for the antigens they recognised (5.7 & 5.8) and targeted two different viral structural proteins (Table 5.2). The same trends in antigen expression were evident with both approaches (Fig. 5.19) whereby much greater capsid antigen (ORF84) abundance was detected in infected cells compared to envelope glycoprotein (ORF56, Gotesman *et al*., 2013). The findings from these assays may not only have provided useful information for the purpose of the current study, but also suggest that the application of capsid antigen for IF diagnostic testing for KHV may also be useful. The OIE (2012) accepts confirmation of KHV infection in the absence of mortalities based on ISH, PCR and IFAT, but there is no definition of which MAbs should be used for IFAT. Often MAbs are screened by ELISA to determine which are the most suitable for diagnostics, but from the current study it appears that the sensitivity of antibody-based assays can differ depending on the MAb applied for that specific assay. Despite the capsid MAb (20F10) producing stronger signals to fixed infected cells, weaker signals were obtained compared to the glycoprotein MAb (10A9) after screening on whole KHV ELISA (Fig. 5.3). Perhaps alternative MAbs, such as the capsid MAb, may improve the sensitivity of KHV titration procedures in cell cultures when CPE is lacking, similar to that undertaken for ISAV using IF

(Falk *et al*., 1998). IFAT carried out with capsid MAbs may also improve KHV detection sensitivity in isolated leukocytes (Bergmann *et al*., 2009a) as infected cells harbour higher levels of capsid antigen compared to other viral antigens according to the results from this study.

The antigen kinetics were not investigated for the other MAbs in Chapter 5 due to time and consumables constraints, which also limited the number of replicates used in the experiments. Further investigations using these techniques are however planned for MAb 10D10 and MAb 7C6. These MAbs recognise a tegument protein encoded by ORF62 and lipoprotein of ORF68 (Aoki *et al*., 2009; 2011; Ilouze *et al*., 2012b). The MAb recognising ORF62 exhibited positive signals in the liver of experimentally infected carp after screening by IHC and IFAT in Chapter 4 (Fig. 4.16 - 4.18), and the MAb recognising ORF68 exhibited both focal nuclear and cytoplasmic staining in Chapter 5 (Fig. 5.20). Both of these antigens are known to be antigenic (Aoki *et al*., 2009; 2011) and were utilised for developing sensitive and specific recombinant ELISAs in Chapter 6. Initially, however, koi serum taken from field cases of a surveillance programme in Singapore (Agri-Food and Veterinary Authority of Singapore) were screened using the KHV whole virus ELISA developed in Chapter 3. The necessity for serological diagnostics was accentuated from these results where 45% of koi were seropositive at a serum dilution of 1/200 (Fig. 6.1), which would otherwise be misdiagnosed as sero-negative because of high cut-off dilutions utilised in published ELISA protocols (Adkison *et al*., 2005; St-Hilaire *et al*., 2009). As many as 69% of the cases that were PCR negative were positive for anti-KHV antibodies at low serum dilutions. There were also seropositive 'negative control' fish at a 1/400 dilution, however, emphasising the need for a more KHV-specific ELISA test. Such a test would be capable of improving the sensitivity of KHV serology without false screening of fish as a result cross reactive antibodies with carp pox virus antigens, which has been reported in a number of studies

(Ronen *et al*., 2003; Adkison *et al*., 2005). Such cross-reactions would be detrimental for DIVA strategies.

The recombinant protein ELISAs developed in Chapter 6 performed well using sera diluted at a 1/200 dilution, with 12/13 of the screened fish being reported as positive for rORF62 and 8/13 positive for rORF68 that had otherwise tested negative for PCR. Furthermore, the recombinant protein ELISAs enabled differentiation between infected and vaccinated carp (Fig. 6.7), using the sera from carp vaccinated with an inactivated vaccine in Chapter 3. Importantly, many infected/exposed fish recognised rORF62 (Fig. 6.6) as did fish that were vaccinated with an attenuated vaccine (Fig. 6.7). Therefore a DIVA strategy appears feasible for KHV, using both of these recombinant proteins in ELISA, although at this stage the assay needs further optimisation as a 1/800 dilution was required to indicate the differential antibody responses above the cutoff (Fig. 6.9). Moreover, a particularly strong responder was positive for both proteins, which may limit the application of this assay to the population level. Previously, the majority of marker vaccines that have been developed for herpesviruses, e.g. PrV and BoHV-1, have been based on envelope glycoprotein deletions (Van Oirschot *et al*., 1986; 1988; 1990; 1991; 1996; Kaashoek *et al*., 1994; 1995; 1996; 1998; Van Oirschot, 1999) and some DIVA approaches have applied companion serology tests for subunit vaccines (Van Drunen Little-van den Hurk *et al*., 1994; 1997; Van Drunen Little-van den Hurk, 2006). However, the application of internal structural proteins of herpesviruses may also be an effective approach for DIVA strategies, similar to those applied for AIV, as noted in the current study. Van Drunen Little-van den Hurk and Babiuk, (1986) observed that polypeptides of BoHV-1 confined to the internal surface of virions and infected cells are only recognised by the immune system following repeated exposure and destruction of infected cells after which, an antibody response to that antigen will be mounted. Based on

this model, an inactivated vaccine cannot infect cells and thus only after exposure to infection should that antigen be recognised, making a DIVA approach possible.

The rORF62 protein produced many breakdown products following dialysis out of urea detergent (Fig. 6.4 & 6.5), but was still detected by the majority of naturally infected and exposed koi that had produced strong and moderate antibody responses to the virus (Fig. 6.6). Therefore this cannot be considered a reason for lack of detection by carp with an inactivated vaccine. Instead the characteristics of the protein encoded by ORF62 in KHV infected fish needs to be considered. The protein encoded by ORF62 is a putative tegument protein with possible homology to the major tegument protein, UL36, of HSV-1 (Michel *et al*., 2010b; Van Beurden *et al*., 2011). The UL36 protein, like that encoded by ORF62 in KHV, is a cysteine protease and during infection of human cells, it is recruited to the capsid and retained following entry into the cell and migration to the nuclear pore via microfilaments (Newcomb and Brown, 2010). The protein is thought to possibly undergo autocleavage in order to release viral DNA into the nucleus by causing conformational changes of the capsid (Jovasevic *et al*., 2008; Pasdeloup *et al*., 2009). The products of autocleavage would only be exposed to fish following cell lysis during viral replication, thus would not be detected by uninfected fish vaccinated with inactivated vaccines. However, the application of such a DIVA system would require highly purified virions as a vaccine strain and the recombinant protein ELISA test to be highly sensitive and specific. At this stage, although promising, the rORF62 and rORF68 recombinant ELISAs do not offer such reliability, which may be attributed to the expression system (*E. coli*) used for their production.

The yeast *Pichia pastoris* has been utilised for the production of antigens for recombinant protein ELISAs for mammalian herpesviruses (Ao *et al*., 2003) as proteins can be expressed with correct folding, disulphide bond formation and post translational modifications such as glycosylation (Macauley-Patrick *et al*., 2005). Other studies developing recombinant ELISAs

for VHSV used *P. pastoris* for generating highly specific fragments of the major antigenic 'G' protein of the virus (Encinas *et al*., 2011a; b). Alternatively, a baculovirus expression system has been used in a number of studies for sensitive detection of notifiable mammalian viral diseases including herpesviruses (Hulst *et al*., 1993; Kimman *et al*., 1996; Oviedo *et al*., 1997; Gut *et al*., 1999; Barderas *et al*., 2000; Chung *et al*., 2002; Pérez-Filgueira *et al*., 2006; Gómez-Sebastián *et al*., 2008) and may prove useful for fish serology.

The advantages of expressing recombinant antigens of interest within baculoviruses in infected insect (e.g. Lepidoptera) larvae, such as *Trichoplusia ni*, is that it enables improved folding and processing of proteins, even over the yeast expression (*P. pastoris*) system. Furthermore, the antigenic, immunogenic and biological characteristics of the native protein have been shown to be reproduced in this expression system for other herpesviruses (Gómez-Sebastián *et al*., 2008; Thomas *et al*., 2009). Thus expression of KHV rORF62 and rORF68 through such a system might reveal more epitopes, associated with the native viral proteins that are conformationally altered through *E. coli*, thus possibly improving the sensitivity of the assay. Differences were noted between recognition of non-structural proteins of FMDV expressed through a baculovirus system compared to recombinant proteins obtained through *E.coli*, expression, which ultimately demonstrated that an alternative antigen was more effective for detecting infected as opposed to vaccinated pigs (Chung *et al*., 2002). As very high yields of protein can be obtained from a single insect larva within 3 days (Pérez-Filgueira *et al*., 2006; Gómez-Sebastián *et al*., 2008) diagnostic test development is also more cost-effective. Expensive tissue culture facilities and high biosecurity measures associated with culturing high profile pathogens is also eliminated from the production line when using baculovirus-insect larvae expression systems (Barderas *et al*., 2000). This makes such diagnostic testing for important pathogens like KHV more broadly available. Recombinant

production systems like this also provide the basis for developing effective and pure subunit vaccines that are useful for DIVA strategies (Pérez-Filgueira *et al*., 2007).

However, alternative internal structural proteins of KHV could perhaps enable a more reliable DIVA approach. The antigen recognised by MAb 20F10 was identified as an uncharacterised KHV protein encoded by ORF84 (Chapter 6; Fig. 6.12). Earlier Western blot analysis had revealed a dominant ~100kDa purified KHV band recognised by this MAb (Chapter 5; Fig. 5.4), which was subsequently found to be antigenic, as it was recognised by >25% of exposed/infected and vaccinated carp (Table 6.7; Fig. 6.11). Interestingly, so far none of the inactivated vaccinated fish were shown to recognise this protein by Western blot, only attenuated vaccinated fish. A 97kDa band of KHV has previously been reported as an immunodominant band from Western blot analysis of experimentally KHV infected carp (Adkison *et al*., 2005), which may constitute the same protein. This protein is expressed to high levels in infected cells (Chapter 5; Fig. 5.7; 5.8; 5.19) and perhaps could provide an effective DIVA approach for an inactivated vaccine similar to the ME2 protein of AIV (Lambrecht *et al*., 2007; Kim *et al*., 2010; Hemmatzadeh *et al*., 2013). The transcript expression of this capsid protein mirrors the expression of the major capsid protein encoded by ORF92 (Ilouze *et al*., 2012a; b). The antibody responses to KHV infection are, however, fundamentally the most important factor to consider for feasible DIVA strategies.

The distinct differences in properties associated with the flexible multimeric IgM of fish (Kaattari *et al*., 1998; 1999) may enable more effective complement fixing activity, as discussed in Chapter 1. As carp possess a high level of natural antibodies (Sinyakov *et al*., 2002; 2006; Sinyakov and Avtalion, 2009), and these are considered an important aspect of fish immunity, these may be very active during KHV infection at an early stage. The importance of natural antibody induced virus neutralisation via complement fixation has been described previously (Bernet *et al*., 2003; Hook *et al*., 2006). As reported by Rakus *et al*.

(2012) the up-regulation of complement associated transcripts and complement activity from sera after only 3 dpi, perhaps implies that both alternative and classical complement pathways are active, resulting in membrane attack complex (MAC) associated lysis of infected cells. Furthermore, proteins involved in the complement host defence pathway (C3-S, C4-1 and C42) have recently been found to be associated with KHV antigens from infected carp tissues after affinity purification (Gotesman *et al*., 2013). The rapid anti-viral activity of natural antibodies immediately post infection may explain the reduced antibody levels reported in experimentally KHV infected carp after 7 dpi (Ronen *et al*., 2003). This may result in the release of abundant capsid antigen, that is expressed to high abundance in infected cells (Chapter 5; Fig. 5.7; 5.8; 5.19) and which is subsequently recognised by MAbs and infected fish sera (Chapter 6; Fig. 5.4; 6.5; 6.11). However, these antibodies were not detectable, even after a highly virulent challenge (Chapter 4) until late stages of infection. Even after 10 wpi the antibody titre of infected fish may be high (1/10,000; Chapter 4), but the level of neutralising antibodies low (1/45; Chapter 4) emphasising the high level of non-neutralising antibodies. Non-neutralising antibodies are thought to represent the majority of antibodies produced to a number of fish viruses and are longer lasting than neutralising antibodies, e.g. SVCV and VHSV (Olesen *et al*., 1991; Dixon *et al*., 1994; Encinas *et al*., 2011a; b). These antibodies may recognise internal viral proteins, e.g. capsid-associated antigens, or tegument-associated antigens (Chapter 6). Therefore, as these appear to be detected predominantly by infected fish, but not vaccinated fish with an inactivated vaccine (Chapter 6; Fig. 6.7 & 6.8) they may constitute useful antigens for serological diagnostics for KHV and DIVA strategies. As capsid antigens constitute TI antigens (Flint *et al*., 2009), they may alleviate issues regarding temperature induced T cell suppression that is characteristic of fish immune responses at low temperatures (Bly and Clem, 1992; Secombes *et al*., 1996). Applying a more immunogenic exogenous marker antigen, than those used in Chapter 3 may enable the

induction of detectable antibodies in vaccinated fish. This could perhaps be applied with plant based protein antigens developed using the synthetic biomarker approach described by Root-Bernstein (2005), which would then possibly allow safe and licensable marker vaccination strategies for food carp as well as ornamental koi. The application of semi-nested PCR or real-time PCR (Gilad *et al*., 2004; Bergmann *et al*., 2010a) on mucus samples enables sensitive detection of KHV DNA in the absence of antibodies during early stages of KHV infection (Chapter 4). Furthermore, Eide *et al*. (2011a) described approaches for utilising the blood leukocytes of latently infected carp for the detection of KHV DNA. Thus, fish with marginal antibody titres on recombinant based ELISA could still be tested non-lethally for viral DNA in mucus. Moreover, blood leukocytes could enable detection of latent stage infection where antibody levels have fallen below the level of detection of the ELISA. Such screening could perhaps be carried out with highly sensitive PCR assays (conventional or semi-nested) detecting the glycoprotein gene of ORF56, (Bergmann *et al*, 2010b) as well as real-time PCR (Gilad *et al*., 2004) (Chapter 4).

7.2 Concluding remarks

In conclusion, DIVA strategies may be feasible for the control of KHVD in carp, but not ISA in Atlantic salmon. Genetic DIVA approaches may be applied for ISAV (Kibenge *et al*., 2009b), although this may not be sensitive enough to detect carrier fish. Utilising non-neutralising antigens of KHV and thus detection of non-neutralising antibodies may enhance the sensitivity of serological tests for KHV, but perhaps not the specificity as carp harbour high levels of natural antibodies. Application of a DNA vaccine that expresses protective envelope glycoproteins, which has been developed (Aoki and Hirono, 2009; 2011), could enable a DIVA approach by applying internal virus antigens of ORF62 or ORF84 to a companion serological diagnostic test. While natural antibodies appear to be important in

carp immunity, they will continue to present a challenge to reliable KHV serology, and thus DIVA strategies against KHVD.

References

Abaitua F., Hollinshead M., Bolstad M., Crump C.M. & O'Hare P. (2012) A nuclear localization signal in the herpesvirus protein VP1-2 is essential for infection via capsid routing to the nuclear pore. *Journal of Virology* **86**(17): 8998-9014.

Abbas A.K., Litchman A.H. & Pober J.S. (2000) Cellular and molecular immunology. USA, W. B. Saunders Company.

Adamek M., Brogden G., Propsting M., Matras M., Rakus K.Ł., Irnazarow I. & Steinhagen D. (2011) Revaluation of the importance of the common carp (*Cyprinus carpio*) skin/fins in the *Cyprinid herpesvirus 3* infection and prospects for diagnostics. European Association of Fish Pathologists (EAFP) 15th International conference of diseases of fish and shellfish; September 12-16; Split, Croatia: Book of Abstracts, 2011. p. 37.

Adamek M., Rakus K.Ł., Chyb J., Brogden G., Huebner A., Irnazarow I. & Steinhagen D. (2012) Interferon type I responses to virus infections in carp cells: *In vitro* studies on *Cyprinid herpesvirus 3* and *Rhabdovirus carpio* infections. *Fish and Shellfish Immunology* **33**: 482-493.

Adamek M., Syakuri H., Harris S., Rakus K.Ł., Brogden G., Matras M., Irnazarow I. & Steinhagen D. (2013) Cyprinid herpesvirus 3 infection disrupts the skin barrier of common carp (*Cyprinus carpio* L.). *Veterinary Microbiology* **162**: 456-470.

Adams A., Aoki T., Berthe F.C.J., Grisez L. & Karunasagar I. (2008) Recent technological advancements on aquatic animal health and their contributions toward reducing disease risks – a review, pp. 71-88. *In* Bondad-Reantaso M. G., Mohan C. V., Crumlish M. and Subasinghe R. P. (Eds.). Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Adams A. & Thompson K.D. (2006) Biotechnology offers revolution to fish health management. *TRENDS in Biotechnology* **24**(5): 201-205.

Adams A. & Thompson K.D. (2008) Recent applications of biotechnology to novel diagnostics for aquatic animals. *Scientific and Technical Review of the Office International des Epizooties* **27**(1): 197-209.

Adkison M.A., Gilad O. & Hedrick R.P. (2005) An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi *Cyprinus carpio*. *Fish Pathology* **40**(2): 53-62.

Aebischer A., Müller M. & Hofmann M.A. (2013) Two newly developed E^{RNS}-based ELISAs allow the differentiation of Classical swine fever virus-infected from marker-vaccinated animals and the discrimination of pestivirus antibodies. *Veterinary Microbiology* **161**(3-4): 274-285.

Afolabi A.S. & Thottappilly G. (2008) Comparative studies on alkaline phosphatase (ALP), alkaline phosphatase amplification (AMP), horse radish peroxidase (HRP), penicillinase (PNC) and avidin-biotin penicillinase (PNC) amplification ELISA in detection of maize streak virus (MSV) in maize plants and Cicadulina mbila (leafhoppers) insect vector. *Science Research Essays* **3**(11): 524-530.

Ahlqvist J., Fotheringham J., Akhyani N., Yao K., Fogdell-Hahn A. & Jacobson S. (2005) Differential tropism of human herpesvirus 6 (HHV-6) variants and induction of latency by HHV-6A in oligodendrocytes. *Journal of Neurovirology* **11**: 384-394.

Albina E., Kwiatek O., Minet C., Lancelot R., Serven de Almeida R. & Libeau G. (2013) Peste des petits ruminants, the next eradicated animal disease? *Veterinary Microbiology* **165**(1-2): 38-44.

Alcorn S.W. and Pascho R.J. (2000) Single-dilution enzyme-linked immunosorbent assay for quantification of antigen-specific salmonid antibody. *Journal of Veterinary Diagnostic Investigation* **12**: 245-252.

Alcorn S.W. and Pascho R. J. (2002) Antibody responses by chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) to various protein antigens. *Fish and Shellfish Immunology* **13**: 327-333.

Allnutt F., Bowers R.M., Rowe C.G., Vakharia V.N., LaPatra S.E. & Dhar A.K. (2007) Antigenicity of infectious pancreatic necrosis virus VP2 subviral particles expressed in yeast. *Vaccine* **25**: 4880-4888.

References

Alonso M., Cano I., Castro D., Perez-Prieto S. & Borrego J. (2004) Development of an *in situ* hybridisation procedure for the detection of sole aquabirnavirus in infected fish cell cultures. *Journal of Virological Methods* **116**: 133-138.

Amend D.F. and Smith L. (1974) Pathophysiology of infectious hematopoietic necrosis virus disease in rainbow trout *Salmo gairdneri*: early changes in blood and aspects of the immune response after injection of IHN virus. *Journal of the Fisheries Research Board of Canada* **31**: 1371-1378.

Anderson D.P. (1992) Immunostimulants, adjuvants and vaccine carriers in fish: Applications to aquaculture. *Annual Review of Fish Diseases* **2**: 281-307.

Anderson E.D., Mourich D.V., Fahrenkrug S.C., Shepherd J. & Leong J.A. (1996a) Genetic immunisation of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. *Molecular Marine Biology and Biotechnology* **5**(2): 114-122.

Anderson E.D., Mourich D.V. & Leong J.A. (1996b) Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. *Molecular Marine Biology and Biotechnology* **5**: 105-113.

Anderson J. & McKay J.A. (1994) The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implications to rinderpest control programmes. *Epidemiology and Infection* **112**(1): 225-231.

Andrew M.E., Morrissy C.J., Lenghaus C., Oke P.G., Sproat K.W., Hodgson A.L.M., Johnson M.A. & Coupar B.E.H. (2000) Protection of pigs against classical swine fever with DNA-delivered gp55. *Vaccine* **18**(18): 1932-1938.

Andrew S.M. & Jasani B. (1987) An improved method for the inhibition of endogenous peroxidase non-deleterious to lymphocyte surface markers. Application to immunoperoxidase studies on eosinophil-rich tissue preparations. *The Histochemical Journal* **19**: 426-430.

Antychowicz J., Reichert M., Matras M., Bergmann S.M. & Haenen O. (2005) Epidemiology, pathogenicity and molecular biology of Koi herpesvirus isolated in Poland. *Bulletin of the Veterinary Institute in Pulawy* **49**: 367-373.

Anziliero D., Santos C.M.B., Brum M.C.S., Weiblen R., Chowdhury S.I. & Flores E.F. (2011) A recombinant bovine herpesvirus 5 defective in thymidine kinase and glycoprotein E is immunogenic for calves and confers protection upon homologous challenge and BoHV-1 challenge. *Veterinary Microbiology* **154**(1-2): 14-22.

Ao J., Wang J., Chen X., Wang X. & Long Q. (2003) Expression of pseudorabies virus gE epitopes in *Pichia pastoris* and its utilization in an indirect PRV gE-ELISA. *Journal of Virological Methods* **114**: 145-150.

Aoki T. & Hirono I. (2009) Patent application no. 20090060951: DNA vaccine for Koi herpesvirus (KHV) disease. http://www.faqs.org/patents/app/20090060951 (accessed 23/04/12).

Aoki T. & Hirono I. (2011) Patent application no. US8052977 B2. *DNA vaccine for Koi herpes virus (KHV) disease*. http://www.google.co.uk/patents/US8052977 (accessed 01/04/13).

Aoki T., Hirono I., Kurokawa K., Fukuda H., Nahary R., Eldar A., Davison A.J., Waltzek T.B., Bercovier H. & Hedrick R.P. (2007) Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. *Journal of Virology* **81**: 5058-5065.

Aoki T., Takano T., Unajak S., Kondo H., Hirono I., Hikima J. & Jung T.S. (2009) Major antigenic region screening of koi herpesvirus from genome library. *Presented at* European Association of Fish Pathologists (EAFP) 14th International conference of diseases of fish and shellfish; September 14-19; Prague, Czech Republic, 2009.

Aoki T., Takano T., Unajak S., Takagi M., Kim Y.R., Park S.B., Kondo H., Hirono I., Saito-Taki T., Hikima J.-I. & Jung T.S. (2011) Generation of monoclonal antibodies specific for ORF68 of koi herpesvirus. *Comparative Immunology, Microbiology and Infectious Diseases* **34**: 209-216.

Arason G.J. (1996) Lectins as defence molecules in vertebrates and invertebrates. *Fish and Shellfish Immunology* **6**: 277-289.

References

Ariav R., Tinman S., Paperna I. & Bejerano I. (1999) First report of newly emerging viral disease of *Cyprinus carpio* species in Israel. *In* Diseases of Fish and Shellfish, 9th International Conference of the European Association of Fish Pathologists, Rodos Palace Congress Center, Rhodes, Greece, 19th to 24th September 1999.

Arkoosh M.R. & Kaattari S.L. (1991) Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*). I. An immunochemical and cellular analysis of the B cell response. *Developmental and Comparative Immunology* **15**: 279-293.

Asche F., Hansen H., Tveteras R. & Tveteras S. (2010) The salmon disease crisis in Chile. *Marine Resource Economics* **24**(4): 405-411.

Aspehaug V., Mikalsen A.B., Snow M., Biering E. & Villoing S. (2005) Characterization of the infectious salmon anemia virus fusion protein. *Journal of Virology* **79**: 12544-12553.

Avellaneda G., Mundt E., Lee C.-W., Jadhao S. and Suarez D.L. (2010) Differentiation of infected and vaccinated animals (DIVA) using the NS1 protein of Avian influenza. *Avian Diseases* **54**: 278-286.

Avtalion R.R. & Milgrom L. (1976) I. Influence of hapten density on the immunological and serological properties of penicilloyl-carrier conjugates. *Immunology* **31**: 589-594.

Azila A., Way K., Wood G., Ainol Y.M.Y., Kamisa A., Norfauzana M.A., Jafrizah A.R. & Sabri M.Y. (2012) Detection of Koi herpesvirus (KHV) in *Cyprinus carpio* (Koi) stocks using enzyme-linked immunosorbent assay (ELISA). *Pertanika Journal of Tropical Agricultural science* **35**(1): 21-25.

Babiuk L. A. (1999) Broadening the approaches to developing more effective vaccines. *Vaccine* **17**: 1587-1595.

Barderas M., Wigdorovitz A., Merelo F., Beitia F., Alonso C., Borca M. & Escribano J.M. (2000) Serodiagnosis of African swine fever using the recombinant protein p30 expressed in insect larvae. *Journal of Virological Methods* **89**: 129-136.

Barros S.C., Cruz B.T., Luis T.M., Ramos F., Fagulha T., Duarte M., Henriques M. & Fevereiro M. (2009). A DIVA system based on the detection of antibodies to non-structural protein 3 (NS3) of bluetongue virus. *Veterinary Microbiology* **137**: 252-259.

Beer M., Reimann I., Hoffmann B. & Depner K. (2007) Novel marker vaccines against classical swine fever. *Vaccine* **25**: 5665-5670.

Benjamin D.C., Berzofsky J.A., East L.J., Gurd F.R.N., Hannum C., Leach S.J., Margoliash E., Michael J.G., Miller A., Prager E.M., Reichlin M., Sercarz E.E., Smith-Gill S.J., Todd P.E. & Wilson A.C. (1984) The antigenic structure of proteins: a reappraisal. *Annual Review of Immunology* **2**: 67-101.

BenMohamed L., Bertrand G., McNamara C.D., Gras-Masse H., Hammer J., Wechsler S.L. & Nesburn A.B. (2003) Identification of novel immunodominant CD4 Th1-type T-cell peptide epitopes from herpes simplex virus glycoprotein D that confer protective immunity. *Journal of Virology* **77**: 9463-9473.

Ben-Porat T., DeMarchi J., Pendrys J., Veach R. & Kaplan A.S. (1986) Proteins specified by the short unique region of the genome of pseudorabies virus play a role in the release of virions from certain cells. *Journal of Virology* **57**: 191-196.

Ben-Porat T. & Veach R.A. (1980) Origin of replication of the DNA of a herpesvirus (pseudorabies). *Proceedings of the National Academy of Sciences* **77**: 172-175.

Bercovier H., Fishman Y., Nahary R., Sinai S., Zlotkin A., Eyngor M., Gilad O., Eldar A. & Hedrick R.P. (2005). Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. *BMC Microbiology* **5**: 1–9.

Bergelson J.M. (2003) Virus interactions with mucosal surfaces: alternative receptors, alternative pathways. *Current Opinion in Microbiology* **6**: 386-391.

Bergmann I.E., de Mello P.A., Neitzert E., Beck E. & Gomes I. (1993) Diagnosis of persistent aphthovirus infection and its differentiation from vaccination response in cattle by use of enzyme-linked

immunoelectrotransfer blot analysis with bioengineered non-structural viral antigens. *American Journal of Veterinary Research* **54**(6): 825-831.

Bergmann S.M. & Kempter J. (2011) Detection of Koi herpesvirus (KHV) after re-activation in persistently infected common carp (*Cyprinus carpio* L.) using non-lethal sampling methods. *Bulletin of the European Association of Fish Pathologists* **31**(3): 92-100.

Bergmann S.M., Kempter J. & Fichtner D. (2009b) How host specific is infection with koi herpesvirus (KHV) for real? The *Israeli Journal of Aquaculture – Bamidgeh* **61**(3): 270.

Bergmann S.M., Kempter J., Sadowski J. & Fichtner D. (2006) First detection, confirmation and isolation of Koi herpesvirus (KHV) in cultured common carp (*Cyprinus carpio* L.) in Poland. *Bulletin of the European Association of Fish Pathologists* **26**(2): 97-104.

Bergmann S.M., Lutze P., Schütze H., Fischer U., Dauber M., Fichtner D. & Kempter J. (2010c) Goldfish (*Carassius auratus*) is a susceptible species for koi herpesvirus (KHV) but not for KHV disease (KHVD). *Bulletin of the European Association of Fish Pathologists* **29**(5): 145-152.

Bergmann S.M., Riechardt M., Fichtner D., Lee P. & Kempter J. (2010a) Investigation on the diagnostic sensitivity of molecular tools used for detection of Koi herpesvirus. *Journal of Virological Methods* **163**(2): 229-233.

Bergmann S.M., Sadowski J., Kielpinski M., Bartlomiejczyk M., Fichtner D., Riebe R., Lenk M. and Kempter J. (2010b) Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *Journal of Fish Diseases* **33**: 267-272.

Bergmann S.M., Schütze H., Fischer U., Fitchner D., Riechardt M., Meyer K., Schrudde D. & Kempter K. (2009a) Detection of koi herpesvirus (KHV) genome in apparently healthy fish. *Bulletin of the European Association of Fish Pathologists* **29**(5): 145-152.

Bergmann S.M., Stumpf P., Schütze H., Fichtner D., Sadowski J. & Kempter J. (2007) Similarities and heterogenicity of koi herpesvirus (KHV) genome detected in ornamental fish without clinical signs. *Aquaculture: Abstracts* **272S1**: S238-S321.

Bernet J., Mullick J., Singh A.K. & Sahu A. (2003) Viral mimicry of the complement system. *Journal of Bioscience* **28**: 249-264.

Biacchesi S., Thoulouze M.-I., Béarzotti M., Yu Y.-X. & Brémont M. (2000) Recovery of NV knockout infectious haematopoietic necrosis virus expressing foreign genes. *Journal of Virology* **74**(23): 11247-11253.

Biering E., Falk K., Hoel E., Thevarajan J., Joerink M., Nylund A., Endresen C. & Krossøy B. (2002) Segment 8 encodes a structural protein of infectious salmon anaemia virus (ISAV); the co-linear transcript from Segment 7 probably encodes a non-structural or minor structural protein. *Diseases of Aquatic Organisms* **49**: 117-122.

Bigarré L., Baud M., Cabon J., Antychowicz J., Bergmann S., Engelsma M., Pozet F., Reichert M. & Castric J. (2009) Differentiation between Cyprinid herpesvirus type-3 lineages using duplex PCR. *Journal of Virological Methods* **158**: 51-57.

Birch-Machin I., Rowan A., Pick J., Mumford J. & Binns M. (1997) Expression of the non-structural protein NS1 of equine influenza A virus: detection of anti-NS1 antibody in post infection equine sera. *Journal of Virological Methods* **65**: 255-263.

Björnsson B.T., Stefansson S.O. & McCormick S.D. (2011) Environmental endocrinology of salmon smoltification. *General and Comparative Endocrinology* **170**: 290-298.

Blome S., Gabriel C., Staubach C., Leifer I., Strebelow G. & Beer M. (2011) Genetic differentiation of infected from vaccinated animals after implementation of an emergency vaccination strategy against classical swine fever in wild boar. *Veterinary Microbiology* **153**: 373-376.

Bly J.E. & Clem L.W. (1992) Temperature and teleost immune functions. *Fish and Shellfish Immunology* **2**: 159-171.

References

Bondad-Reantaso M., Sunarto A., Subasinghe R., Dodet B. & Vicari M. (2007) Managing the koi herpesvirus disease outbreak in Indonesia and the lessons learned. *Developments in Biologicals* **129**: 21-28.

Borsos T., Chapius R.M. & Langore J.J. (1981) Distinction between fixation of C1 and the activation of C by natural IgM anti-hapten antibody: Effect of cell surface hapten density. *Molecular Immunology* **18**: 863-868.

Bosch J.C., Kaashoek M.J., Kroese A.H. & Van Oirschot (1996) An attenuated bovine herpesvirus 1 marker vaccine induces a better protection than two inactivated marker vaccines. *Veterinary Microbiology* **52**: 223-234.

Bosch J.C., Kaashoek M.J. & Van Oirschot J.T. (1997) Inactivated bovine herpesvirus 1 marker vaccines are more efficacious in reducing virus excretion after reactivation than a live marker vaccine. *Vaccine* **15**(10): 1512-1517.

Bosman K.J., Mourits M.C.M., Oude Lansink A.G.J.M. & Saatkamp H.W. (2012) Minimisation of the impact of Aujeszky's Disease outbreaks in the Netherlands: A conceptual framework. *Transboundary and Emerging Diseases*: 1-12.

Bouchard D.A., Brockway K., Giray C., Keleher W. and Merrill P.L. (2001) First report of infectious salmon anaemia (ISA) in the United States. *Bulletin of the European Association of Fish Pathologists* **21**: 86–88.

Bouma A. (2005) Determination of the effectiveness of Pseudorabies marker vaccines in experiments and field trials. *Biologicals* **33**: 241-245.

Bouma A., de Smit A. J., de Kluijver E. P., Terpstra C. & Moormann R.J.M. (1999) Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever. *Veterinary Microbiology* **66**(2): 101-114.

Brahmakshatriya V.R., Lupiani B. & Reddy S.M. (2010) Characterisation and evaluation of avian influenza NS1 mutant virus as a potential live and killed DIVA (differentiating between infected and vaccinated animals) vaccine for chickens. *Vaccine* **28**: 2388-2396.

Bretzinger A., Fischer-Scherl T., Oumouna M., Hoffman R. & Truyen U. (1999) Mass mortalities in koi carp, *Cyprinus carpio*, associated with gill and skin disease. *Bulletin of the European Association of Fish Pathologists* **19**(5): 182-185.

Bricknell I.R., Bruno D.W., Cunningham C., Hastings T.S., McVicar A.H., Munro P.D., Raynard R. & Stagg R.M. (1998) Report on the first occurrence of infectious salmon anemia (ISA) in Atlantic salmon in Scotland, United Kingdom. *In* Proceedings of the Third International Symposium on Aquatic Animal Health, Baltimore, MD, 30 August – 3 September, 1998.

Brody N.I. & Siskind G.W. (1972) Studies on antigenic competition II. Evidence for effect at level of antigen 'processing'. *Immunology* **22**: 75-85.

Bromage E.S., Ye J. & Kaattari S.L. (2006) Antibody structural variation in rainbow trout fluids. *Comparative Biochemistry and Physiology* **Part B 143**: 61-69.

Brown L.L., Sperker S.A., Clouthier S., & Thornton J.C. (2000) Development of a vaccine against infectious salmon anaemia virus (ISAV) *Bulletin of the Aquaculture Association of Canada* **100**: 4-7.

Buczkowski H., Parida S., Bailey D., Barrett T. & Banyard A.C. (2012) A novel approach to generating morbillivirus vaccines: Negatively marking the rinderpest vaccine. *Vaccine* **30**: 1927-1935.

Burleson F.G., Chambers T.M. & Wiedbrauk D.L. (1992) Plaque assays. *In* Virology: A laboratory manual. U.S.A., Academic Press, Inc., Chapter 16, pp. 74-84.

Cain K.D., Jones D.R. & Raison R.L. (2002) Antibody-antigen kinetics following immunisation of rainbow trout (*Oncorhynchus mykiss*) with a T-cell dependent antigen. *Developmental and Comparative Immunology* **26**: 181-190.

Campadelli-Fiume G., Arsenakis M., Farabegoli F. & Roizman B. (1988) Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. *Journal of Virology* **62**: 159-167.

References

Campadelli-Fiume G. & Roizman B. (2006) The egress of herpesviruses from cells: the unanswered questions. *Journal of Virology* **80**: 6716-6719.

Cancel-Tirado S.M., Evans R.B. & Yoon K.-J. (2004) Monoclonal antibody analysis of Porcine Reproductive and Respiratory Syndrome Virus epitopes associated with antibody-dependent enhancement and neutralisation of virus infection. *Veterinary Immunology and Immunopathology* **102**: 249-262.

Cann A.J. (2005) Principles of Molecular Virology, Fourth Edition. Elsevier Academic Press, USA.

Capua I., Cattoli G. & Marangon S. (2004) DIVA – A vaccination strategy enabling the detection of field exposure to Avian influenza. *In* Control of infectious animal diseases by vaccination. Eds. Schudel A. and Lombard M. *Developments in Biologicals (Basel)* **119**: 229-233.

Capua I., Terregino C., Cattoli G., Mutinelli G. & Rodriguez J.F. (2003) Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. *Avian Pathology* **32**(1): 47-55.

Cardoso T.C., Novais J.B., Antello T.F., Silva-Frade C., Ferrarezi M.C., Ferrari H.F., Gameiro R. & Flores E.F. (2012) Susceptibility of neuron-like cells derived from bovine Wharton's jelly to bovine herpesvirus type 5 infections. *BMC Veterinary Research* **8**: 242.

Castillo-Olivares J., Wieringa R., Bakonyi T., de Vries A.F., Davis-Poynter N.J. & Rottier P.J.M. (2003) Generation of a candidate live marker vaccine for Equine arteritis virus by deletion of the major virus neutralisation domain. *Journal of Virology* **77**(15): 8470-8480.

Castrucci M.R., Bilsel P. & Kowaoka Y. (1992) Attenuation of influenza A virus by insertion of a foreign epitope into the neuraminidase. *Journal of Virology* **66**(8): 4647-4653.

Caswell-Reno P., Reno P. & Nicholson B.L. (1986) Monoclonal antibodies to Infectious Pacreatic Necrosis Virus: Analysis of viral epitopes and comparison of different isolates. *Journal of General Virology* **67**: 2193-2205.

Cavoy ® KHV vaccine. http://www.cavoy.com/Certification/3000.html (Last accessed 1 April 2013).

Chalfie M., Tu Y., Euskirchen G., Ward W.W. & Prasher D.C. (1994) Green fluorescent protein as a marker for gene expression. *Science* **263**: 802-805.

Cheng L., Chen C.Y., Tsai M.A., Wang P.C., Hsu J.P., Chern J.P., Chern R.S. & Chen S.C. (2011) Koi herpesvirus epizootic in cultured carp and koi, *Cyprinus carpio* L., in Taiwan. *Journal of Fish Diseases* **34**: 547-554.

Choi D.L., Sohn S.G., Bang J.D., Do J.W. & Park M.S. (2004) Ultrastructural identification of a herpes-like virus infection in common carp *Cyprinus carpio* in Korea. *Diseases of Aquatic Organisms* **61**: 165-168.

Choi J.-G., Kim M.-C., Kang H.-M., Kim K.-I., Lee K.-J., Park C.-K., Kwon J.-H., Kim J.-h. & Lee Y.-J. (2013) Protective efficacy of baculovirus-derived influenza virus-like particles bearing H5 HA alone or in combination with M1 in chickens. *Veterinary Microbiology* **162**: 623-630.

Chu W.-H. & Lu C.-P. (2008) *In vivo* fish models for visualising *Aeromonas hydrophila* invasion pathway using GFP as a biomarker. *Aquaculture* **277**: 152-155.

Chung W.-B., Sorensen K. J., Liao P.-C., Yang P.-C. & Jong M.-H. (2002) Differentiation of foot-and-mouth disease virus-infected from vaccinated pigs by enzyme-linked immunosorbent assay using non-structural protein 3AB as the antigen and application to an eradication program. *Journal of Clinical Microbiology* **40**(8): 2843-2848.

Cipriano R. C. (2009) Antibody against infectious salmon anaemia virus among feral Atlantic salmon (*Salmo salar*). *ICES Journal of Marine Science* **66**: 865-870.

Cipriano R.C. & Miller O. (2003) Infectious salmon anemia: the current state of our knowledge. *In* Miller O and Cipriano RC, technical coordinators (2003). International Response to Infectious Salmon Anemia: Prevention, Control, and Eradication: Proceedings of a Symposium, 3–4 September 2002, New Orleans, *LA.Technical*

*Bulletin **1902***. Washington, DC: US Department of Agriculture, Animal and Plant Health Inspection Service, pp. 1-10.

Clarke M. (2009) Henderson Morley announces KHV vaccine results. Practical fish keeping: http://www.practicalfishkeeping.co.uk/content.php?sid=2143 (Accessed 16/04/2012).

Clavijo A., Wright P. & Kitching P. (2004) Developments in diagnostic techniques for differentiating infection from vaccination in foot-and-mouth disease. *The Veterinary Journal* **167**: 9-22.

Clem L.W. & McLean W.E. (1975) Phylogeny of immunoglobulin structure and function. *Immunology* **29**: 791-799.

Clem L.W. & Small P.A. (1970) Phylogeny of immunoglobulin structure and function. V. Valencies and association constants of teleost antibodies to a haptenic determinant. *Journal of Experimental Medicine* **132**: 385-400.

Clouthier S.C., Rector T., Brown N.E. & Anderson E.D. (2002) Genomic organization of infectious salmon anaemia virus. *Journal of General Virology* **83**: 421-428.

Coberley S.S., Condit R.C., Herbst L.H. & Klein P.A. (2002) Identification and expression of immunogenic proteins of a disease-associated marine turtle herpesvirus. *Journal of Virology* **76**: 10553-10558.

Companjen A.R., Florack D.E.A., Slootweg T., Borst J.W. & Rambout J.H.W.M. (2006) Improved uptake of plant-derived LTB-linked proteins in carp gut and induction of specific humoral immune responses upon infeed delivery. *Fish and Shellfish Immunology* **21**: 251-260.

Cone R.A. (2009) Barrier properties of mucus. *Advanced Drug Delivery Reviews* **61**: 75-85.

Cook-Versloot M., Griffiths S., Cusack R., McGeachy S. & Ritchie R. (2004) Identification and characterisation of infectious salmon anaemia virus (ISAV) haemagglutinin gene highly polymorphic region (HPR) type 0 in North America. *Bulletin-European Association of Fish Pathologists* **24**: 203-208.

Corchero J.L., Mar E., Spira T.J., Pellett P.E. & Inoue N. (2001). Comparison of serologic assays for detection of antibodies against human herpesvirus 8. *Clinical and Diagnostic Laboratory Immunology* **8**: 913-921.

Cornwell E.R., Bellmund C.A., Groocock G.H., Wong P.T., Hambury K.L., Getchell R.G. & Bowser P.R. (2013) Fin and gill biopsies are effective nonlethal samples for detection of *Viral hemorrhagic septicaemia virus* genotype IVb. *Journal of Veterinary Diagnostic Investigation* **25(**2): 203-209.

Cossarini-Dunier M., Desvaux F.-X. & Dorson M. (1986) Variability in humoral responses to DNP-KLH of rainbow trout (*Salmo gairdneri*). Comparison of antibody kinetics and immunoglobulins spectrotypes between normal trouts and trouts obtained by gynogenesis or self-fertilisation. *Developmental and Comparative Immunology* **10**: 207-217.

Costa J.Z. d.G. (2005) B cell epitopes in fish nodavirus. Ph.D Doctoral thesis, University of Stirling, Scotland.

Costa J., Adams A., Bron J., Thompson K., Starkey W. & Richards R. (2007) Identification of B-cell epitopes on the betanodavirus capsid protein. *Journal of Fish Diseases* **30**: 419-426.

Costes B., Fournier G., Michel B., Delforge C., Raj V.S., Dewals B., Gillet L., Drion P., Body A., Schynts F., Lieffrig F. & Vanderplasschen A. (2008) Cloning of the Koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*. *Journal of Virology* **82**(10): 4955-4964.

Costes B., Lieffrig F. & Vanderplasschen A. (2012) Patent application EP20080803235: A recombinant koi herpesvirus (KHV) or cyprinid herpesvirus 3 (CyHV-3) and a vaccine for the prevention of a disease caused by KHV/CyHV-3 in *Cyprinus carpio carpio* or *Cyprinus carpio koi*.

Costes B., Raj V.S., Michel B., Fournier G., Thirion M., Gillet L., Mast J., Lieffrig F., Bremont M. & Vanderplasschen A. (2009) The major portal of entry of koi herpesvirus in *Cyprinus carpio* is the skin. *Journal of Virology* **83**(7): 2819-30.

References

Cox J.C. & Coulter A.R. (1997) Adjuvants - a classification and review of their modes of action. *Vaccine* **15**: 248-256.

Crabb B.S. & Studdert M.J. (1990) Comparative studies of the proteins of equine herpesviruses 4 and 1 and asinine herpesvirus 3: antibody response of the natural hosts. *Journal of General Virology* **71**: 2033-2041.

Cross P.C. & Mercer K.L. (1993) Cell. *In* Cell and tissue ultrastructure: A functional perspective, Second edition, W.H. Freeman and Co. Chapter 1, pp. 1-42.

Cunningham C.O., Gregory A., Black J., Simpson I. & Raynard R.S. (2002) A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. *Bulletin of the European Association of Fish Pathologists* **22**: 366-374.

Cunningham C. & Snow M. (2000) Genetic analysis of infectious salmon anaemia virus (ISAV) from Scotland. *Diseases of Aquatic Organisms* **41**: 1-8.

Dale O.B., Ørpetveit I., Lyngstad T.M., Kahns S., Skall H.F., Olesen N.J. & Dannevig B.H. (2009) Outbreak of viral haemorrhagic septicaemia (VHS) in seawater-farmed rainbow trout in Norway caused by VHS virus Genotype III. *Diseases of Aquatic Organisms* **85**: 93-103.

Dannevig B.H., Brudeseth B.E., Gjøen T., Rode M., Weregeland H.I., Evensen Ø. & Press McL. (1997) Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). *Fish and Shellfish Immunology* **7**: 213-226.

Dannevig B.H., Falk K. & Namork E. (1995) Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *Journal of General Virology* **76**: 1353-1359.

Dannevig B.H., Falk K. & Krogsrud J. (1993) Leukocytes from Atlantic salmon, *Salmo salar* L., experimentally infected with infectious salmon anaemia (ISA) exhibit an impaired response to mitogens. *Journal of Fish Diseases* **16**: 351-359.

Das S.C., Baron M.D. & Barrett T. (2000) Recovery and characterization of a chimeric rinderpest virus with the glycoproteins of peste-des-petits-ruminants virus: homologous F and H proteins are required for virus viability. *Journal of Virology* **74**: 9039-9047.

Davidovich M., Dishon A., Ilouze M. & Kotler M. (2007) Susceptibility of cyprinid cultured cells to cyprinid herpesvirus 3. *Archives of Virology* **152**: 1541-1546.

Davis A.C., Roux K.H. & Shulman M.J. (1989) On the structure of polymeric IgM. European *Journal of Immunology* **18**: 1001-1008.

Davison A.J. & Davison M.D. (1995) Identification of structural proteins of Channel catfish virus by mass spectrometry. *Virology* **206**: 1035-1043.

Davison A.J., Eberle R., Ehlers B., Hayward G.S., McGeoch D.J., Minson A.C., Pellet P.E., Roizman B., Studdert M.J. & Thiry E. (2009) The order Herpesvirales. *Archives of Virology* **154**: 171-177.

De Diego M., Brocchi E., Mackay D. & De Simone F. (1997) The non-structural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. *Archives of Virology* **142**: 2021-2033.

De Paschale M. & Clerici P. (2012) Serological diagnosis of Epstein-Barr virus infection: Problems and solutions. *World Journal of Virology* **1**(1): 31-43.

Delhon G., Moraes M.P., Lu Z., Afonso C.L., Flores E.F., Weiblen R., Kutish G.F. & Rock D.L. (2003) Genome of Bovine herpesvirus 5. *Journal of Virology* **77**(19): 10339-10347.

Denzin N. & Staak C. (2000) Fish immunoglobulin – A sero-diagnostician's perspective. *Bulletin of the European Association of Fish Pathologists* **20**(2): 60-64.

References

De Smit A.J., Bouma A., de Kluijver E.P., Terpstra C. & Moorman R.J.M. (2001) Duration of the protection of an E2 subunit marker vaccine against classical swine fever after a single vaccination. *Veterinary Microbiology* **78**(4): 307-317.

Devold M., Krossøy B., Aspehaug V. & Nylund A. (2000) Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. *Diseases of Aquatic Organisms* **40**: 9-18.

Dhar A.K. & Allnutt T. (2011) Challenges and opportunities in developing oral vaccines against viral diseases of fish. *Journal of Marine Science: Research and Development* **S**: 1-6.

Dhar A.K., Bowers R.M., Rowe C.G. & Allnutt T.F.C. (2010) Expression of a foreign epitope on infectious pancreatic necrosis virus VP2 capsid protein subviral particle (SVP) and immunogenicity in rainbow trout. *Antiviral Research* **85**: 525-531.

Diallo A., Minet C., Le Goff C., Berhe G., Albina E., Libeau G. & Barrett T. (2007) The threat of peste des petits ruminants: progress in vaccine development for disease control. *Vaccine* **25**: 5591-5597.

Dimmock N.J. (1984) Mechanisms of neutralization of animal viruses. *Journal of General Virology* **65**: 1015-1022.

Dishon A., Davidovich M., Ilouze M. & Kotler M. (2007) Persistence of *Cyprinid herpesvirus 3* in infected cultured carp cells. *Journal of Virology* **81**(9): 4828-4836.

Dishon A., Perelberg A., Bishara-Shieban J., Ilouze M., Davidovich M., Werker S. & Kotler M. (2005) Detection of carp Interstitial Nephritis and Gill Necrosis virus in fish droppings. *Applied Environmental Microbiology* **71**(11): 7285-7291.

Dixon P.F., Hattenberger-Baudouy & Way K. (1994). Detection of carp antibodies to spring viraemia of carp virus by a competitive immunoassay. *Diseases of Aquatic Organisms* **19**: 181-186.

Dixon P., Joiner C., Way K., Reese R., Jeney G. & Jeney Z. (2009) Comparison of the resistance of selected families of common carp, *Cyprinus carpio* L., to koi herpesvirus: preliminary study. *Journal of Fish Diseases* **32**: 1035-1039.

Doel T.R. (2003) FMD vaccines. *Virus Research* **91**(1): 81-99.

Donaldson A.I. & Kitching R.P. (1989) Transmission of foot-and-mouth disease by vaccinated cattle following natural challenge. *Research in Veterinary Science* **46**(1): 9-14.

Dong C., Li X., Weng S., Xie S. & He J. (2013) Emergence of fatal European genotype CyHV-3/KHV in mainland China. *Veterinary Microbiology* **162**: 239-244.

Dong C., Weng S., Li W., Li X., Yi Y., Liang Q. & He J. (2011) Characterisation of a new cell line from caudal fin of koi, *Cyprinus carpio koi*, and first isolation of cyprinid herpesvirus 3 in China. *Virus Research* **161**: 140-149.

Dong X.N. & Chen Y.H. (2006) Candidate peptide-vaccines induced immunity against CSFV and identified sequential neutralising determinants in antigenic domain A of glycoprotein E2. *Vaccine* **24**(11): 1906-1913.

Dong X.N., Chen Y., Wu Y. & Chen Y.H. (2005) Candidate multi-peptide vaccine against classical swine fever virus induced potent immunity with serological marker. *Vaccine* **23**(28): 3630-3633.

Doszpoly A., Benkő M., Csaba G., Dán Á., Lang M. & Harrach B. (2011) Introduction of the family Alloherpesviridae: The first molecular detection of herpesviruses of cyprinid fish in Hungary. *Magyar Allatorvosok Lapja* **133**(3): 174-181.

Dougherty W.G. & Semler B.L. (1993) Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes. *Microbiological reviews* **57**: 781.

Du Pasquier L. (1982) Antibody diversity in lower vertebrates – why is it so restricted? *Nature* **296**: 311-313.

Du Pasquier L., Wilson M., Greenberg A.S. & Flajnik M.F. (1998) Somatic mutation in ectothermic vertebrates: Musings on selection and origins. Somatic Diversification of immune responses: Current Topics in Microbiology and Immunology, 229, Kelsoe G. and Flajnik M. F. Eds. Spring Berlin Heidelberg, pp. 199-216.

Dulbecco R. & Ginsberg H.S. (1988) Virology (2nd Ed.). *Assays of infectivity*, J. B. Lippincott Company pp. 19-26.

Dulbecco R. & Vogt M. (1953) Some problems of animal virology as studied by the plaque technique. Cold Spring Harbor Symp. *Quantitative Biology* **18**: 273-279.

Eblé P.L., Geurts Y., Quak S., Moonen-Leusen H.W., Blome S., Hofmann M.A., Koenen F., Beer M. & Loeffen W.L.A. (2013) Efficacy of chimeric pestivirus vaccine candidates against classical swine fever: protection and DIVA characteristics. *Veterinary Microbiology* **162**: 437-446.

Eggset G., Mikkelsen H. & Killie J.-E.A. (1997a) Immunocompetence and duration of immunity against *Vibrio salmonicida* and *Aeromonas salmonicida* after vaccination of Atlantic salmon (*Salmo salar* L.) at low and high temperatures. *Fish and Shellfish Immunology* **7**: 247-260.

Eggset G., Mortensen A., Johansen L.-H. & Sommer A.-I. (1997b) Susceptibility to furunculosis, cold water vibriosis, and infectious pancreatic necrosis (IPN) in post-smolt salmon (*Salmon salar* L.) as a function of smolt status by seawater transfer. *Aquaculture* **158**: 179-191.

Eggset G., Mortensen A. & Loken S. (1999) Vaccination of Atlantic salmon (*Salmo salar* L.) before and during smoltification; effects on smoltification and immunological protection. *Aquaculture* **170**: 101-112.

Eide D.M., Linder R.D., Strømsheim A., Fjalestad K., Larsen H.J.S. & Røed K.H. (1994) Genetic variation in antibody response to diphtheria toxoid in Atlantic salmon and rainbow trout. *Aquaculture* **127**: 103-113.

Eide K.E., Miller-Morgan T., Heidel J., Bildfell R. & Jin L. (2011b) Results of total DNA measurement in koi by tissue koi herpesvirus real-time PCR. *Journal of Virological Methods* **172**: 81-84.

Eide K.E., Miller-Morgan T., Heidel J.R., Kent M.L., Bildfell R.J., LaPatra S., Watson G. & Jin L. (2011a). Investigation of koi herpesvirus latency in koi. *Journal of Virology* **85** (10): 4954-4962.

Einer-Jensen K., Krogh T.N., Roepstorff P. & Lorenzen N. (1998) Characterisation of intramolecular disulphide bonds and secondary modifications of the glycoprotein from viral haemorrhagic septicaemia virus (VHSV), a fish rhabdovirus. *Journal of Virology* **72**: 10189-10196.

Elcombe B.M., Chang R.J., Taves C.J. & Winkelhake J.L. (1985) Evolution of antibody structure and effector functions: comparative haemolytic activities of monomeric and tetrameric IgM from rainbow trout, *Salmo gairdnerii*. *Comparative Biochemistry and Physiology B, Comparative Biochemistry* **80**(4): 697-706.

El-Din M.M. (2011) Histopathological Studies in Experimentally Infected Koi Carp (*Cyprinus carpio* koi) with Koi Herpesvirus in Japan. *World Journal of Fish and Marine Sciences* **3**(3): 252-259.

Eliassen T.M., Frøystad M.K., Dannevig B.H., Jankowska M., Brech A., Falk K., Romøren K. & Gjøen T. (2000) Initial events in infectious salmon anemia virus infection: evidence for the requirement of a low-pH step. *Journal of Virology* **74**: 218-227.

Ellis A. (1982) *Differences between the immune mechanisms of fish and higher vertebrates*. Academic Press, New York.

El-Matbouli M., Rucker U. & Soliman H. (2007) Detection of *Cyprinid herpesvirus-3* (CyHV-3) DNA in infected fish tissues by nested polymerase chain reaction. *Diseases of Aquatic Organisms* **78**(1): 23-28.

El-Matbouli M. & Soliman H. (2011) Transmission of Cyprinid herpesvirus-3 (CyHV-3) from goldfish to naïve common carp by cohabitation. *Research in Veterinary Science* **90**: 536-539.

Emmenegger E.J. & Kurath G. (2008) DNA vaccine protects ornamental koi (*Cyprinus carpio* koi) against North American spring viraemia of carp virus. *Vaccine* **26**: 6415-6421.

References

Encinas P., Gomez-Casado E., Estepa A. & Coll J.M. (2011a) An ELISA for detection of rainbow trout antibodies to viral haemorrhagic septicaemia virus using recombinant fragments of their viral G protein. *Journal of Virological Methods* **176**: 14-23.

Encinas P., Gomez-Casado E., Fregeneda-Grandes J.M., Olesen N.J., Lorenzen N., Estepa A. & Coll J.M. (2011b) Rainbow trout surviving infections of viral haemorrhagic septicaemia virus (VHSV) show lasting antibodies to recombinant G protein fragments. *Fish and Shellfish Immunology* **30**: 929-935.

Engel-Herbert I., Werner O., Teifke J.P., Mebatsion T., Metternleiter T.C. & Römer-Oberdörfer A. (2003) Characterisation of a recombinant Newcastle disease virus expressing the green fluorescent protein. *Journal of Virological Methods* **108**: 19-28.

Enzmann P.J., Fichtner D., Schütze H. & Walliser G. (1998) Development of vaccines against VHS and IHN: Oral application, molecular marker and discrimination of vaccinated fish from infected populations. *Journal of Applied Ichthyology* **14**: 179-183.

Enzmann P.J. & Konrad M. (1993) Longevity of antibodies in brown trout and rainbow trout following experimental infection with VHS-virus. *Bulletin of the European Association of Fish Pathologists* **13**(6): 193-194.

Espinoza J.C. & Kuznar J. (2002) Rapid simultaneous detection and quantitation of infectious pancreatic necrosis virus (IPNV). *Journal of Virological Methods* **105**: 81-85.

Esteban A.M. (2012) An overview of the immunological defenses in fish skin. *ISRN Immunology* **2012**: 1-29.

Etlinger H.M., Chiller J. & Hodgins H.O. (1979) Evolution of the lymphoid system. IV. Murine T-independent but not T dependent antigens are very immunogenic in rainbow trout *Salmo Gaidneri*. *Cellular Immunology* **47**: 400-406.

Evelyn T.P.T. (1997) A historical review of fish vaccinology. *In* 'Fish Vaccinology' Eds. Gudding R., Lillehaug A., Midtlyng P. J. and Brown F. *Developments in Biological Standardisation* **90**: 3-12.

Evensen O., Thorud K. & Olsen Y. (1991) A morphological study of the gross and light microscopic lesions of infectious anaemia in Atlantic salmon (*Salmo salar*). *Research in Veterinary Science* **51**: 215-222.

Fabian M., Baumer A. & Steinhagen D. (2013) Do wild fish species contribute to the transmission of koi herpesvirus to carp in hatchery ponds? *Journal of Fish Diseases* **36**(5): 505-514.

Falk K., Aspehaug V., Vlasak R. & Endresen C. (2004) Identification and characterization of viral structural proteins of infectious salmon anemia virus. *Journal of Virology* **78**: 3063-3071.

Falk K. & Dannevig B.H. (1995) Demonstration of a protective immune response in infectious salmon anaemia (ISA)-infected Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms* **21**: 1-5.

Falk K., Namork E. & Dannevig B.H. (1998) Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus. *Diseases of Aquatic Organisms* **34**: 77-85.

Falk K., Namork E., Rimstad E., Mjaaland S. & Dannevig B.H. (1997) Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.). *Journal of Virology* **71**: 9016-9023.

Fang Y., Christopher-Hennings J., Brown E., Lui H., Chen Z., Lawson S.R., Breen R., Clement T., Gao X., Bao J., Knudsen D., Daly R. & Nelsen E. (2008) Development in genetic markers in the non-structural protein 2 region of a US type-1 porcine reproductive and respiratory syndrome virus: implications for future recombinant marker vaccine development. *Journal of General Virology* **89**: 3086-3096.

Feinstein A., Richardson N. & Taussig M.J. (1986) Immunoglobulin flexibility in complement activation. *Immunology Today* **7**: 169-173.

Fernandez-Alonso M., Lorenzo G., Perez L., Bullido R., Estepa A., Lorenzen N. & Coll J.M. (1998) Mapping of linear antibody epitopes of the glycoprotein of VHSV, a salmonid rhabdovirus. *Diseases of Aquatic Organisms* **34**: 167-176.

Fichtner D., Bergmann S., Riechardt M., Teifke J.P. & Fuchs. W. (2007) Evaluation of the virulence of an EGFP-expressing, thymidine kinase-negative KHV mutant. 13th European Association of Fish Pathologists (EAFP) International Conference on Diseases of Fish and Shellfish, 2007, Sept 17-21, Grado, Italy, Book of abstracts, p. 145.

Flajnik M.F. (1996) The immune system of ectothermic vertebrates. *Veterinary Immunology and Immunopathology* **54**: 145-150.

Flint S., Enquist L., Racaniello V. & Skalka A. (2004) Principles of Virology, 2nd Edition, ASM Press, Washington.

Flint S.J., Enquist L.W., Racaniello V.R. & Skalka A.M. (2009) Principles of Virology, Volume II: Pathogenesis and Control, Chapter 4: Immune Defenses, 3rd Edition, ASM Press, pp. 87-132.

Floegel-Niesmann G. (2001) Classical swine fever (CSF) marker vaccine trial III. Evaluation of discriminatory ELISAs. *Veterinary Microbiology* **83**: 121-136.

Flores E.F., Osorio F.A., Zanella E.L., Kit S. & Kit M. (1993) Efficacy of a deletion mutant bovine herpesvirus-1 (BHV-1) vaccine that allows serologic differentiation of vaccinated from naturally infected animals. *Journal of Veterinary Diagnostic Investigation* **5**: 534-540.

Flynn D.C., Meyer W.J., Mackenzie J.M. & Johnston R.E. (1990) A conformational change in Sindbis virus glycoproteins E1 and E2 is detected at the plasma membrane as a consequence of early virus-cell interaction. *Journal of Virology* **64**(4): 3643-3653.

Fournier G., Boutier M., Raj V.S., Mast J., Parmentier E., Vanderwalle P., Peeters D., Lieffrig F., Farnir F., Gillet L. & Vanderplasschen A. (2012) Feeding *Cyprinus carpio* with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. *Veterinary research* **43**: 1-10.

Fournier G. & Vanderplasschen A. (2011) Cyprinid Herpesvirus 3: an Interesting Virus for Applied and Fundamental Research. *Bulletin De l' Academie Veterinaire De France* **164**: 353-358.

Franti M., Aubin J., De Saint-Maur G., Kosuge H., Yamanishi K., Gautheret-Dejean A., Garbarg-Chenon A., Huraux J-M. & Agut H. (2002) Immune reactivity of human sera to the glycoprotein B of human herpesvirus 7. *Journal of Clinical Microbiology* **40**: 44-51.

Fregeneda-Grandes J.M. & Olesen N.J. (2007) Detection of rainbow trout antibodies against viral haemorrhagic septicaemia virus (VHSV) by neutralization test is highly dependent on the virus isolate used. *Diseases of Aquatic Organisms* **74**: 151-158.

Fregeneda-Grandes J.M., Skall H.F. & Olesen N.J. (2008) Antibody response of rainbow trout with single or double infections involving viral haemorrhagic septicaemia virus and infectious haematopoietic necrosis virus. *Diseases of Aquatic Organisms* **83**: 23-29.

Fuchs W., Fichtner D., Bergmann S.M. & Mettenleiter T.C. (2011) Generation and characterization of koi herpesvirus recombinants lacking viral enzymes of nucleotide metabolism. *Archives of Virology* **156**: 1059-1063.

Fuchs W., Veits J., Helferich D., Granzow H., Teifke J.P. & Mettenleiter T.C. (2007) Molecular biology of avian infectious laryngotracheitis virus. *Veterinary Research* **38**: 261-279.

Fujiki K., Shin D.-H., Nakao M. & Yano T. (2000) Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1β, high affinity immunoglobulin E Fc receptor γ subunit and serum amyloid A. *Fish and Shellfish Immunology* **10**: 229-242.

Gao W., Soloff A.C., Lu X., Montecalvo A., Nguyen D.C., Matsuoka Y., Robbins P.D., Swayne D.E., Donis R.O., Katz J.M., Barratt-Boyes S.M. & Gambotto A. (2006) Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. *Journal of Virology* **80**: 1959-1964.

Garver K.A., Al-Hussinee L., Hawley L.M., Schroeder T., Edes S., LePage V., Contador E., Russell S., Lord S., Stevenson R.M., Souter B., Wright E. & Lumsden J.S. (2010) Mass mortality associated with koi herpesvirus in wild common carp in Canada. *Journal of Wildlife Diseases* **46**: 1242-1251.

Garver K.A., Hawley L.M., McClure C.A., Schroeder T., Aldous S., Doig F., Snow M., Edes S., Baynes C. & Richard J. (2011) Development and validation of a reverse transcription quantitative PCR for quantitative detection of viral hemorrhagic septicaemia virus. *Diseases of Aquatic Organisms* **95**: 97-112.

Gaudin Y., de Kinkelin P. & Benmansour A. (1999) Mutations in the glycoprotein of viral haemorrhagic septicaemia virus that affect virulence for fish and the pH threshold for membrane fusion. *Journal of General Virology* **80**: 1221-1229.

Geada M.M., Galindo I., Lorenzo M.M., Perdiguero B. & Blasco R. (2001) Movements of vaccinia virus intracellular enveloped virions with GFP tagged to the F13L envelope protein. *Journal of General Virology* **82**: 2747-2760.

Ghadially F. (1997). Mitochondria. *In* Ultrastructural pathology of the cell and matrix, Fourth Edition, (Ghadially F. Ed.) Butterworth-Heinemann **1**: 195-328.

Gibson W. & Roizman B. (1972) Proteins specified by herpes simplex virus VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. *Journal of Virology* **10**: 1044-1052.

Gilad O., Yun S., Adkison M.A., Way K., Willits N.H., Bercovier H. & Hedrick R.P. (2003) Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *Journal of General Virology* **84**: 2661-2668.

Gilad O., Yun S., Andree K., Adkison M., Zlotkin A., Bercovier H., Eldar A. & Hedrick R.P. (2002) Initial characteristics of Koi Herpesvirus and development of a polymerase chain reaction assay to detect the virus in Koi, *Cyprinus carpio* koi. *Diseases of Aquatic Organisms* **48**: 101-108.

Gilad O., Yun S., Zagmutt-Vergara F.J., Leutenegger C.M., Bercovier H. & Hedrick R.P. (2004) Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio* koi as assessed by real-time TaqMan PCR. *Diseases of Aquatic Organisms* **60**: 179-187.

Gilbert R. & Ghosh H.P. (1993) Immunoelectron microscopic localization of herpes simplex virus glycoprotein gB in the nuclear envelope of infected cells. *Virus research* **28**: 217-231.

Gilbert R., Ghosh K., Rasile L. & Ghosh H.P. (1994) Membrane anchoring domain of Herpes simplex virus glycoprotein gB is sufficient for nuclear envelope localisation. *Journal of Virology* **68**(4): 2272-2285.

Ginsberg H.S. (1988) Herpesviruses. *In* Virology, (Dulbecco R. and Ginsberg H.S. Eds.), Second Edition, J. B. Lippincott Co. Chapter 53, pp. 161-177.

Giugni T.D., Churchill M.A., Pande H., Campo K., Guha M. & Zaia J.A. (1992) Expression in insect cells and immune reactivity of a 28K tegument protein of human cytomegalovirus. *Journal of General Virology* **73**: 2367-2374.

Glazov E.A., Horwood P.F., Assavalapsakul W., Kongsuwan K., Mitchell R.W., Mitter N. & Mahony T.J. (2010) Characterisation of microRNAs encoded by the bovine herpesvirus 1 genome. *Journal of General Virology* **91**(1): 32-41.

Godoy M.G., Aedo A., Kibenge M.J., Groman D.B., Yason C.V., Grothusen H., Lisperguer A., Calbucura M., Avendano F., Imilan M., Jarpa M. & Kibenge F.S. (2008) First detection, isolation and molecular characterisation of infectious salmon anaemia virus associated with clinical disease in farmed salmon (S*almo salar*) in Chile. *BMC Veterinary Research* **4**: 28-41.

Goethe R., González O.F., Linder T. & Gerlach G.-F. (2001) A novel strategy for protective *Actinobacillus pleuropneumoniae* subunit vaccines: detergent extraction of cultures induced by iron restriction. *Vaccine* **19**: 966-975.

Gomez D.K., Joh S.J., Jang H., Shin S.P., Choresca C.H., Han J.E., Kim J.H., Jun J.W. & Park S.C. (2011) Detection of koi herpesvirus (KHV) from koi (*Cyprinus carpio koi*) broodstock in South Korea. *Aquaculture* **311**: 42-47.

References

Gomez-Casado E., Estepa A. & Coll J.M. (2011) A comparative review on European-farmed finfish RNA viruses and their vaccines. *Vaccine* **29**: 2657-2671.

Gómez-Sebastián S., Pérez-Filgueira D.M., Gómez-Casado E., Nuñez M.C., Sánchez-Ramos I., Tabarés E. & Escribano J.M. (2008) DIVA diagnostic of Aujeszky's disease using an insect-derived virus glycoprotein E. *Journal of Virological Methods* **153**: 29-35.

Gonzalez R., Charlemagne J., Mahana W. & Avrameas S. (1988) Specificity of natural serum antibodies present in phylogenetically distinct fish species. *Immunology* **63**: 31-36.

Gotesman M., Soliman H. & El-Matbouli M. (2013) Antibody screening identifies 78 putative host proteins involved in Cyprinid herpesvirus 3 infection or propagation in common carp, *Cyprinus carpio* L. *Journal of Fish Diseases*, Published ahead of print, doi: 10.1111/jfd.12073.

Gou D., Kubota H., Onuma M. & Kodama H. (1991) Detection of salmonid herpesvirus (*Oncorhynchus masou* virus) in fish by Southern-blot technique. *The Journal of veterinary medical science/the Japanese Society of Veterinary Science* **53**(1): 43-48.

Granzow H., Klupp B.G., Fuchs W., Veits J., Osterrieder N. & Mettenleiter T.C. (2001) Egress of alphaherpesviruses: Comparative ultrastructural study. *Journal of Virology* **75**(8): 3675-3684.

Granzow H., Klupp B.G. & Mettenleiter T.C. (2004) The pseudorabies virus US3 protein is a component of primary and of mature virions. *Journal of Virology* **78**: 1314-1323.

Granzow H., Weiland F., Jöns A., Klupp B.G., Karger A. & Mettenleiter T.C. (1997) Ultrastructural analysis of the replication cycle of Pseudorabies virus in cell culture: A reassessment. *Journal of Virology* **71**(3): 2072-2082.

Grave K., Engelstad M., Søli N.E., & Håstein T. (1990) Utilisation of antibacterial drugs in salmonid farming in Norway during 1980-1988. *Aquaculture* **86**: 347-358.

Gray W.L., Mullis L., LaPatra S.E., Groff J.M. & Goodwin A. (2002) Detection of Koi herpesvirus DNA in tissues of infected fish. *Journal of Fish Diseases* **25**: 171-178.

Gray W., Williams R. & Griffin B. (1999) Detection of channel catfish virus DNA in acutely infected channel catfish, *Ictalurus punctatus* (Rafinesque), using the polymerase chain reaction. *Journal of Fish Diseases* **22**: 111-116.

Grefte J., Van der Gun B., Schmolke S., Van der Giessen M., Van Son W., Plachter B., Jahn G. & The T.H. (1992). The lower matrix protein pp65 is the principal viral antigen present in peripheral blood leukocytes during an active cytomegalovirus infection. *The Journal of General Virology* **73**: 2923-2932.

Gregory A. (2002) Detection of infectious salmon anaemia virus (ISAV) by *in situ* hybridisation. *Diseases of Aquatic Organisms* **50**: 105-110.

Grimmett S.G., Warg J.V., Getchell R.G., Johnson D.J. & Bowser P.R. (2006) An unusual koi herpesvirus associated with a mortality event of common carp *Cyprinus carpio* in New York State, USA. *Journal of Wildlife Diseases* **42**: 658-662.

Gunimaladevi I., Kono T., Venugopal M. & Sakai M. (2004) Detection of koi herpesvirus in common carp, *Cyprinus carpio* L., by loop-mediated isothermal amplification. *Journal of Fish Diseases* **27**: 583-589.

Gustafson L.L., Ellis S.K., Beattie M.J., Chang B.D., Dickey D.A., Robinson T.L., Marenghi F.P., Moffett P.J. & Page F.H. (2007) Hydrographics and the timing of infectious salmon anemia outbreaks among Atlantic salmon (*Salmo salar* L.) farms in the Quoddy region of Maine, USA and New Brunswick, Canada. *Preventive Veterinary Medicine* **78**: 35-56.

Gut M., Jacobs L., Tyborowska J., Szewczyk B. & Bienkowska-Szewczyk K. (1999) A highly specific and sensitive competitive enzyme-linked immunosorbent assay (ELISA) based on baculovirus expressed pseudorabies virus glycoprotein gE and gI complex. *Veterinary Microbiology* **69**: 239-249.

References

Haenen O.L.M., Way K., Bergmann S.M. & Ariel E. (2004) The emergence of koi herpesvirus and its significance to European aquaculture. *Bulletin of the European Association of Fish Pathologists* **24**(6): 293-307.

Hahn J., Park S.H., Song J.Y., An S.H. & Ahn B.Y. (2001) Construction of recombinant swinepox viruses and expression of the classical swine fever virus E2 protein. *Journal of Virological Methods* **93**(1-2): 49-56.

Halvorson D.A. (2002) The control of H5 or N7 mildly pathogenic avian influenza: a role for inactivated vaccine. *Avian Pathology* **31**(1): 5-12.

Hames B.D. & Rickwood D. (1990) Gel electrophoresis of proteins: a practical approach. Oxford, IRL Press.

Hammond J.M., Jansen E.S., Morrissy C.J., Williamson M.M., Hodgson A.L.M. & Johnson M.A. (2001) Oral and subcutaneous vaccination of commercial pigs with a recombinant porcine adenovirus expressing the classical swine fever virus gp55 gene. *Archives of Virology* **146**: 1787-1793.

Hammond J.M., McCoy R.J., Jansen E.S., Morrissy C.J., Hodgson A.L.M. & Johnson M.A. (2000) Vaccination of a single dose of a recombinant porcine adenovirus expressing the classical swine fever virus gp55 (E2) gene protects pigs against classical swine fever. *Vaccine* **18**: 1040-1050.

Han J.E., Kim J.H., Renault T., Choresca Jr.C., Shin S.P., Jun J.W. & Park S.C. (2013) Identifying the viral genes encoding envelope glycoproteins for differentiation of *Cyprinid herpesvirus 3* isolates. *Viruses* **5**: 568-576.

Hangartner L., Zinkernagel R.M. & Hengartner H. (2006) Antiviral antibody responses: the two extremes of a wide spectrum. *Nature Reviews Immunology* **6**: 231-243.

Hansen J.D., Landis E.D. & Phillips R.B. (2005) Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *PNAS* **102** (19): 6919-6924.

Hanson L., Dishon A. & Kotler M. (2011) Herpesviruses that infect fish. *Viruses* **3**: 2160-2191.

Harmache A., LeBerre M., Droineau S., Giovannini M. & Brémont M. (2006) Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. *Journal of Virology* **80**: 3655-3659.

Hasselaar P., Triplett D., LaRue A., Derksen R., Blokzijl L., De Groot P.G., Wagenknecht D.R. & McIntyre J.A. (1990) Heat treatment of serum and plasma induces false positive results in the antiphospholipid antibody ELISA. *Journal of Rheumatology* **17**: 186-191.

Hastings T., Olivier G., Cusack R., Bricknell I., Nylund A., Binde M., Munro P. & Allan C. (1999) Infectious salmon anaemia. *Bulletin of the European Association of Fish Pathologists* **19**: 286–288.

Hayat M.A. (1989) Principles and techniques of electron microscopy – biological applications. 3rd edition. Macmillan Press. Scientific and Medical, Basingstoke and London.

Hedge N.R., Chevalier M.S. & Johnson D.C. (2003) Viral inhibition of MHC class II antigen presentation. *Trends in Immunology* **24**(5): 278-285.

Hedrick R.P. (1996) Movement of pathogens with the international trade of live fish: problems and solutions. *Revue Scientifique et Technique (Paris)* **15**: 523-531.

Hedrick R.P., Gilad O., Yun S.C., McDowell T.S., Waltzek T.B., Kelley G.O. & Adkison M.A. (2005) Initial isolation and characterization of a herpes-like virus (KHV) from koi and common carp. *Bulletin of the Fisheries Research Agency* **2**: 1–7.

Hedrick R.P., Gilad O., Yun S., Spangenberg J.V., Marty G.D., Nordhausen R.W., Kebus M.J., Bercovier H. & Eldar A. (2000) A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *Journal of Aquatic Animal Health* **12**: 44-57.

Hedrick R.P., Waltzek T.B. & McDowell T.S. (2006) Susceptibility of koi carp, common carp, goldfish, and goldfish× common carp hybrids to Cyprinid herpesvirus-2 and herpesvirus-3. *Journal of Aquatic Animal Health* **18**: 26-34.

References

Heffner S., Kovács F., Klupp B.G. & Mettenleiter T.C. (1993) Glycoprotein gp50-Negative Pseudorabies virus: a novel approach toward a nonspreading live herpesvirus vaccine. *Journal of Virology* **67**(3): 1529-1537.

Hema M., Nagendrakumar S.B., Yamini R., Chandran D., Rajendra L., Thiagarajan D., Parida S., Paton D.J. and Srinivasan V.A. (2007) Chimeric tyomovirus-like particles displaying foot-and-mouth disease virus non-structural protein epitopes and its use for detection of FMD-NSP antibodies. *Vaccine* **25**: 4784-4794.

Hemmatzadeh F., Sumarningsih S., Tarigan S., Indriana R., Dharmayanti I., Ebrahimie E. & Igniatovic J. (2013) Recombinant M2e protein-based ELISA: A novel and inexpensive approach for differentiating Avian influenza infected chickens from vaccinated ones. *PLoS One* **8**(2): e56801.

Henderson L.M. (2005) Overview of marker vaccine and differential diagnostic test technology. *Biologicals* **33**(4): 203-209.

Herath T.K. (2010) Cellular and molecular pathogenesis of Salmonid alphavirus 1 in Atlantic salmon, *Salmo salar* L. Ph.D Doctoral thesis, University of Stirling, Scotland.

Hilleman M.R. (2000) Vaccines in historic evolution and perspective: a narrative of vaccine discoveries. *Vaccine* **18**: 1436-1447.

Hoar W.S. (1976) Smolt transformation-evolution, behaviour, and physiology. *Journal of the Fisheries Research Board of Canada* **33**: 1233-1252.

Hodgins H.O., Weiser R.S. & Ridgway G.J. (1967) The nature of antibodies and the immune response in rainbow trout (*Salmo gairdneri*). *The Journal of Immunology* **99**: 534-544.

Hoffmann B., Beer M., Schelp C., Schirrmeier H. & Depner K. (2005) Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *Journal of Virological Methods* **130**(1-2): 36-44.

Hoffman B., Depner K., Schirrmeier H. & Beer M. (2006) A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. *Journal of Virological Methods* **136**: 200-209.

Holland M.C.H. & Lambris J.D. (2002) The complement system in teleosts. *Fish and Shellfish Immunology* **12**: 399-420.

Hook L.M., Lubinski J.M., Jiang M., Pangburn M.K. & Friedman H.M. (2006) Herpes Simplex virus Type 1 and 2 glycoprotein C prevents complement-mediated neutralisation induced by natural immunoglobulin M antibody. *Journal of Virology* **80**(8): 4038-4046.

Hovland T., Nylund A., Watanabe K. & Endresen C. (1994) Observation of infectious salmon anaemia virus in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* **17**: 291-296.

Huang B., Tan C., Chang S.F., Munday B., Mathew J.A., Hgoh G.H. & Kwang J. (2001) Detection of nodavirus in barramundi, *Lates calcarifer* (Bloch), using recombinant coat protein-based ELISA and RT-PCR. *Journal of Fish Diseases* **24**: 135-141.

Huang C., Chien M.-S., Hu C.-M., Chen C.-W. & Hsieh P.-C. (2006) Secreted expression of the classical swine fever virus glycoprotein E^{RNS} in yeast and application to a sandwich blocking ELISA. *Journal of Virological Methods* **132**: 40-47.

Huang C., Zhang X., Gin K.Y.H. & Qin Q.W. (2004) *In situ* hybridisation of a marine fish virus, Singapore grouper iridovirus with a nucleic acid probe of major capsid protein. *Journal of Virological Methods* **117**: 123-128.

Hulst M.M., Westera D.F., Wensvoort G. & Moorman R.J.M. (1993) Glycoprotein E1 of Hog cholera virus expressed in insect cells protects swine from Hog cholera. *Journal of Virology* **67**(9): 5435-5442.

Hutoran M., Ronen A., Perelberg A., Ilouze M., Dishon A., Bejerano I., Chen N. & Kotler M. (2005) Description of an as yet unclassified DNA virus from diseased *Cyprinus carpio* species. *Journal of Virology* **79**(4): 1983-1991.

References

Iida T. & Sano M. (2005) Koi herpesvirus disease. *Uirusu* **55**: 145-151.

Ilouze M., Davidovich M., Diamant A., Kotler M. & Dishon A. (2011) The outbreak of carp disease caused by CyHV-3 as a model for new emerging viral diseases in aquaculture: a review. *Ecological Research* **26**: 885-892.

Ilouze M., Dishon A. Kahan T. & Kotler M. (2006b) Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant DNA viruses. *FEBS Letters* **580**: 4473-4478.

Ilouze M., Dishon A. & Kotler M. (2006a) Characterisation of a novel virus causing a lethal disease in carp and koi. *Microbiology and Molecular Biology Reviews* **70**(1): 147-156.

Ilouze M., Dishon A. & Kotler M. (2012a) Coordinated and sequential transcription of the cyprinid herpesvirus-3 annotated genes. *Virus Research* **169**: 98-106.

Ilouze M., Dishon A. & Kotler M. (2012b) Down-regulation of the cyprinid herpesvirus-3 annotated genes in cultured cells maintained at restrictive high temperature. *Virus Research* **169**: 289-295.

Ishioka T., Yoshizumi M., Izumi S., Suzuki K., Suzuki H., Kozawa K., Arai M., Nobusawa K., Morita Y., Kato M., Hoshino T., Iida T., Kosuge K. & Kimura H. (2005) Detection and sequence analysis of DNA polymerase and major envelope protein genes in koi herpesviruses derived from *Cyprinus carpio* in Gunma prefecture, Japan. *Veterinary Microbiology* **110**: 27-33.

Jacobs L. & Kimman T.G. (1994) Epitope-specific antibody response against glycoprotein E of Pseudorabies virus. *Clinical and Diagnostic Laboratory Immunology* **1**(5): 500-505.

Jadhao S.J., Lee C.-W., Sylte M. & Suarez D.L. (2009) Comparative efficacy of North American and antigenically matched reverse genetics derived H5N9 DIVA marker vaccines against highly pathogenic Asian H5N1 avian influenza viruses in chickens. *Vaccine* **27**: 6247-6260.

James C.M., Foong Y.Y., Mansfield J.P., Fenwick S.G. & Ellis T.M. (2007) Use of tetanus toxoid as a differentiating infected from vaccinated animals (DIVA) strategy for sero-surveillance of avian influenza virus vaccination in poultry. *Vaccine* **25**: 5892-5901.

James C.M., Foong Y.Y., Mansfield J.P., Vind A.R., Fenwick S.G. & Ellis T.M. (2008) Evaluation of a positive marker of avian influenza vaccination in ducks for use in H5N1 surveillance. *Vaccine* **26**: 5345-5351.

Jang H., Kim Y., Cha I., Noh S., Park S., Ohtani M., Hikima J., Aoki T. & Jung T.S. (2011) Detection of antigenic proteins expressed by lymphocystis virus as vaccine candidates in olive flounder, *Paralichthys olivaceus* (Temminck & Schlegel). *Journal of Fish Diseases* **34**: 555-562.

Jones C. & Chowdhury S. (2008) A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the Bovine respiratory disease complex and development of improved vaccines. *Animal Health Research Reviews* **8**(2): 187-205.

Jones D.R., Hannan C.M., Russel-Jones G.J. & Raison R.L. (1999a) Selective B cell non-responsiveness in the gut of the rainbow trout - *Oncorhynchus mykiss*. *Aquaculture* **172**: 29-39.

Jones S.R.M., Mackinnon A.M. & Salonius K. (1999b) Vaccination of freshwater-reared Atlantic salmon reduces mortality associated with infectious salmon anaemia virus. *Bulletin of the European Association of Fish Pathologists* **19**(3): 98-101.

Jonstrup S.P., Kahns S., Skall H.F., Boutrup T.P. & Olesen N.J. (2012) Development and validation of a novel Taqman based real time RT-PCR assay suitable for demonstrating freedom from viral haemorrhagic septicaemia virus. *Journal of Fish Diseases* **36**(1): 9-23.

Jorgensen P.E.V., Olsen N.J., Lorenzen N., Winton J.R. & Ristow S.S. (1991) Infectious hematopoietic necrosis (IHN) and viral hemorrhagic septicaemia (VHS): detection of trout antibodies to the causative viruses by means of plaque neutralisation, immunofluorescence, and enzyme-linked immunosorbent assay. *Journal of Aquatic Animal Health* **3**: 100-108.

References

Joseph T., Cepica A., Brown L., Ikede B.O. & Kibenge F.S. (2004) Mechanism of cell death during infectious salmon anemia virus infection is cell type-specific. *Journal of general virology* **85**: 3027-3036.

Jovasevic V., Liang L. & Roizman B. (2008) Proteolytic cleavage of VP1-2 is required for release of herpes simplex virus 1 DNA into the nucleus. *Journal of Virology* **82**: 3311-3319.

Kaashoek M.J., Moerman A., Madić J., Rijsewijk F.A., Quak J., Gielkejns A.L. & Van Oirschot J.T. (1994) A conventionally attenuated glycoprotein E-negative strain of bovine herpesvirus type-1 is an efficacious and safe vaccine. *Vaccine* **12**: 439-444.

Kaashoek M.J., Moerman A., Madic J., Weerdmeester K., Maris-Veldhuis M., Rijsewijk F.A.M. & Van Oirschot J.T. (1995) An inactivated vaccine based on a glycoprotein E-negative strain of bovine herpesvirus-1 induces protective immunity and allows serological differentiation. *Vaccine* **13**(4): 342-346.

Kaashoek M.J., Rijsewijk F.A.M., Ruuls R.C., Keil G.M., Thiry E., Pastoret P.P. & Van Oirschot J.T. (1998) Virulence, immunogenicity and reactivation of bovine herpesvirus 1 mutants with a deletion in the gC, gG, gI, gE or in both the gI and gE gene. *Vaccine* **16**: 802-809.

Kaashoek M.J., Van Engelenburg F.A., Moerman A., Gielkens A.L., Rijsewijk F.A.M. & Van Oirschot J.T. (1996) Virulence and immunogenicity in calves of thymidine kinase and glycoprotein E-negative bovine herpesvirus-1 mutants. *Veterinary Microbiology* **48**: 143-153.

Kaattari S.L. (1994) Development of a piscine paradigm of immunological memory. *Fish and Shellfish Immunology* **4**: 447-457.

Kaattari S.L., Evans D. & Klemer J. (1998) Varied redox forms of teleost IgM: an alternative to isotypic diversity? *Immunological Reviews* **166**: 133-142.

Kaattari S.L., Klemer J.V. & Evans D.A. (1999) Teleost antibody structure: Simple prototype or elegant alternative. *Bulletin of the European Association of Fish Pathologists* **19**(6): 245-249.

Kaattari S.L. & Piganelli J.D. (1996) The specific immune system: humoral defense. *In* Chapter 5: The Fish Immune System: organism, pathogen and environment. Eds. Iwama G. and Nakanishi T. (1996), Academic Press, pp. 207-243.

Kachamakova N.M., Irnazarow I., Parmentier H.K., Savelkoul H.F.J., Pilarczyk A. & Wiegertjes G.F. (2006) Genetic differences in natural antibody levels in common carp (*Cyprinus carpio* L.). *Fish and Shellfish Immunology* **21**: 404-413.

Kaelin K., Dezélée S., Masse M.J., Bras F. & Flamand A. (2000) The UL25 protein of Pseudorabies virus associates with capsids and localises to the nucleus and to microtubules. *Journal of Virology* **74**(1): 474-482.

Kalthoff D., König P., Trapp S. & Beer M. (2010) Immunization and challenge experiments with a new modified live bovine herpesvirus type 1 marker vaccine prototype adjuvanted with a co-polymer. *Vaccine* **28**: 5871-5877.

Kancharla S.R. & Hanson L. (1996) Production and shedding of channel catfish virus (CCV) and thymidine kinase negative CCV in immersion exposed channel catfish fingerlings. *Diseases of Aquatic Organisms* **27**: 25-34.

Kao C., Wu M., Chiu Y., Lin J., Wu Y., Yueh Y., Chen L.K., Shaio M.F. & King C.C. (2001) Flow cytometry compared with indirect immunofluorescence for rapid detection of dengue virus type 1 after amplification in tissue culture. *Journal of Clinical Microbiology* **39**: 3672-3677.

Kärber G. (1931) Beitrag zur kollectiven Behandlung pharmakologischer Reihenversuche. Naunyn-Schmiedeberg's *Archives of Pharmacology* **162**(4): 480-483.

Kasai H., Muto Y. & Yoshimizu M. (2005) Virucidal effects of ultraviolet, heat treatment and disinfectants against koi herpesvirus (KHV). *Fish Pathology* **40**: 137-138.

Kempter J., Sadowski J., Schutze H., Fischer U., Dauber M., Fichtner D., Panicz R. & Bergmann S.M. (2009) Koi herpesvirus: Do acipinserid restitution programs pose a threat to carp farms in the disease-free zones? *Acta Ichthyologica et Piscatoria* **39**(2): 119-126.

References

Kibenge F.S.B., Godoy M.G., Wang Y., Kibenge M.J.T., Gherardelli V., Mansilla S., Lisperger A., Jarpa M., Larroquete G., Avendaño F., Lara M. & Gallardo A. (2009a) Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around 1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. *Virology Journal* **6**: 88-104.

Kibenge F.S., Kibenge M.J., Groman D. & McGeachy S. (2006) *In vivo* correlates of infectious salmon anemia virus pathogenesis in fish. *Journal of General Virology* **87**: 2645-2652.

Kibenge F., Kibenge M., Simard N., Riveroll A., Pallapothu M. & Salonius K. (2009b) Development of a DIVA system for an infectious salmon anaemia (ISA) virus vaccine using a qRT-PCR test based on segment 6 of the virus. 14th European Association of Fish Pathologists International Conference on Diseases of Fish and Shellfish, Prague, Czech Republic, September 14-19.

Kibenge F.S., Kibenge M.J., Wang Y., Qian B., Hariharan S. & McGeachy S. (2007) Mapping of putative virulence motifs on infectious salmon anemia virus surface glycoprotein genes. *Journal of General Virology* **88**: 3100-3111.

Kibenge F.S., Munir K., Kibenge M.J., Joseph T. & Moneke E. (2004) Infectious salmon anemia virus: causative agent, pathogenesis and immunity. *Animal Health Research Reviews* **5**: 65-78.

Kibenge F., Whyte S., Hammell K., Rainnie D., Kibenge M. & Martin C. (2000) A dual infection of infectious salmon anaemia (ISA) virus and a togavirus-like virus in ISA of Atlantic salmon *Salmo salar* in New Brunswick, Canada. *Diseases of Aquatic Organisms* **42**(1): 11-15.

Kibenge M.J., Munir K. & Kibenge F.S. (2005) Constitutive expression of Atlantic salmon Mx1 protein in CHSE-214 cells confers resistance to infectious salmon anaemia virus. *Virology Journal* **2**: 75.

Kibenge M.T., Opazo B., Rojas A.H. & Kibenge F.S.B. (2002) Serological evidence of infectious salmon anaemia virus (ISAV) infection in farmed fishes, using an indirect enzyme-linked immunosorbent assay (ELISA). *Diseases of Aquatic Organisms* **51**: 1-11.

Kielpinski M., Kempter J., Panicz R., Sadowski J., Schütze H., Ohlemeyer S. & Bergmann S.M. (2010) Detection of KHV in freshwater mussels and crustaceans from ponds with KHV history in common carp (*Cyprinus carpio*). *Israeli Journal of Aquaculture - Bamidgeh* **62**(1): 28-37.

Killie J.A. & Jørgensen T.Ø. (1994) Immunoregulation in fish I: Intramolecular-induced suppression of antibody responses to haptenated protein antigens studied in Atlantic salmon (*Salmo salar* L.) *Developmental and Comparative Immunology* **18**(2): 123-136.

Killie J.A. & Jørgensen T.Ø. (1995) Immunoregulation in fish II: Intermolecular-induced suppression of antibody responses studied by haptenated antigens in Atlantic salmon (*Salmo salar* L.) *Developmental and Comparative Immunology* **19**(5): 389-404.

Kim M.-C., Choi J.-G., Kwon J.-S., Kang H.-M., Paek M.-R., Jeong O.-M., Kwon J.-H. & Lee Y.-J. (2010) Field application of the H9M2e enzyme-linked immunosorbent assay for differentiation of H9N2 avian influenza virus-infected chickens from vaccinated chickens. *Clinical and Vaccine Immunology* **17**(12): 1977-1984.

Kim T.J., Jung T.S. & Lee J.I. (2007b) Expression and serological application of a capsid protein of an iridovirus isolated from rock bream, *Oplegnathus fasciatus* (Temminck & Schlegel). *Journal of Fish Diseases* **30**: 691-699.

Kim W.S., Nishizawa T. & Yoshimizu M. (2007a) Non-specific adsorption of fish immunoglobulin M (IgM) to blocking reagents on ELISA plate wells. *Diseases of Aquatic Organisms* **78**: 55-59.

Kim Y.R., Hikima J., Jang H.B., Nho S.W., Park S.B., Cha I.S., Ohtani M., Eom AH., Aoki T. & Jung T.S. (2011) Identification and determination of antigenic proteins of Korean ranavirus-1 (KRV-1) using MALDI-TOF/TOF MS analysis. *Comparative Immunology, Microbiology and Infectious Diseases* **34**: 237-245.

Kimman T.G., De Leeuw O., Kochan G., Szewczyk B., van Rooij E., Jacobs L. Kramps J.A. & Peeters B. (1996) An indirect double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) using baculovirus-expressed antigen for the detection of antibodies to glycoprotein E of pseudorabies virus and comparison of the method with blocking ELISAs. *Clinical and Diagnostic Laboratory Immunology* **3**: 167-174.

References

Kinker D.R., Swenson S.L., Wu L.L. & Zimmerman J.J. (1997) Evaluation of serological tests for the detection of pseudorabies gE antibodies during early infection. *Veterinary Microbiology* **55**(1-4): 99-106.

Kirkland P.D. & Delbridge G. (2011) Use of blocking ELISA for antibodies to equine influenza virus as a test to distinguish between naturally infected and vaccinated horses: proof of concept studies. *Australian Veterinary Journal* **89** Supp 1: 45-46.

Klupp B.G., Granzow H. & Mettenleiter T.C. (2000) Primary envelopment of pseudorabies virus at the nuclear membrane requires the UL34 gene product. *Journal of Virology* **74**: 10063-10073.

Klupp B.G., Granzow H., Mundt E. & Mettenleiter T.C. (2001) Pseudorabies virus UL37 gene product is involved in secondary envelopment. *Journal of Virology* **75**(19): 8927-8936.

Klupp B.G., Hengartner C.J., Mettenleiter T.C. & Enquist L.W. (2004) Complete annotated sequence of the Pseudorabies virus genome. *Journal of Virology* **78**(1): 424-440.

Koenig P., Lange E., Reimann I. & Beer M. (2007) CP7_E2alf: A safe and efficient marker vaccine strain for oral immunization of wild boar against Classical swine fever virus (CSFV). *Vaccine* **25**: 3391-3399.

Kool M., Soullié T., van Nimwegen M., Willart M.A., Muskens F., Jung S., Hoogsteden H.C., Hammad H. & Lambrecht B.N. (2008) Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *Journal of Experimental Medicine* **205**(4): 869-882.

Koren C. & Nylund A. (1997) Morphology and morphogenesis of infectious salmon anaemia virus replicating in the endothelium of Atlantic salmon *Salmo salar*. *Diseases of aquatic organisms* **29**: 99-109.

Koumans-van Diepen J.C.E., van de Lisdonk M.H., Taverne-Thiele A.J., Verburg-van Kemenade B.M. & Rombout J.H. (1994) Characterisation of immunoglobulin-binding leukocytes in carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology* **18**(1): 45-56.

KoVax KV3 KHV vaccine. http://www.kovax.co.il/products.asp (last accessed 1 April 2013).

Kowalski J., Gilbert S.A., Van Drunen Little-van den Hurk S., Van den Hurk J., Babiuk L.A. & Zamb T. (1993) Heat shock promotor-driven synthesis of secreted bovine herpesvirus glycoproteins in transfected cells. *Vaccine* **11**: 1100-1108.

Kramer T., Greco T.M., Enquist L.W. & Cristea I.M. (2011) Proteomic characterisation of Pseudorabies virus extracellular virions. *Journal of Virology* **85**(13): 6427-6441.

Krossøy B., Devold M., Sanders L., Knappskog P.M., Aspehaug V., Falk K., Nylund A., Koumans S., Endresen C. & Biering E. (2001) Cloning and identification of the infectious salmon anaemia virus haemagglutinin. *Journal of General Virology* **82**: 1757-1765.

Krossøy B., Hordvik I., Nilsen F., Nylund A. & Endresen C. (1999) The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the orthomyxoviridae. *Journal of Virology* **73**: 2136-2142.

Kuby J. (1994) Antigen-antibody interactions. *In* Immunology. 2nd edition, W. H. Freeman and Co., New York.

Kurita J., Yuasa K., Ito T., Sano M., Hedrick R.P., Engelsma M.Y., Haenen O.L.M., Sunarto A., Kholidin E. B., Chou H.-Y., Tung M.-C., de la Peňa L., Lio-Po G., Tu C., Way K. & Iida T. (2009) Molecular epidemiology of koi herpesvirus. *Fish Pathology* **44**(2): 59-66.

Kwon J.-S., Kim M.-C., Jeong O.-M., Kang H.-M., Song C.-S., Kwon J.-H. & Lee Y.-J. (2009) Novel use of a N2-specific enzyme-linked immunosorbent assay for differentiation of infected from vaccinated animals (DIVA)-based identification of avian influenza. *Vaccine* **27**: 3189-3194.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**(5259): 680-685.

References

Lai S.K., Hida K., Shukair S., Wang Y., Figueiredo A., Cone R., Hope T.J. & Hanes J. (2009) Human immunodeficiency virus type 1 is trapped by acidic but not by neutralized human cervicovaginal mucus. *Journal of Virology* **83**: 11196-11200.

Lambrecht B., Steensels M., Van Borm S., Meulemans G. & Van Den Berg T. (2007) Development of an M2e-specific enzyme-linked immunosorbent assay for differentiating infected from vaccinated animals. *Avian Diseases* **51**(1): 221-226.

Langhorne P. & Simpson T. (1981) Natural changes in serum cortisol in Atlantic salmon (*Salmo salar* L.) during parr-smolt transformation. *In* Pickering A. D. (ed.) Stress and Fish. Academic Press, London pp. 349-350.

La Patra S.E. (1996) The use of serological techniques for virus surveillance and certification of finfish. *Annual Review of Fish Diseases* **6**: 15-28.

La Patra S.E., Roberti K.A., Rohovec J.S. & Fryer J.L. (1989a) Fluorescent antibody test for the rapid diagnosis of infectious hematopoietic necrosis. *Journal of Aquatic Animal Health* **1**(1): 29-36.

La Patra S.E., Rohovec J.S. & Fryer J.L. (1989b) Detection of infectious hematopoietic necrosis virus in fish mucus. *Fish Pathology* **24**: 197-202.

Laurent S., Blondeau C., Belghazi M., Remy S., Esnault E., Rasschaert P. & Rasschaert D. (2007) Sequential autoprocessing of Marek's disease herpesvirus protease differs from that of other herpesviruses. *Journal of Virology* **81**(11): 6117-6121.

Lauscher A., Krossøy B., Frost P., Grove S., König M., Bohlin J., Falk K., Austbø L. & Rimstad E. (2011) Immune responses in Atlantic salmon (*Salmo salar*) following protective vaccination against infectious salmon anaemia (ISA) and subsequent ISA virus infection. *Vaccine* **29**: 6392-6401.

Lee N.-S., Jung S.H., Park J.W. & Do J.W. (2012) *In situ* hybridisation detection of koi herpesvirus in paraffin-embedded tissues of Common carp *Cyprinus carpio* collected in 1998 in Korea. *Fish Pathology* **47**(3): 100-103.

Lee S.-W., Markham P.F., Markham J.F., Peterman I., Noormohammadi A.H., Browning G.F., Ficorilli B.P., Hartley C.A. & Delvin J.M. (2011) First complete genome sequence of infectious laryngotracheitis virus. *BMC Genomics* **12**: 197.

Le Morvan C., Troutaud D. & Deschaux P. (1998) Differential effects of temperature on specific and nonspecific immune defences in fish. *The Journal of Experimental Biology* **201**: 165-168.

Lequin R.M. (2005) Enzyme-immunoassay (EIA)/Enzyme-linked immunosorbent assay (ELISA). *Clinical Chemistry* **51**(12): 2415-2418.

Leung A.K.C. (2011) "Variolation" and vaccination in late imperial China, Ca 1570-1911. *In* History of vaccine development, Ed. Plotkin S. A., Springer, New York. pp. 5-12.

Li C., Ping J., Jung B., Deng G., Jiang Y., Li Y., Tian G., Yu K., Bu Z. & Chen H. (2008) H5N1 influenza marker vaccine for serological differentiation between vaccinated and infected chickens. *Biochemical and Biophysical Research Communications* **372**: 293-297.

Li D., Liu Z.X., Sun P., Li Y.L., Tian M.N., Chen Y.L., Xie B.X., Bao H.F., Fu Y.F., Cao Y.M., Li P.H., Bai X.W., Sun J.C., Guo J.H., Liu X.T. & Xie Q.G. (2010) The efficacy of FMD vaccine reduced non-structural proteins with a mAB against 3B protein. *Veterinary Research Communications* **34**(5): 445-457.

Li Z., Woo C.J., Iglesias-Ussel M.D., Ronai D. & Scharff M.D. (2004) The generation of antibody diversity through somatic hypermutation and class switch recombination. *Genes and Development* **18**: 1-11.

Lillehaug A., Lunestad B.T. & Grave K. (2003) Epidemiology of bacterial diseases in Norwegian aquaculture – a description based on antibiotic prescription data for the ten-year period 1991-2000. *Diseases of Aquatic Organisms* **53**: 115-125.

Lillehaug A., Ramstad A., Bækken K. & Retain L.J. (1993) Protective immunity in Atlantic salmon (*Salmo salar* L.) vaccinated at different water temperatures. *Fish and Shellfish Immunology* **3**: 143-156.

References

Ling S.H.M., Wang X.H., Xie L., Lim T.M. & Leung K.Y. (2000) Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in *in vivo* and *in vitro* fish models. *Microbiology* **146**: 7-19.

Lio-Po G. D. (2011) Recent developments in the study and surveillance of koi herpesvirus (KHV) in Asia, pp. 13-28. *In* Bondad-Reantaso M.G., Jones J.B., Corsin F. & Aoki T. (Eds.). Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Selangor, Malaysia.

Liu H.-S., Jan M.-S., Chou C.-K., Chen C.-K. & Ke N.-J. (1999) Is green fluorescent protein toxic to the living cells? *Biochemical and Biophysical Research Communications* **260**: 712-717.

Liu M., Wood J.M., Ellis T., Krauss S., Seiler P., Johnson C., Hoffmann E., Humberd J., Hulse D., Zhang Y., Webster R.G. & Perez D.R. (2003) Preparation of a standardised, efficacious agricultural H5N3 vaccine by reverse genetics. *Virology* **314**(2): 580-590.

Liu Y., Yuan J., Wang W., Chen X., Tang R., Wang M. & Li L. (2011) Identification of envelope protein ORF10 of channel catfish herpesvirus. *Canadian Journal of Microbiology* **58**: 271-277.

Lobb C.J. & Clem L.W. (1983) Distinctive subpopulations of catfish serum antibody and immunoglobulin. *Molecular Immunology* **20**: 811-818.

Lopez-Jimena B., Alonso d-M.C., Thompson K.D., Adams A., Infante C., Castro D., Borrego J.J. & Garcia-Rosado E. (2011) Tissue distribution of Red spotted grouper Nervous Necrosis Virus (RGNNV) genome in experimentally infected juvenile European seabass (*Dicentrarchus labrax*). *Veterinary Microbiology* **154** (1-2): 86-95.

Lopez-Jimena B., Garcia-Rosado E., Thompson K.D., Adams A., Infante C., Borrego J.J. & Alonso M.d.-C. (2012) Distribution of red-spotted grouper nervous necrosis (RGNNV) antigens in nervous and non-nervous organs of European seabass (*Dicentrarchus labrax*) during the course of an experimental challenge. *Journal of Veterinary Science* **13**(4): 355-362.

Lorenzen N. & La Patra S.E. (1999) Immunity to rhabdoviruses in rainbow trout: the antibody response. *Fish and Shellfish Immunology* **9**: 345-360.

Lorenzen N. & La Patra S.E. (2005) DNA vaccines for aquaculture. *Revue Scientifique et Technique de L' Office des Epizooties* **24**(1): 201-213.

Lorenzen N., Lorenzen E., Einer-Jensen K., Heppell J., Wu T. & Davis H. (1998) Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. *Fish and Shellfish Immunology* **8**: 261-270.

Lorenzen N. & Olsen N.J. (1997) Immunisation with viral antigens: Viral haemorrhagic septicaemia. *In* Fish Vaccinology (R. Gudding, A. Lillehaug, P. J. Midtlyng and F. Brown, eds). *Developments in Biological Standardisation* **90**: 201-209.

Lorenzen N., Olesen N.J. & Jørgensen P.E.V. (1988) Production and characterisation of monoclonal antibodies to four Egtved virus structural proteins. *Diseases of Aquatic Organisms* **4**: 35-42.

Lorenzo G., Estepa, A., Chilmonczyk S. & Coll J. (1995) Different peptides from hemorrhagic septicemia rhabdoviral proteins stimulate leucocyte proliferation with individual fish variation. *Virology* **212**: 348-355.

Loret S., Guay G. & Lippé R. (2008) Comprehensive characterisation of extracellular Herpes simplex virus type 1 virions. *Journal of Virology* **82**(17): 8605-8618.

Lu Y., Klein P.J., Xu L.C., Santhapuram H.K., Bloomfield A., Howard S.J., Vlahov I.R., Ellis P.R., Low P.S. & Leamon C.P. (2009) Strategy to prevent drug-related hypersensitivity in folate-targeted hapten immunotherapy of cancer. *The AAPS Journal* **11**(3): 628-638.

Luo Y., Yuan Y., Ankenbauer R.G., Nelson L.D., Witte S.B., Jackson J.A. & Welch S.K. (2012) Construction of chimeric bovine viral diarrhoea viruses containing glycoprotein E rns of heterologous pestiviruses and evaluation of the chimeras as potential marker vaccines against BVDV. *Vaccine* **30**(26): 3843-3848.

References

Lydyard P.M., Whelam A. & Fanger M.W. (2000) Instant notes in Immunology, UK, Bios Scientific Publishers Lda.

Lydyard P.M., Whelam A. & Fanger M.W. (2004) Instant notes in immunology, Second Edition, U. K., Bios. Scientific Publishers Lda.

Lyngøy C. (2003) Infectious salmon anaemia in Norway and the Faroe Islands: An industrial approach. *In* Miller O and Cipriano RC, technical coordinators (2003) International Response to Infectious Salmon Anemia: Prevention, Control, and Eradication: Proceedings of a Symposium, 3–4 September 2002, New Orleans, *LA.Technical Bulletin **1902***. Washington, DC: US Department of Agriculture, Animal and Plant Health Inspection Service, pp. 97-109.

Lyngstad T.M., Jansen P.A., Sindre H., Jonassen C.M., Hjortaas M.J., Johnsen S. & Brun E. (2008) Epidemiological investigation of infectious salmon anaemia (ISA) outbreaks in Norway 2003-2005. *Preventive Veterinary Medicine* **84**: 213-227.

Maas A., Meens J., Baltes N., Hennig-Pauka I. & Gerlach G. (2006) Development of a DIVA subunit vaccine against *Actinobacillus pleuropneumoniae* infection. *Vaccine* **24**: 7226-7237.

Macauley-Patrick S., Fazenda M.L., McNeil B. & Harvey L.M. (2005) Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* **22**: 249-270.

Machiels B., Lété C., de Fays K., Mast J., Dewals B., Stevenson P.G., Vanderplasschen A. & Gillet L. (2011) The bovine herpesvirus 4 Bo10 gene encodes a nonessential viral envelope protein that regulates viral tropism through both positive and negative effects. *Journal of Virology* **85**: 1011-1024.

Mackay D.K.J., Forsyth M.A., Davies P.R., Berlinzani A., Belsham G.J., Flint M. & Ryan M.D. (1998) Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant non-structural proteins in ELISA. *Vaccine* **16**(5): 446-459.

Magnadottir B., Gudmundsdottir S., Gudmundsdottir B.K. & Helgason S. (2009) Natural antibodies of cod (*Gadus morhua* L.): Specificity, activity and affinity. *Comparative Biochemistry and Physiology*. *Part B: Biochemistry and Molecular Biology* **154**(3): 309-316.

Makkay A.M., Krell P.J. & Nagy É. (1999) Antibody detection-based differential ELISA for NDV-infected or vaccinated chickens versus NDV HN-subunit vaccinated chickens. *Veterinary Microbiology* **66**: 209-222.

Manis J.P., Tian M. & Alt F.W. (2002) Mechanism and control of class-switch recombination. *Trends in Immunology* **23**: 31-39.

Manning M.J. & Nakanishi T. (1996) The specific immune system: Cellular defences. *In* The Fish Immune System: organism, pathogen and environment. Eds. Iwama G. and Nakanishi T. (1996), Academic Press, Chapter 4, pp. 159-205.

Marcos-Lopez M., Waltzek T.B., Hedrick R.P., Baxa D.V., Garber A.F., Liston R., Johnsen E., Forward B.S., Backman S. & Ferguson H.W. (2011) Characterization of a novel alloherpesvirus from Atlantic cod (*Gadus morhua*). *Journal of Veterinary Diagnostic Investigation* **24**: 65-73.

Mardones F.O., Perez A.M. & Carpenter T.E. (2009) Epidemiological investigation of the re-emergence of infectious salmon anaemia virus in Chile. *Diseases of Aquatic Organisms* **84**: 105-114.

Mardones F.O., Perez A.M., Valdes-Donoso P. & Carpenter T.E. (2011) Farm-level reproduction number during an epidemic of infectious salmon anaemia virus in southern Chile in 2007-2009. *Preventive Veterinary Medicine* **102**: 175-184.

Markussen T., Jonassen C.M., Numanovic S., Braaen S., Hjortaas M., Nilsen H. & Mjaaland S. (2008) Evolutionary mechanisms involved in the virulence of infectious salmon anaemia virus (ISAV), a piscine orthomyxovirus. *Virology* **374**: 515-527.

Mata E., Carcaboso A.M., Hernández R.M., Igartua M., Corradin G. & Pedraz J.L. (2007) Adjuvant activity of polymer microparticles and Montanide ISA 720 on immune responses to *Plasmodium falciparum* MSP2 long synthetic peptides in mice. *Vaccine* **25**: 877-885.

Matras M., Antychowicz J., Castric J. & Bergmann S.M. (2012) CyHV-3 infection dynamics in common carp (*Cyprinus carpio*) – evaluation of diagnostic methods. *Bulletin of the Veterinary Institute in Pulawy* **56**: 127-132.

Matsui K., Honjo M., Kohmatsu Y., Uchii K., Yonekura R. & Kawabata Z. (2008) Detection and significance of koi herpesvirus (KHV) in freshwater environments. *Freshwater Biology* **53**: 1262-1272.

Maule A.G., Schreck C.B. & Kaattari S.L. (1987) Changes in the immune system of coho salmon (*Oncorhynchus kisutch*) during the parr-to-smolt transformation and after implantation of cortisol. *Canadian Journal of Fisheries and Aquatic Sciences* **44**(1): 161-166.

McGeoch D.J., Rixon F.J. & Davison A.J. (2006) Topics in herpesvirus genomics and evolution. *Virus Research* **117**: 90-104.

McGill R. (2005) The salmon farm monitor. 'Northern climes, March 2005'. http://www.salmonfarmmonitor.org/ncmarch2005.shtml (accessed 01 April 2013).

McVoy M.A., Nixon D.E., Hur J.K. & Adler S.P. (2000) The ends on herpesvirus DNA replicative concatemers contain pac2 cis cleavage/packaging elements and their formation is controlled by terminal cis sequences. *Journal of Virology* **74**: 1587-1592.

Mebatsion T., Koolen M.J.M., de Vaan L.T.C., de Haas N., Braber M., Römer-Oberdörfer A., vab den Elzen P. & van der Marel P. (2002) Newcastle disease virus (NDV) marker vaccine: an immunodominant epitope on the nucleoprotein gene of NDV can be deleted or replaced by a foreign epitope. *Journal of Virology* **76**(20): 10138-10146.

Meeusen E.N.T., Walker J., Peters A., Pastoret P.-P. & Jungersen G. (2007) Current status of veterinary vaccines. *Clinical Microbiology Reviews* **20**(3): 489-510.

Melingen G.O., Nilsen F. & Wergeland H.I. (1995a) The serum antibody levels in Atlantic salmon (*Salmo salar* L.) after vaccination with *Vibrio salmonicida* at different times during the smolting and early post-smolt period. *Fish and Shellfish Immunology* **5**(3): 223-235.

Melingen G.O., Stefansson S.O., Berg A. & Wergeland H.I. (1995b) Changes in serum protein and IgM concentration during smolting and early post-smolt period in vaccinated and unvaccinated Atlantic salmon (*Salmo salar* L.) *Fish and sellfish Immunology* **5**: 211-221.

Mettenleiter T.C. (2002) Herpesvirus assembly and egress. *Journal of Virology* **76**(4): 1537-1547.

Mettenleiter T.C. (2004) Budding events in herpesvirus morphogenesis. *Virus Research* **106**: 167-180.

Mettenleiter T.C., Klupp B.G. & Granzow H. (2009) Herpesvirus assembly: An update. *Virus Research* **143**: 222-234.

Meuwissen M.P., Horst S.H., Huirne R.B. & Dijkhuizen A.A. (1999) A model to estimate the financial consequences of classical swine fever outbreaks: principles and outcomes. *Preventative Veterinary Medicine* **42**(3-4): 249-270.

Meyer K., Bergmann S.M., Van der Marel M. & Steinhagen D. (2011) Detection of koi herpesvirus: Impact of extraction method, primer set and DNA polymerase on the sensitivity of polymerase chain reaction examinations. *Aquaculture Research* **42**(11): 1-8.

Michel B., Fournier G., Lieffrig F., Costes B. & Vanderplasschen A. (2010a) Cyprinid herpesvirus 3. *Emerging Infectious Diseases* **16**(12): 1835-1843.

Michel B., Leroy B., Raj V.S., Lieffrig F., Mast J., Wattiez R., Vanderplasschen A.F. & Costes B. (2010b) The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions. *Journal of General Virology* **91**: 452-462.

Mijnes J., Van der Horst L., Van Anken E., Horzinek M., Rottier P. & De Groot R. (1996) Biosynthesis of glycoproteins E and I of feline herpesvirus: gE-gI interaction is required for intracellular transport. *Journal of Virology* **70**: 5466-5475.

Mikalsen A.B., Sindre H., Torgersen J. & Rimstad E. (2005) Protective effects of a DNA vaccine expressing the infectious salmon anaemia virus hemagglutinin-esterase in Atlantic salmon. *Vaccine* **23**: 4895-4905.

Millar D.G., Hirst T.R. & Snider D.P. (2001) *Echerichia coli* heat-labile enterotoxin B subunit is a more potent mucosal adjuvant than its closely related homologue, the B subunit of Cholera toxin. *Infection and Immunity* **69**(5): 3476-3482.

Miller O. (2003) Design and Implementation of an Infectious Salmon Anemia Program *In* Miller O. and Cipriano R.C., technical coordinators (2003). International Response to Infectious Salmon Anemia: Prevention, control, and eradication: Proceedings of a Symposium, 3–4 September 2002, New Orleans, *LA.Technical Bulletin **1902***. Washington, DC: US Department of Agriculture, Animal and Plant Health Inspection Service, pp. 167-174.

Miller O. & Cipriano R.C. (2003) International Response to Infectious Salmon Anemia: Prevention, Control, and Eradication: Proceedings of a Symposium, 3–4 September 2002, New Orleans, *LA.Technical Bulletin **1902***. Washington, DC: US Department of Agriculture, Animal and Plant Health Inspection Service.

Minamoto T., Honjo M.N., Yamanaka H., Tanaka N., Itayama T. & Kawabata Z. (2011) Detection of Cyprinid herpesvirus-3 DNA in lake plankton. *Research in Veterinary Science* **90**: 530-532.

Minamoto T., Honjo M.N., Yamanaka H., Uchii K. & Kawabata Z. (2012) Nationwide Cyprinid herpesvirus 3 contamination in natural rivers of Japan. *Research in Veterinary Science* **93**: 508-514.

Minke J.M., Audonnet J.C. & Fischer L. (2004) Equine viral vaccines: the past, present and future. *Veterinary Research* **35**: 425-443.

Mitchison N. (1971) The carrier effect in the secondary response to hapten-protein conjugates. I. Measurement of the effect with transferred cells and objections to the local environment hypothesis. *European Journal of Immunology* **1**: 10-17.

Miwa S., Ito T. & Sano M. (2007) Morphogenesis of koi herpesvirus observed by electron microscopy. *Journal of Fish Diseases* **30**: 715-722.

Miyazaki T., Kuzuya Y., Yasumoto S., Yasuda M. & Kobayashi T. (2008) Histopathological and ultrastructural features of koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of KHV. *Diseases of Aquatic Organisms* **80**(1): 1-11.

Mjaaland S., Markussen T., Sindre H., Kjøglum S., Dannevig B.H., Larsen S. & Grimholt U. (2005) Susceptibility and immune responses following experimental infection of MHC compatible Atlantic salmon (*Salmo salar* L.) with different infectious salmon anaemia virus isolates. *Archives of Virology* **150**(11): 2195-2216.

Mjaaland S., Rimstad E. & Cunningham C.O. (2002) Molecular diagnosis of infectious salmon anaemia. *In*: Molecular Diagnosis of Salmonid Diseases, Cunningham C.O., ed. Kluwer Academic Publishers, Dordrecht, The Netherlands, 1–22.

Mjaaland S., Rimstad E., Falk K. & Dannevig B.H. (1997) Genomic characterisation of the virus causing infectious salmon anaemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. *Journal of Virology* **71**: 7681-7686.

Mocarski E.S., Shenk T. & Pass R.F. (2007) Cytomegaloviruses. *In* Fields Virology 5th Edition, Knipe D.M., Howley P.M., Griffin D.E., Lamb R.A., Martin M.A., Roizman B. and Straus S.E. (Eds.) Philadelphia: Lippincott, Williams and Wilkins, pp. 2701-2772.

Mochida K., Lou Y.-H., Hara A. & Yamauchi K. (1994) Physical biochemical properties of IgM from a teleost fish. *Immunology* **8**: 675-680.

Moennig V. (2005) Eradicating versus vaccination strategies to control infectious diseases – some lessons to be learned from terrestrial animals. *In* Midtlyng P.J. (Ed.): Progress in Fish Vaccinology. *Developments in Biologicals* **121**: 13-19.

References

Mohan C.M., Dey S., Rai A. & Kataria J. (2006) Recombinant haemagglutinin neuraminidase antigen-based single serum dilution ELISA for rapid serological profiling of Newcastle disease virus. *Journal of Virological Methods* **138**: 117-122.

Mohapatra J.K., Pandey L.K., Sanyal A. & Pattnaik B. (2011) Recombinant non-structural polyprotein 3AB-based serodiagnostic strategy for FMD surveillance in bovines irrespective of vaccination. *Journal of Virological Methods* **177**: 184-192.

Molloy S., Thomas E., Hoyt K. & Bouchard D. (2013) Enhanced detection of infectious salmon anaemia virus using a low-speed centrifugation technique in three fish cell lines. *Journal of Fish Diseases* **36**: 35-44.

Monier J.C. (1975) Antigenic competition between two sequentially acting antigens: Immunosuppressive effect of T cells in spleen and lymph nodes of mouse. *Journal of Immunology* **115**(3): 644-647.

Montero J., Garcia J., Ordas M.C., Casanova I., Gonzalez A., Villena A., Coll J. & Tafalla C. (2011) Specific regulation of the chemokine response to viral haemorrhagic septicaemia virus at the entry site. *Journal of Virology* **85**(9): 4046-4056.

Moormann R.J.M., De Rover T., Briaire J., Peeters B.P.H., Gielkens A.L.I. & Van Oirschot J.T. (1990) Inactivation of the thymidine kinase gene of a gI deletion mutant of pseudorabies virus generates a safe but still highly immunogenic vaccine strain. *Journal of General Virology* **71**: 1591-1595.

Morrison R.N. & Nowak B.F. (2002) The antibody response of teleost fish. *Seminars in Avian and Exotic Pet Medicine* **11**: 46-54.

Müller A., Solem S.T., Karlsen C.R. & Jørgensen T.Ø. (2008) Heterologous expression and purification of the infectious salmon anemia virus hemagglutinin esterase. *Protein expression and purification* **62**: 206-215.

Muller J.D., Wilkins M., Foord A.J., Dolezal O., Yu M., Heine H.G. & Wang L.-F. (2010) Improvement of a recombinant antibody-based serological assay for foot-and-mouth disease virus. *Journal of Immunological Methods* **352**: 81-88.

Mullins J.E., Groman D. & Wadowska D. (1998) Infectious salmon anaemia in salt water Atlantic salmon (*Salmo salar* L.) in New Brunswick, Canada. *Bulletin of the European Association of Fish Pathologists* **18**: 110–114.

Munro L.A. & Gregory A. (2009) Application of network analysis to farmed salmonid movement data from Scotland. *Journal of Fish Diseases* **32**: 641-644.

Munro P.D., Murray A.G., Fraser D.I. & Peeler E.J. (2003) An evaluation of the relative risks of infectious salmon anaemia transmission associated with different salmon harvesting methods in Scotland. *Ocean & Coastal Management* **46**: 157-174.

Murray A.G., Munro L.A., Wallace I.S., Berx B., Pendrey D., Fraser D. & Raynard R.S. (2010) Epidemiological investigation into the re-emergence and control of an outbreak of infectious salmon anaemia in the Shetland Islands, Scotland. *Diseases of Aquatic Organisms* **91**: 189-200.

Murwantoko (2009) Cloning ORF2 membrane protein of Koi herpesvirus Lake Toba, Indonesian isolate. *Hayati Journal of Biosciences* **16**(2): 49-53.

Nagashima Y., Kikuchi N., Shimakura K. & Shiomi K. (2003) Purification and characterization of an antibacterial protein in the skin secretion of rockfish *Sebastes schlegeli*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **136**: 63-71.

Nakao M., Kajiya T., Sato Y., Somamoto T., Kato-Unoki Y., Matsushita M., Nakata M., Fujita T. & Yano T. (2006) Lectin pathway of bony fish complement: identification of two homologs of the mannose-binding lectin associated with MASP2 in the common carp (*Cyprinus carpio*). *Journal of Immunology* **177**: 5471-5479.

Nelson P., Reynolds G., Waldron E., Ward E., Giannopoulos K. & Murray P. (2000) Demystified: Monoclonal antibodies. *Molecular Pathology* **53**: 111-117.

Neukirch M., Böttcher K. & Bunnajirakul S. (1999) Isolation of a virus from koi with altered gills. *Bulletin of the European Association of Fish Pathologists* **19**(5): 221-224.

Neukirch M. & Kunz U. (2001) Isolation and preliminary characterization of several viruses from koi (*Cyprinus carpio*) suffering gill necrosis and mortality. *Bulletin of the European Association of Fish Pathologists* **21**: 125-135.

Newcomb W.W. & Brown J.C. (2009) Time-dependent transformation of the herpesvirus tegument. *Journal of Virology* **83**: 8082-8089.

Newcomb W.W. & Brown J.C. (2010) Structure and capsid association of the herpesvirus large tegument protein UL36. *Journal of Virology* **84**(19): 9408-9414.

Nicola A.V., McEvoy A.M. & Straus S.E. (2003) Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells. *Journal of Virology* **77**: 5324-5332.

Nii S. (1991) Electron microscopic study on the development of herpesviruses. *Journal of Electron Microscopy* **41**: 414-423.

Nii S., Morgan C. & Rose H.M. (1968) Electron microscopy of herpes simplex virus: II. Sequence of development. *Journal of Virology* **2**: 517-536.

Nonaka M. & Smith S.L. (2000) Complement system of bony and cartilaginous fish. *Fish and Shellfish Immunology* **10**: 215-228.

Novotny L., Pokorova D., Reschova S., Vicenova M., Axmann R., Vesely T. & Mikler J.R. (2010) First clinically apparent Koi herpesvirus infection in the Czech Republic. *Bulletin of the European Association of Fish Pathologists* **30**: 85-91.

Nylund A., Alexandersen S., Lovik P. & Jakobsen P. (1994) The response of brown trout (*Salmo trutta* L.) to repeated challenge with infectious salmon anaemia (ISA). *Bulletin of the European Association of Fish Pathologists* **14**(5): 167-170.

Nylund A., Devold M., Mullins J. & Plarre H. (2002) Herring (*Clupea harengus*): a host for infectious salmon anaemia virus (ISAV). *Bulletin of the European Association of Fish Pathologists* **22**: 311-318.

Nylund A., Hovland T., Watanabe K. & Endresen C. (1995) Presence of infectious salmon anaemia virus (ISAV) in tissues of Atlantic salmon, *Salmo salar* L., collected during three separate outbreaks of the disease. *Journal of Fish Diseases* **18**: 135-145.

Nylund A. & Jakobsen P. (1995) Sea trout as a carrier of infectious salmon anaemia virus. *Journal of Fish Biology* **47**: 174-176.

Nylund A., Kvenseth A., Krossøy B. & Hodneland K. (1997) Replication of the infectious salmon anaemia virus (ISAV) in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **20**: 275-279.

Nylund A., Wallace C. & Hovland T. (1993) The possible role of *Lepeophtheirus salmonis* (Krøyer) in the transmission of infectious salmon anaemia. In Pathogens of wild and farmed fish: Sea lice (Boxshall G. A. and Defaye D. Eds.), pp. 367-373. London: Ellis Horwood.

O'Dowd A.M., Ellis A.E. & Secombes C.J. (1998) Binding of immune complexes to Atlantic salmon peripheral blood leucocytes. *Developmental & Comparative Immunology* **22**: 439-448.

Oh M.J., Jung S.J., Choi T.J., Kim H.R., Rajendran K.V., Kim Y.J., Park M.A. & Chun S.K. (2001) A viral disease occurring in cultured carp *Cyprinus carpio* in Korea. *Fish Pathology* **36**(3): 147-151.

OIE (2009) World Organisation for Animal Health. (2009) Manual of diagnostic tests for aquatic animals.Â http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2010/A_summry.htm. Office International Des Epizooties. (Last accessed 16 March 2013).

OIE (2012) World Organisation for Animal Health. (2012) Manual of diagnostic tests for aquatic animals.Â http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2010/A_summry.htm. Office International Des Epizooties. (Last accessed 16 March 2013).

References

Olesen N.J. (1998) Sanitation of viral haemorrhagic septicaemia (VHS). *Journal of Applied Ichthyology* **14**: 173-177.

Olesen N.J. & Korsholm H. (1997) Control measures for viral diseases in aquaculture: eradication of VHS and IHN. *Bulletin of the European Association of Fish Pathologists* **17**: 229-233.

Olesen N.J., Lorenzen N. & Jørgensen P.E.V. (1991) Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence (IF) and plaque neutralisation tests (50% PNT). *Diseases of Aquatic Organisms* **10**: 31-38.

Omori R. & Adams B. (2011) Disrupting seasonality to control disease outbreaks: the case of koi herpes virus. *Journal of Theoretical Biology* **271**: 159-165.

Øster B. & Höllsberg P. (2002) Viral gene expression patterns in human herpesvirus 6B-infected T cells. *Journal of Virology* **76**: 7578-7586.

O'Toole R., von Hofsten J., Rosqvist R., Olsson P.-E. & Wolf-Watz H. (2004) Visualisation of Zebrafish infection by GFP-labelled *Vibrio anguillarum*. *Microbial Pathogenesis* **37**: 41-46.

Ourth D.D. (1982) Neutralisation of bacterial exotoxin (tetanus toxin) by channel catfish IgM antibody. *Immunology* **45**: 49-53.

Oviedo J., Rodriguez F., Gomez-Puertas P., Brun A., Gomez N., Alonso C. & Escribano J.M. (1997) High level expression of the major antigenic African swine fever virus proteins p54 and p30 in baculovirus and their potential use as diagnostic reagents. *Journal of Virological Methods* **64**(1): 27-35.

Paarlberg P.L., Lee J.G. & Seitzinger A.H. (2002) Potential revenue impact of an outbreak of foot-and-mouth disease in the United States. *Journal of the American Medical Association* **22**(7): 988-992.

Parida S., Anderson J., Cox S.J., Barnett P.V. & Paton D.J. (2006) Secretory IgA as an indicator of oro-pharyngeal foot-and-mouth disease virus replication and as a tool for post vaccination surveillance. *Vaccine* **24**: 1107-1116.

Parida S., Mahapatra M., Kumar S., Das S.C., Baron M.D., Anderson J. & Barrett T. (2007) Rescue of a chimeric rinderpest virus with the nucleocapsid protein derived from peste-des-petits-ruminants virus: use as a marker vaccine. *Journal of General Virology* **88**: 2019-2027.

Pasdeloup D., Blondel D., Isidro A.L. & Rixon F.J. (2009) Herpesvirus capsid association with the nuclear pore complex and viral DNA release involve the nucleoporin CAN/Nup214 and the capsid protein pUL25. *Journal of Virology* **83**: 6610-6623.

Pasick J. (2004) Application of DIVA vaccines and their companion diagnostic tests to foreign animal disease eradication. *Animal Health Research Reviews* **5**(2): 257-262.

Pastoret P.P., Thiry E., Brochier B. & Derboven G. (1982) Bovid herpesvirus 1 infection of cattle: pathogenesis, latency, consequences of latency. *Annals de Recherches Veterinaires* **13**: 221-235.

Paterson W.D. & Fryer J.L. (1974) Effect of temperature and antigen dose on the antibody response of juvenile coho salmon (*Oncorhynchus kisutch*) to *Aeromonas salmonicida* endotoxin. *Journal of the Fisheries Research Board of Canada* **31**: 1743-1749.

Pau C., Lam L.L., Spira T.J., Black J.B., Stewart J.A., Pellett P.E. & Respess R.A. (1998) Mapping and serodiagnostic application of a dominant epitope within the human herpesvirus 8 ORF 65-encoded protein. *Journal of Clinical Microbiology* **36**(6): 1574-1577.

Pearson H. (2004) Carp virus crisis prompts moves to avert global spread. *Nature* **427**: 577.

Peeler E., Way K. & Oidtmann B. (2009) An assessment of the impact of importing carp (*Cyprinus carpio*) vaccinated against KHV on the site level prevalence of Koi herpesvirus in England and Wales. Koi herpesvirus risk assessment, CEFAS, pp 1-55.

Penkert R.R. & Kalejta R.F. (2011) Tegument protein control of latent herpesvirus establishment and animation. *Herpesviridae* **2**: 3-23.

References

Pensaert M.B., De Smet K. & De Waele K. (1990) Extent and duration of virulent virus excretion upon challenge of pigs vaccinated with different glycoprotein-deleted Aujeszky's disease vaccines. *Veterinary Microbiology* **22**: 107-117.

Pensaert M., Labarque G., Favoreel H. & Nauwynck H. (2004) Aujeszky's disease vaccination and differentiation of vaccinated from infected pigs. *Developments in Biologicals (Basel)* **119**: 243-254.

Pereira L. (1994) Function of glycoprotein B homologues of the family herpesviridae. *Infectious Agents and Disease* **3**: 9-28.

Perelberg A., Ilouze M., Kotler M. & Steinitz M. (2008) Antibody response and resistance of *Cyprinus carpio* immunised with Cyprinid herpes virus 3 (CyHV-3). *Vaccine* **26**: 3750-3756.

Perelberg A., Ronen A., Hutoran M., Smith Y. & Kotler M. (2005) Protection of cultured *Cyprinus carpio* against a lethal viral disease by an attenuated virus vaccine. *Vaccine* **23**: 3396-3403.

Perelberg A., Smirnov M., Hutoran M., Diamant A., Bejerano Y. & Kotler M. (2003) Epidemiological description of a new viral disease afflicting cultured *Cyprinus carpio* in Israel. *The Israeli Journal of Aquaculture – Bamidgeh* **55**(1): 5-12.

Pérez-Filgueira D.M., González-Camacho F., Gallardo C., Resino-Talaván P., Blanco E., Gómez-Casado E., Alonso C. & Escribano J.M. (2006) Optimisation and validation of recombinant serological tests for African Swine Fever diagnosis based on detection of the p30 protein produced in *Trichoplusia ni larvae*. *Journal of Clinical Microbiology* **44**(9): 3114-3121.

Pérez-Filgueira D.M., Resino-Talaván P., Cubillos C., Angulo I., Barderas M.G., Barcena J. & Escribano J.M. (2007) Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV. *Virology* **364**: 422-430.

Perkins J., Clavijo A., Ortiz J.I., Salo T.J., Holland H.J., Hindson B.J. & McBride M.T. (2007a) Toward a multiplexed serotyping immunoassay for foot-and-mouth disease virus. *Journal of Veterinary Diagnostic Investigation* **19**: 180-184.

Perkins J., Parida S. & Clavijo A. (2007b) Use of a standardised bovine serum panel to evaluate a multiplexed non-structural protein antibody assay for serological surveillance of foot-and-mouth disease. *Clinical and Vaccine Immunology* **14**(11): 1472-1482.

Piačková V., Flajšhans M., Pokorová D., Reschová S., Gela D., Čížek A. & Veselỳ T. (2013) Sensitivity of common carp, *Cyprinus carpio* L., strains and crossbreeds reared in the Czech Republic to infection by cyprinid herpesvirus 3 (CyHV-3; KHV). *Journal of Fish Diseases* **36**: 75-80.

Pikarsky E., Ronen A., Abramowitz J., Levavi-Sivian B., Hutoran M., Shapira Y., Steinitz M., Perelberg A., Soffer D. & Kotler M. (2004) Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *Journal of Virology* **78**: 9544-9551.

Pikulkaew S., Meeyam T. & Banlunara W. (2009) The outbreak of koi herpesvirus (KHV) in koi (*Cyprinus carpio* koi) from Chiang Mai province, Thailand. *Thai Journal of Veterinary Medicine* **39**: 53-58.

Pilström L & Bengtén E. (1996) Immunoglobulin in fish – genes, expression and structure. *Fish and Shellfish Immunology* **6**: 243-262.

Plarre H., Devold M., Snow M. & Nylund A. (2005) Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. *Diseases of Aquatic Organisms* **66**(1): 71-79.

Plotkin J.B. (2010) Transcriptional regulation is only half the story. *Molecular Systems Biology* **6**: 406.

Plotkin S.A. (2011) History of vaccine development, Ed. Plotkin S. A., Springer, New York. pp. 5-12.

Plumb J.A. (1973) Neutralisation of channel catfish virus by serum of channel catfish. *Journal of Wildlife Diseases* **9**: 324-330.

References

Podolski S.H. (2012) Metchnikoff and the microbiome. *The Lancet* **380**(9856): 1810-1811.

Pokorova D., Piačková V., Cizek A., Reschova S., Hulova J., Vicenova M. & Vesely T. (2007) Tests for the presence of koi herpesvirus (KHV) in common carp (*Cyprinus carpio carpio*) and koi carp (*Cyprinus carpio* koi) in the Czech Republic. *Veterinarni Medicina Praha* **52**: 562-568.

Pokorova D. Reschova S., Hulova J., Vicenova M. & Vesely T. (2010) Detection of *Cyprinid herpesvirus-3* in field samples of common and koi carp by various single-round and nested PCR methods**.** *Journal of the World Aquaculture Society* **41**(5): 773-779.

Pokorova D., Vesely T., Piačková V., Reschova S. & Hulova J. (2005) Current knowledge on koi herpesvirus (KHV): a review. *Veterinarni Medicina - Czech* **50**: 139-147.

Porta C., Xu X., Loureiro S., Paramasivam S., Ren J., Al-Khalil T., Burman A., Jackson T., Belsham G.J., Curry S., Lomonossoff G. P., Parida S., Paton D., Li Y., Wilsden G., Ferris N., Owens R., Kotecha A., Fry E., Stuart D.I., Charleston B. & Jones I.M. (2013) Efficient production of foot-and-mouth disease virus empty capsids in insect cells following down regulation of 3C protease activity. *Journal of Virological Methods* **187**: 406-412.

Press C.McL. & Evensen Ø. (1999) The morphology of the immune system of teleost fishes. *Fish and Shellfish Immunology* **9**: 309-318.

Press C.McL., Evensen Ø., Retain L.J. & Landsverk T. (1996) Retention of furunculosis vaccine components in Atlantic salmon *Salmo salar* L., following different routes of administration. *Journal of Fish Diseases* **19**: 215-224.

Pross H.F. & Eidinger D. (1974) Antigenic competition: A review of non-specific antigen-induced suppression. *Advances in Immunology* **18**: 133-168.

Quint W.G.V., Gielkens A.L.J., Van Oirschot J.T., Berns A.I.M. & Cuypers H.T. (1987) Construction and characterisation of deletion mutants of pseudorabies virus: a new generation of 'live' vaccines. *Journal of General Virology* **68**: 523-534.

Rabilloud T., Vuillard L., Gilly C. & Lawrence J.J. (1994) Silver staining of proteins in polyacrylamide gels: a general overview. *Cellular and Molecular Biology* **40**(1): 57-75.

Raj. V.S., Fournier G., Rakus K., Ronsmans M., Ouyang P., Michel B., Delforges C., Costes B., Farnir F., Leroy B., Wattiez R., Melard C., Mast J., Lieffrig F. & Vanderplasschen A. (2011) Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Veterinary Research* **42**: 92-100.

Rakus K.Ł., Irnazarow I., Adamek M., Palmeria L., Kawana Y., Hirono I., Kondo H., Matras M., Steinhagen D., Flasz B., Brogden G., Vanderplasschen A. & Aoki T. (2012) Gene expression analysis of common carp (*Cyprinus carpio* L.) lines during Cyprinid herpesvirus 3 infection yields insights into differential immune responses. *Developmental and Comparative Immunology* **37**: 65-76.

Rakus K.Ł., Wiegertjes G.F., Adamek M., Siwicki A.K., Lepa A. & Irnazarow I. (2009) Resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II *B* gene polymorphism. *Fish and Shellfish Immunology* **26**: 737-743.

Ramachandran S., Davoli K.A., Yee M.B., Hendricks R.L. & Kinchington P.R. (2010) Delaying the expression of herpes simplex virus type 1 glycoprotein B (gB) to a true late gene alters neurovirulence and inhibits the gB-CD8 T-cell response in the trigeminal ganglion. *Journal of Virology* **84**: 8811-8820.

Raynard R., Murray A. & Gregory A. (2001) Infectious salmon anaemia virus in wild fish from Scotland. *Diseases of Aquatic Organisms* **46**: 93-100.

Reed L.J. & Muench H. (1938) A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology* **27**: 493-497.

Reid D.P., Szanto A., Glebe B., Danzmann R.G., & Ferguson M.M. (2005) QTL for body weight and condition factor in Atlantic salmon (*Salmo salar*): Comparative analysis with rainbow trout (*Oncorhynchus mykiss*) and Arctic charr (*Salvelinus alpinus*). *Heredity* **94**: 166-172.

References

Ren X., Harms J.S. & Splitter G.A. (2001) Bovine herpesvirus 1 tegument protein VP22 interacts with histones, and the carboxyl terminus of VP22 is required for nuclear localization. *Journal of Virology* **75**: 8251-8258.

Rey F.A. (2006). Molecular gymnastics at the herpesvirus surface. *EMBO reports* **7**: 1000-1005.

Rijkers G.T., Frederix-Wolters E.M.H. & Van Muiswinkel W.B. (1980) The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). *Immunology* **41**: 91-97.

Rijsewijk F., Pritz-Verschuren S., Kerkhoff S., Botter A., Willemsen M., Nieuwstadt T. & Haenen O. (2005) Development of a polymerase chain reaction for the detection of Anguillid herpesvirus DNA in eels based on the herpesvirus DNA polymerase gene. *Journal of Virological Methods* **124**: 87-94.

Rikardsen A.H. & Elliot J.M. (2000) Variations in juvenile growth, energy allocation and life-history strategies of two populations of arctic charr in North Norway. *Journal of Fish Biology* **56**: 328-246.

Rimstad E. & Mjaaland S. (2002) Infectious salmon anaemia virus. *APMIS* **110**: 273-282.

Rimstad E., Mjaaland S., Snow M., Mikalsen A.B. & Cunningham C.O. (2001) Characterization of the infectious salmon anemia virus genomic segment that encodes the putative hemagglutinin. *Journal of Virology* **75**: 5352-5356.

Ritchie R.J., Bardiot A., Melville K., Griffiths S., Cunningham C.O. & Snow M. (2002) Identification and characterisation of the genomic segment 7 of the infectious salmon anaemia virus genome. *Virus Research* **84**(1-2): 161-170.

Ritchie R.J., Cook M., Melville K., Simard N., Cusack R. & Griffiths S. (2001) Identification of infectious salmon anaemia virus in Atlantic salmon in Nova Scotia (Canada): evidence for functional strain differences. *Diseases of Aquatic Organisms* **44**: 171–178.

Rodger H.D., Turnbull T., Muir F., Millar S. & Richards R.H. (1998) Infectious salmon anaemia (ISA) in the United Kingdom. *Bulletin of the European Association of Fish Pathologists* **18**: 115–116.

Rodriguez L.L. & Gay C.G. (2011) Development of vaccines towards the global control and eradication of foot-and-mouth disease. *Expert Review of Vaccines* **10**: 377-387.

Roitt I. (1997) Roitt's Essential Immunology, 9th Edition. Blackwell Science Ltd., Chapter 3, Antibodies: pp. 43-62.

Rolland J.B., Bouchard D., Coll J. & Winton J.R. (2005) Combined use of the ASK and SHK-1 cell lines to enhance the detection of infectious salmon anaemia virus. *Journal of Veterinary Diagnostic Investigation* **17**: 151-157.

Rolland J.B., Bouchard D.A. & Winton J.R. (2003) Improved diagnosis of infectious salmon anaemia virus by use of a new cell line derived from Atlantic salmon kidney tissue. *In* Miller O. and Cipriano R.C., technical coordinators (2003). International Response to Infectious Salmon Anemia: Prevention, Control, and Eradication: Proceedings of a Symposium, 3–4 September 2002, New Orleans, *LA.Technical Bulletin **1902***. Washington, DC: US Department of Agriculture, Animal and Plant Health Inspection Service, pp. 63-68.

Rolland J.B. & Nylund A. (1999) Sea running brown trout: carrier and transmitter of the infectious salmon anemia virus (ISAV). *Bulletin of the European Association of Fish Pathologists* **18**: 50–55.

Rombout J.H., Huttenhuis H.B., Picchietti S. & Scapigliati G. (2005) Phylogeny and ontogeny of fish leukocytes. *Fish and Shellfish Immunology* **19**: 441-455.

Ronen A., Perelberg A., Abramowitz J., Hutoran M., Tinman S., Bejerano I., Steinitz M. & Kotler M. (2003) Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. *Vaccine* **21**: 4677-4684.

Root-Bernstein R.S. (2005) Vaccination markers: designing unique antigens to be added to vaccines to differentiate between natural infection and vaccination. *Vaccine* **23**: 2057-2059.

References

Rosenkranz D., Klupp B.G., Teifke J.P., Granzow H., Fichtner D., Mettenleiter T.C. & Fuchs W. (2008) Identification of envelope protein pORF81 of koi herpesvirus. *Journal of General Virology* **89**: 896-900.

RSE, The Royal Society of Edinburgh (2002) The scientific issues surrounding the control of infectious salmon anaemia (ISA) in Scotland. A report of the Royal Society of Edinburgh working party on infectious salmon anaemia. http://www.royalsoced.org.uk/cms/files/advice-papers/inquiry/salmon/report.pdf (last accessed 24 April 2013).

Ruben L.N., Warr G.W., Decker J.M. & Marchalonis J.J. (1977) Phylogenetic origins of immune recognition: Lymphoid heterogeneity and the hapten/carrier effect in the goldfish, *Carassius auratus*. *Cellular Immunology* **31**: 266-283.

Sadler J., Marecaux E. & Goodwin A. (2008) Detection of koi herpes virus (CyHV-3) in goldfish, *Carassius auratus* (L.), exposed to infected koi. *Journal of Fish Diseases* **31**: 71-72.

Salonius K., Simard N., Harland R. & Ulmer J.B. (2007) The road to licensure of a DNA vaccine. *Current Opinion in Investigational Drugs* **8**(8): 635-641.

Sandmeier F.C., Tracy C.R., DuPré S. & Hunter K. (2012) A trade-off between natural and acquired antibody production in a reptile: implications for long-term resistance to disease. *Biology Open* **0**: 1-5.

Sano M., Ito T., Kurita J., Yanai T., Watanabe N., Miwa S. & Iida T. (2004) First detection of koi herpesvirus in cultured common carp *Cyprinus carpio* in Japan. *Fish Pathology* **39**(3): 165-167.

Sano N., Moriwake M., Hondo R. & Sano T. (1994) *Herpesvirus cyprini*: a search for viral genome in infected fish by in situ hybridisation. *Journal of Fish Diseases* **16**: 495-499.

Sano N., Sano M., Sano T. & Hondo R. (1992) *Herpesvirus cyprini*: detection of the viral genome by in situ hybiridisation. *Journal of Fish Diseases* **15**: 153-162.

Sano T., Morita N., Shima N. & Akimoto M. (1991) *Herpesvirus cyprini*: lethality and oncogenicity. *Journal of Fish Diseases* **14**: 533-543.

Schiøtz B.L., Bækkevold E.S., Poulsen L.C., Mjaaland S. & Gjøen T. (2009) Analysis of host-and strain-dependent cell death responses during infectious salmon anemia virus infection *in vitro*. *Virology Journal* **6**: 91.

Schynts F., Baranowski E., Lemaire M. & Thiry E. (1999) A specific PCR to differentiate between gE negative vaccine and wildtype bovine herpesvirus type 1 strains. *Veterinary Microbiology* **66**: 187-195.

Secombes C.J., Hardie L.J. & Daniels G. (1996) Cytokines in fish: an update. *Fish and Shellfish Immunology* **6**: 291-304.

Serero M. & Avtalion R.R. (1978) Regulatory effect of temperature and antigen upon immunity in ectothermic vertebrates. III. Establishment of immunological suppression in fish. *Developmental and Comparative Immunology* **2**: 87-94.

Shao J.-J., Wong C.K., Lin T., Lee S.K., Cong G.-Z., Sin F.W.Y., Du J.-Z., Gao S.-D., Liu X.-T., Cai X.-P., Xie Y., Chang H.-Y. & Liu J.-X. (2011) Promising multiple-epitope recombinant vaccine against Foot-and-Mouth Disease virus type O in swine. *Clinical and Vaccine Immunology* **18**(1): 143-151.

Shapira Y., Magen Y., Zak T., Kotler M., Hulata G. & Levavi-Sevan B. (2005) Differential resistance to koi herpesvirus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (*Cyprinus carpio* L.) strains and crossbreds. *Aquaculture* **245**: 1-11.

Shen L., Stuge T.B., Zhou H., Khayat M., Barker K.S., Quiniou S.M.A., Wilson M., Bengtén E., Chinchar V. G., Clem L.W. & Miller N.W. (2002) Channel catfish cytotoxic cells: a minireview. *Developmental and Comparative Immunology* **26**(2): 141-149.

Shih H.H., Hu C.W. & Wang C.S. (2003) Detection of *Herpesvirus anguillae* infection in eel using *in situ* hybridisation. *Journal of Applied Ichthyology* **19**: 99-103.

References

Shil N.K., Markham P.F., Noormohammadi A.H., O'Rourke D. & Delvin J.M. (2012) Development of an enzyme-linked immunosorbant assay to detect chicken serum antibody to glycoprotein g of infectious laryngotracheitis virus. *Avian Diseases* **56**(3): 509-515.

Shimizu T., Yoshida N., Kasai H. & Yoshimizu M. (2006) Survival of koi herpesvirus (KHV) in environmental water. *Fish Pathology* **41**(4): 153-157.

Shin G., Palaksha K., Yang H., Shin Y., Kim Y., Lee E., Oh M. & Jung T.S. (2006) Partial two-dimensional gel electrophoresis (2-DE) maps of *Streptococcus iniae* ATCC29178 and *Lactococcus garvieae* KG9408. *Diseases of Aquatic Organisms* **70**: 71-79.

Siddiqui M. (2010) Monoclonal antibodies as diagnostics; an appraisal. *Indian Journal of Pharmaceutical Sciences* **72**: 12-17.

Sigholt T., Staurnes M., Jakobsen H.J. & Åsgård T. (1995) Effects of continuous light and short-day photoperiod on smolting, seawater survival and growth in Atlantic salmon (*Salmo salar*). *Aquaculture* **130**: 373-388.

Simko E., Brown L.L., MacKinnon A.M., Byrne P.J., Ostland V.E. & Ferguson H.W. (2000) Experimental infection of Atlantic salmon, *Salmo salar* L., with infectious salmon anaemia virus: a histopathological study. *Journal of Fish Diseases* **23**: 27-32.

Sinyakov M.S. & Avtalion R.R. (2009) Vaccines and natural antibodies: A link to be considered. *Vaccine* **27**: 1985-1986.

Sinyakov M.S., Dror M., Lublin-Tennenbaum T., Salzberg S., Margel S. & Avtalion R.R. (2006) Nano- and microparticles as adjuvants in vaccine design: Success and failure is related to host natural antibodies. *Vaccine* **24**: 6534-6541.

Sinyakov M.S., Dror M., Zhevelev H.M., Margel S. & Avtalion R.R. (2002) Natural antibodies and their significance in active immunisation and protection against a defined pathogen in fish. *Vaccine* **20**: 3668-3674.

Siwicki A.K., Kazuń K., Kazuń B. & Majewicz-Zbikowska E. (2012) Impact of cyprinid herpesvirus-3, which causes interstitial nephritis and gill necrosis, on the activity of carp (*Cyprinus carpio* L.) macrophages and lymphocytes. *Archives of Polish Fisheries* **20**: 123-128.

Skall H.F., Olesen N.J. & Mellergaard S. (2005) Viral hemorrhagic septicaemia virus in marine fish and its implications for fish farming – a review. *Journal of Fish Diseases* **28**: 509-529.

Skepper J.N., Whiteley A., Browne H. & Minson A. (2001) Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment → deenvelopment → reenvelopment pathway. *Journal of Virology* **75**(12): 5697-5702.

Smith P., Krohn R.I., Hermanson G., Mallia A., Gartner F., Provenzano, M., Fujimoto E., Goeke N., Olson B. & Klenk D.C. (1985) Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**(1): 76-85.

Snow M., Raynard R. & Bruno D. (2001) Comparative susceptibility of Arctic char (*Salvelinus alpinus*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) to the Scottish isolate of infectious salmon anaemia virus. *Aquaculture* **196**: 47-54.

Snow M., Raynard R., Murray A., Bruno D., King J., Grant, R., Bricknell I., Bain N. & Gregory A. (2003) An evaluation of current diagnostic tests for the detection of infectious salmon anaemia virus (ISAV) following experimental water-borne infection of Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* **26**: 135-145.

Soliman H. & El-Matbouli M. (2005) An inexpensive and rapid diagnostic method of Koi Herpesvirus (KHV) infection by loop-mediated isothermal amplification. *Virology Journal* **2**: 1-8.

Soliman H. & El-Matbouli M. (2010) Loop mediated isothermal amplification combined with nucleic acid lateral flow strip for diagnosis of cyprinid herpesvirus-3. *Molecular and Cellular Probes* **24**: 38-43.

Sommer A. & Mennen S. (1996) Propagation of infectious salmon anaemia virus in Atlantic saimon, *Salmo salar* L., head kidney macrophages. *Journal of Fish Diseases* **19**: 179-183.

References

Sommerset I., Krossøy B., Biering E. & Frost P. (2005a) Vaccines for fish in aquaculture. *Expert Review of Vaccines* **4**(1): 89-101.

Sommerset I., Skern R., Biering E., Bleie H., Fiksdal I.U., Grove S. & Nerland A.H. (2005b) Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. *Fish and Shellfish Immunology* **18**: 13-29.

Sørensen H.P. & Mortensen K.K. (2005) Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microbial Cell Factories* **4**: 1-8.

Sørensen K.J., De Stricker K., Dyrting K.C., Grazioli S. & Haas B. (2005) Differentiation of foot-and-mouth disease virus infected animals from vaccinated animals using a blocking ELISA based on baculovirus expressed FMDV 3ABC antigen and a 3ABC monoclonal antibody. *Archives of Virology* **150**(4): 805-814.

Sørensen K., Madsen K., Madsen E., Salt J., Nqindi J. & Mackay D. (1998) Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. *Archives of Virology* **143**: 1461-1476.

Spear P.G. & Longnecker R. (2003) Herpesvirus entry: an update. *Journal of Virology* **77**: 10179-10185.

Specker J.L. & Schreck C.B. (1982) Changes in plasma corticosteroids during smoltification of coho salmon, *Oncorhynchus kisutch*. *General and comparative endocrinology* **46**: 53-58.

Spencer J.V., Newcomb W.W., Thomsen D.R., Homa F.L. & Brown J.C. (1998) Assembly of the herpes simplex virus capsid: preformed triplexes bind to the nascent capsid. *Journal of Virology* **72**: 3944-3951.

Stafford J.L., Bengtén E., Du Pasquier L., McIntosh R.D., Quiniou S.M., Clem L.W., Millaer N.W. & Wilson M. (2006) A novel family of diversified immunoregulatory receptors in teleosts is homologous to both mammalian Fc receptors and molecules encoded within the leukocyte receptor complex. *Immunogenetics* **58**(9): 758-773.

Stagg R.M. (2003) The eradication of an outbreak of clinical infectious salmon anaemia from Scotland. *In* Miller O. and Cipriano R.C., technical coordinators (2003). International Response to Infectious Salmon Anemia:Prevention, Control, and Eradication: Proceedings of a Symposium, 3–4 September 2002, New Orleans, *LA.Technical Bulletin **1902***. Washington, DC: US Department of Agriculture, Animal and Plant Health Inspection Service, pp. 111-124.

Stagg R.M., Bruno D.W., Cunningham C.O., Raynard R.S., Munro P.D., Murray A.G., Allan C.E.T., Smail D. A., McVicar A.H. & Hastings T.S. (2001) Epizootiological investigations into an outbreak of infectious salmon anaemia (ISA) in Scotland. FRS Marine Laboratory Report No. 13/01.

Stefansson S.O., Björnsson B.T., Ebbesson L.O. & McCormick S.D. (2008) Smoltification. *In* Fish Physiology (Finn R. N. and Kapoor B. G. Eds.). Science Publishers, Inc. Enfield (NH) & IBH Publishing Co. Pvt. Ltd., New Delhi. Chapter 20, pp. 639 – 681.

Stegeman A. (1995) Pseudorabies virus eradication by area-wide vaccination is feasible. *Veterinary Quarterly* **17**(4): 150-156.

Stettler P., Devos R., Moser C., Tratschin J.-D. & Hoffmann M.A. (2002) Establishment and application of bicistronic classical swine fever virus genomes for foreign gene expression and complementation of E2 deletion mutants. *Virus Research* **85**: 173-185.

St-Hilaire S., Beevers N., Joiner C., Hedrick R.P. & Way K. (2009) Antibody response of two populations of common carp, *Cyprinus carpio* L, exposed to koi herpesvirus. *Journal of Fish Diseases* **32**: 311-320.

St-Hilaire S., Beevers N., Way K., Le Deuff R.M., Martin P. & Joiner C. (2005) Reactivation of koi herpesvirus infections in common carp *Cyprinus carpio*. *Diseases of Aquatic Organisms* **67**: 15-23.

Stils H.F. (2005) Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. *ILAR journal* **46**: 280-293.

Stolen J. & Mäkelä O. (1975) Carrier preimmunisation in the anti-hapten response of a marine fish. *Nature* **254**(5502): 718-719.

Stone D.M., Ferguson H.W., Tyson P.A., Savage J., Wood G., Dodge M.J., Woolford G., Dixon P.F., Feist S.W. & Way K. (2008) The first report of viral haemorrhagic septicaemia in farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), in the United Kingdom. *Journal of Fish Diseases* **31**: 775-784.

Strebel K., Beck E., Strohmaier K. & Schaller H. (1986) Characterisation of foot-and-mouth disease virus gene products with antisera against bacterially synthesised fusion proteins. *Journal of Virology* **57**(3): 983-991.

Stripecke R., del Carmen Villacres M., Skelton D.C., Satake N., Halene S. & Kohn D.B. (1999) Immune response to green fluorescent protein: implications for gene therapy. *Gene therapy* **6**: 1305-1312.

Suarez D.L. (2005) Overview of avian influenza DIVA test strategies. *Biologicals* **33**: 221-226.

Suarez D.L. (2012) DIVA vaccination strategies for avian influenza virus. *Avian Diseases* **56**(4 SUPPL 1.): 836-844.

Sunarto A., Liongue C., McColl K.A., Adams M.M., Bulach D., Crane M.S.J., Schat K.A., Slobedman B., Barnes A.C., Ward A.C. & Walker P.J. (2012) Koi herpesvirus encodes and expresses a functional interleukin-10. *Journal of Virology* **86**: 11512-11520.

Sunarto A., Rukyani A. & Itami T. (2005) Indonesian experience on the outbreak of koi herpesvirus in koi and carp (*Cyprinus carpio*). *Bulletin of the Fisheries Research Agency* **2**: 15-21.

Sunyer J.O., Zarkadis I.K. & Lambris J.D. (1998) Complement diversity: a mechanism for generating immune diversity? *Immunology Today* **19**(11): 519-523.

Swan C.M., Lindstrom N.M. & Cain K.D. (2008) Identification of a localised mucosal immune response in rainbow trout, *Oncorhynchus mykiss* (Walbaum), following immunisation with a protein-hapten antigen. *Journal of Fish Diseases* **31**: 383-393.

Swayne D.E., Garcia M., Beck J.R., Kinney N. & Suarez D.L. (2000) Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. *Vaccine* **18**: 1088-1095.

Syakuri H., Adamek M., Brogden G., Rakus K.Ł., Matras M., Irnazarow I. & Steinhagen D. (2013) Intestinal barrier of carp (*Cyprinus carpio* L.) during cyprinid 3-infection: Molecular identification and regulation of the mRNA expression of claudin genes. *Fish and Shellfish Immunology* **34**: 305-314.

Tadiso T.M., Lie K.K. & Hordvik I. (2011) Molecular cloning of IgT from Atlantic salmon, and analysis of the relative expression of τ, μ and δ in different tissues. *Veterinary Immunology and Immunopathology* **139**: 17-26.

Takashima J., Watanabe N., Takanori Y. & Nakamura T. (2005) The status of koi herpesvirus disease outbreaks in Lake Kasumigaura and Kitaura. *Bulletin of the Fisheries Research Agency* **2**: 65-71.

Tatner M.F. (1986) The ontogeny of humoral immunity in rainbow trout, *Salmo gairdneri*. *Veterinary Immunology and Immunopathology* **12**: 93-105.

Taussig M.J. (1977) Antigenic competition. *In* The antigens, volume IV, Sela M. (ed.), Academic Press, New York. Chapter 5, pp. 333-368.

Taylor N., Dixon P., Jeffery K., Peeler E., Denham K. & Way K. (2010) Koi herpesvirus: distribution and prospects for control in England and Wales. *Journal of Fish Diseases* **33**: 221-230.

Taylor N.G., Norman R.A., Way K. & Peeler E.J. (2011) Modelling the Koi herpesvirus (KHV) epidemic highlights the importance of active surveillance within a national control policy. *Journal of Applied Ecology* **48**: 348-355.

Teo C.G. & Griffin B.E. (1990) Visualisation of single copies of the Epstein-Barr virus genome by *In situ* Hybridisation. *Analytical Biochemistry* **186**: 78-85.

Thiry E., Dubuisson J. & Pastoret P.P. (1986) Pathogenesis, latency and reactivation of infections by herpesviruses. *Scientific and Technical Review of the Office International des Epizooties* **5**(4): 809-819.

Thomas C., Young N.J., Heaney J., Collins M.E. & Brownlie J. (2009) Evaluation of efficacy of mammalian and baculovirus expressed E2 subunit vaccine candidates to bovine viral diarrhoea virus. *Vaccine* **27**: 2387-2393.

Thorud K.E. & Djupvik H.O. (1988) Infectious anaemia in Atlantic salmon (*Salmo salar* L.). *Bulletin of the European Association of Fish Pathologists* **8**: 109–111.

Thorud K.E. & Håstein T. (2003) Experiences with regulatory responses to infectious salmon anaemia in Norway. *In* Miller O and Cipriano RC, technical coordinators (2003). International Response to Infectious Salmon Anemia:Prevention, Control, and Eradication: Proceedings of a Symposium, 3–4 September 2002, New Orleans, *LA.Technical Bulletin **1902***. Washington, DC: US Department of Agriculture, Animal and Plant Health Inspection Service, pp. 155-159.

Tian J., Sun B., Luo Y., Zhang Y. & Nie P. (2009) Distribution of IgM, IgD and IgZ in mandarin fish, *Siniperca chuatsi* lymphoid tissues and their transcriptional changes after *Flavobacterium columnare* stimulation. *Aquaculture* **288**: 14-21.

Tobar J.A., Contreras F.C., Betz Y., Bravo C., Caruffo M., Jerez S., Goodrich T. & Dhar A.K. (2010) Oral vaccination against infectious salmon anaemia in Atlantic salmon (*Salmo salar*) induces specific immunity and provides protection against infectious salmon anaemia virus challenge. World Aquaculture Society Meeting. WAS, San Diego, CA. https://www.was.org/wasmeetings/meetings/ShowAbstract.aspx?Id=20064 (Last accessed 16 March 2013).

Tomlinson S., Stanley K.K. & Esser A.F. (1993) Domain structure, functional activity, and polymerisation of trout complement protein C9. *Developmental and Comparative Immunology* **17**: 67-76.

Tonegawa S. (1983) Somatic generation of antibody diversity. *Nature* **302**: 575-581.

Tonheim T.C., Bøgwald J. & Dalmo R.A. (2008) What happens to the DNA vaccine in fish? A review of current knowledge. *Fish and Shellfish Immunology* **25**: 1-18.

Tonpitak W., Baltes N., Hennig-Pauka I. & Gerlach G.-F. (2002) Construction of an *Actinobacillus pleuropneumoniae* serotype 2 prototype live negative-marker vaccine. *Infection and Immunity* **70**(12): 7120-7125.

Toplak I., Grilc Fajfar A., Hostnik P. & Jenčič V. (2011) The detection and molecular characterisation of koi herpesvirus (KHV) in Slovenia. *Bulletin of the European Association of Fish Pathologists* **31**(6): 219-226.

Tu C., Weng M., Shiau J. & Lin S. (2004) Detection of koi herpesvirus in koi *Cyprinus carpio* in Taiwan. *Fish Pathology* **39**: 109-110.

Tumpey T.M., Alvarez R., Swayne D.E. & Suarez D.L. (2005) Diagnostic approach for differentiating infected from vaccinated poultry on the basis of antibodies to NS1, the non-structural protein of Influenza A virus. *Journal of Clinical Microbiology* **43**(2): 676-683.

Uchii K., Matsui K., Iida T. & Kawabata Z. (2009) Distribution of the introduced cyprinid herpesvirus 3 in a wild population of common carp, *Cyprinus carpio* L. *Journal of Fish Diseases* **32**: 857-864.

Uchii K., Telschow T., Minamoto T., Yamanaka H., Honjo M.N., Matsui K. & Kawabata Z. (2011) Transmission dynamics of an emerging infectious disease in wildlife through host reproductive cycles. *The ISME Journal* **5**: 244-251.

Uttenthal Å., Parida S., Rasmussen T.B., Paton D.J., Haas B. & Dundon W.G. (2010) Strategies for differentiating infection in vaccinated animals (DIVA) for foot-and-mouth disease, classical swine fever and avian influenza. *Expert Reviews in Vaccines* **9**(1): 73-87.

Valdenegro-Vega V.A., Crosbie P., Vincent B., Cain K.D. & Nowak B.F. (2013) Effect of immunization route on mucosal and systemic immune response in Atlantic salmon (*Salmo salar*). *Veterinary Immunology and Immunopathology* **151**: 113-123.

References

Van Beurden S.J., Forlenza M., Westphal A.H., Wiegertjes G.F., Haenen O.L.M. & Engelsma M.Y. (2011a) The alloherpesviral counterparts of interleukin 10 in European eel and common carp. *Fish and Shellfish Immunology* **31**: 1211-1217.

Van Beurden S.J., Leroy B., Wattiez R., Haenen O.L.M., Boeren S., Vervoort J.M., Peeters B.P.H., Rottier P.J.M., Engelsma M.Y. & Vanderplasshen A.F. (2011b) Identification and localization of the structural proteins of anguillid herpesvirus 1. *Veterinary Research* **42**: 105-120.

Van der Wal F.J., Achterberg R.P., de Boer S.M., Boshra H., Brun A., Maassen C.B.M. & Kortekaas J. (2012) Bead-based suspension array for simultaneous detection of antibodies against the Rift Valley fever virus nucleocapsid and gN glycoprotein. *Journal of Virological Methods* **183**: 99-105.

Van Drunen Little-van den Hurk S. (2006) Rationale and perspectives on the success of vaccination against bovine herpesvirus-1. *Veterinary Microbiology* **113**: 275-282.

Van Drunen Little-van den Hurk S. & Babiuk L.A. (1986) Polypeptide specificity of the antibody response after primary and recurrent infection with Bovine herpesvirus 1. *Journal of Clinical Microbiology* **23**(2): 274-282.

Van Drunen Little-van den Hurk S., Garzon S., Van Den Hurk J.V., Babiuk L.A. & Tijssen P. (1995) The role of the major tegument protein VP8 of bovine herpesvirus-1 in infection and immunity. *Virology* **206**: 413-425.

Van Drunen Little-van den Hurk S., Tikoo S.K., Van den Hurk J.V., Babiuk L.A. & Van Donkersgoed J. (1997) Protective immunity in cattle following vaccination with conventional and marker bovine herpesvirus-1 (BHV1) vaccines. *Vaccine* **15**(1): 36-44.

Van Drunen Little-van den Hurk S., Van Donkersgoed J., Kowalski J., van den Hurk J.V., Harland R., Babiuk L.A. & Zamb T.J. (1994) A subunit gIV vaccine, produced by transfected mammalian cells in culture, induces mucosal immunity against bovine herpesvirus-1 in cattle. *Vaccine* **12**(14): 1295-1302.

Van Gennip H.G.P., Bouma A., Van Rijn P.A., Widjojoatmodjo M.N. & Moormann R.J.M. (2002) Experimental non-transmissible marker vaccines for classical swine fever (CSF) by trans-complementation of E^{RNS} or E2 of CSFV. *Vaccine* **20**(11-12): 1544-1556.

Van Gennip H.G.P., Van Rijn P.A., Widjojoatmodjo M.N., de Smit A.J. & Moormann R.J.M. (2001) Chimeric classical swine fever viruses containing envelope protein E^{RNS} or E2 of bovine viral diarrhoea virus protect pigs against challenge with CSFV and induce a distinguishable antibody response. *Vaccine* **19**: 447-459.

Van Oirschot J.T. (1999) DIVA vaccines that reduce virus transmission. *Journal of Biotechnology* **73**: 195-205.

Van Oirschot J.T., Daus F., Kimman T.G. & Van Zaane D. (1991) Antibody response to glycoprotein I in maternally immune pigs exposed to a mildly virulent strain of pseudorabies virus. *American Journal of Veterinary Research* **52**: 1788-1793.

Van Oirschot J.T., Gielkens A.L.J., Moormann R.J.M. & Berns A.J.M. (1990) Marker vaccines, virus protein-specific antibody assays and the control of Aujeszky's disease. *Veterinary Microbiology* **23**: 85-101.

Van Oirschot J.T., Houwers D.J., Rziha H J. & Moonen P.J.M.L. (1988) Development of an ELISA for the detection of antibodies to glycoprotein I of Aujeszky's disease virus: a method for the serologic differentiation between infected and vaccinated pigs. *Journal of Virological Methods* **22**: 191-206.

Van Oirschot J.T., Kaashoek M.J., Maris-Veldhuis M.A., Weerdmeester K. & Rijsewijk F.A.M. (1997) An enzyme-linked immunosorbent assay to detect antibodies against glycoprotein gE of bovine herpesvirus 1 allows differentiation between infected and vaccinated animals. *Journal of Virological Methods* **67**: 23-34.

Van Oirschot J.T., Kaashoek M.J., Rijsewijk F.A.M. & Stegeman J.A. (1996) The use of marker vaccines in eradication of herpesviruses. *Journal of Biotechnology* **44**: 75-81.

Van Oirschot J.T., Rziha H.J., Moonen P.J.L.M., Pol J.M.A. & Zaane D. (1986) Differentiation of serum antibodies from pigs vaccinated with Aujeszky's disease virus by a competitive enzyme immunoassay. *Journal of General Virology* **67**: 1179-1182.

References

Van Rijn P.A., Bossers A., Wensvoort G. & Moormann R.J.M. (1996) Classical swine fever virus (CSFV) envelope glycoprotein E2 containing one structural antigenic unit protects pigs from lethal CSFV challenge. *Journal of General Virology* **77**: 2737-2745.

Van Zijl M., Wensvoort G., de Kluyver E., Hulst M., van der Gulden H., Gielkens A., Berns A. & Moormann R. (1991) Live attenuated Pseudorabies virus expressing envelope glycoprotein E1 of Hog cholera virus protects swine against both Pseudorabies and Hog cholera. *Journal of Virology* **65**(5): 2761-2765.

Vannie P., Capua I., Le Potier M.F., Mackay D.K.J., Muylkens B., Parida S., Paton D. J. & Thiry E. (2007) Marker vaccines and the impact of their use on diagnosis and prophylactic measures. *Science and Technical Review – International Office of Epizootics* **26**(2): 351-372.

Vannie P., Hutet E. & Cariolet R. (1991) Levels of virulent virus excreted by infected pigs previously vaccinated with different glycoprotein deleted Aujeszky's disease vaccines. *Veterinary Microbiology* **29**: 213-223.

Vider-Shalit T., Fishbain V., Raffaeli, S. & Louzoun Y. (2007) Phase-dependent immune evasion of herpesviruses. *Journal of Virology* **81**: 9536-9545.

Vike S., Nylund S. & Nylund A. (2009) ISA virus in Chile: evidence of vertical transmission. *Archives of Virology* **154**: 1-8.

Vilain C., Wetzel M., Du Pasquier L. & Charlemagne J. (1984) Structural and functional analysis of spontaneous anti-nitrophenyl antibodies in three cyprinid fish species: Carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and tench (*Tinca tinca*). *Developmental & Comparative Immunology* **8**: 611-622.

Villinger F., Mueller H.K., Bruckner L., Ackermann M. & Kihm U. (1989) Antibodies to foot-and-mouth disease virus infection associated (VIA) antigen: Use of a bioengineered VIA protein as antigen in an ELISA *Veterinary Microbiology* **20**: 235-246.

Vogel C. & Marcotte E.M. (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nature Reviews Genetics* **13**: 227-232.

Voronin Y., Holte S., Overbaugh J. & Emerman M. (2009) Genetic drift of HIV populations in culture. *PloS Genetics* **5**(3): e1000431.

Walsh E.P., Baron M.D., Anderson J. & Barrett T. (2000a) Development of a genetically marked recombinant rinderpest vaccine expressing green fluorescent protein. *Journal of General Virology* **81**: 709-718.

Walsh E.P., Baron M.D., Rennie L.F., Monaghan P., Anderson J. & Barrett T. (2000b) Recombinant Rinderpest vaccines expressing membrane-anchored proteins as genetic markers: Evidence of exclusion of marker protein from the virus envelope. *Journal of Virology* **74**(21):10165-10175.

Walster C. (1999) Clinical observations of severe mortalities in koi carp, *Cyprinus carpio*, with gill disease. *The Journal of the Fish Veterinary Society* (3): 54-58.

Waltzek T.B., Kelley G.O., Alfaro M.E., Kurobe T., Davison A.J. & Hedrick R.P. (2009) Phylogenetic relationships in the family Alloherpesviridae. *Diseases of Aquatic Organisms* **84**: 179-194.

Waltzek T.B., Kelley G.O., Stone D.M., Way K., Hanson L., Fukuda H., Hirono I., Aoki T., Davison A.J. & Hedrick R.P. (2005) Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae**.** *Journal of General Virology* **86**: 1659-1667.

Wang C.Y., Chang T.Y., Walfield A.M., Ye J., Shen M., Chen S.P., Li M. C., Lin Y. L., Jong M. H., Yang P. C., Chyr N., Kramer E. & Brown F. (2002) Effective synthetic peptide vaccine for foot-and-mouth disease in swine. *Vaccine* **20**: 2603-2610.

Wang H., Blair C.D., Olson K. E. & Clem R.J. (2008) Effects of inducing or inhibiting apoptosis on Sindbis virus replication in mosquito cells. *Journal of General Virology* **89**: 2651-2661.

Weatherall D.J. (2011) The specificity of serological reactions by Karl Landstiner (1936), Charles C. Thomas, Springfield, Illinois. *The FASEB Journal* **25**: 2513-2514.

References

Weiss E. & Avtalion R.R. (1977) Regulatory effect of temperature and antigen upon immunity in ectothermic vertebrates. II. Primary enhancement of anti-hapten antibody responses at high and low temperatures. *Developmental and Comparative Immunology* **1**(2): 93-103.

Weli S.C., Aamelfot M., Dale O.B., Koppang E.O. & Falk K. (2013) Infectious salmon anaemia virus infection of Atlantic salmon gill epithelial cells. *Virology Journal* **10**: 5-12.

Welsh R.M., Selin L.K. & Szomolanyi-Tsuda E. (2004) Immunological memory to viral infections. *Annual Review of Immunology* **22**: 711-743.

Wen Y., Fang W., Xiang L.-X., Pan R.-L. & Shao J.-Z. (2011) Identification of Treg-like cells in *Tetraodon*: insight into the origin of regulatory T subsets during early vertebrate evolution. *Cell and Molecular Life Sciences* **68**: 2615-2626.

Wetzel M. & Charlemagne J. (1985) Antibody diversity in fish. Isoelectrofocalisation study of individually-purified specific antibodies in three teleost fish species: Tench, carp and goldfish. *Developmental & Comparative Immunology* **9**: 261-270.

Whyte S.K. (2007) The innate immune response of fin-fish - a review of current knowledge. *Fish and Shellfish Immunology* **23**: 1127-1151.

Wigdorovitz A., Pérez Filueira D.M., Robertson N., Carrillo C., Sadir A.M., Morris T.J. & Borca M.V. (1999) Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant Tobacco mosaic virus expressing the FMDV structural protein VP1. *Virology* **264**: 85-91.

Wilson M.R. & Warr G.W. (1992) Fish immunoglobulins and the genes that encode them. *Annual Review of Fish Diseases* **2**: 201-221.

Wise J.A., Bowser P.R. & Boyle J.A. (1985) Detection of channel catfish virus in asymptomatic adult channel catfish, *Ictalurus punctatus* (Rafinesque). *Journal of Fish Diseases* **8**: 485-493.

Wolf K. & Darlington R.W. (1971) Channel catfish virus: a new herpesvirus of ictalurid fish. *Journal of Virology* **8**: 525-533.

Wolf A., Hodneland K., Frost P., Braaen S. & Rimstad E. (2013) A hemagglutinin-esterase-expressing salmonid alphavirus replicon protects Atlantic salmon (*Salmo salar*) against infectious salmon anaemia (ISA). *Vaccine* **31**: 661-669.

Workenhe S.T., Rise M.L., Kibenge M.J.T. & Kibenge F.S.B. (2010) The fight between the teleost fish immune response and aquatic viruses. *Molecular Immunology* **47**: 2525-2536.

Workenhe S.T., Wadowska D.W., Wright G.M., Kibenge M.J. & Kibenge F.S. (2007) Demonstration of infectious salmon anaemia virus (ISAV) endocytosis in erythrocytes of Atlantic salmon. *Virology journal* **4**: 13.

Xijier, Inagaki M., Kishita K. Yabe T. & Kanamaru Y. (2011) An Assay for Detecting Neutralization of Rotavirus Infection by Quantitative Determination of VP6 Protein Fluorescence Intensity. *Bioscience, biotechnology, and biochemistry* **75**: 2059-2062.

Yamamoto T., Batts W.N., Arakawa C.K. & Winton J.R. (1990) Multiplication of infectious hematopoietic necrosis virus in rainbow trout following immersion infection: whole-body assay and immunohistochemistry. *Journal of Aquatic Animal Health* **2**(4): 271-280.

Yamamoto T. & Clermont T. (1990) Multiplication of infectious hematopoietic necrosis virus in rainbow trout following immersion infection: organ assay and electron microscopy. *Journal of Aquatic Animal Health* **2**: 261-270.

Yang Y., Tung J.W., Ghosn E.E.B., Herzenberg L.A. & Herzenberg L.A. (2007) Division and differentiation of natural antibody-producing cells in mouse spleen. *Proceedings of the National Academy of Sciences of the United States of America* **104**(11): 4542-4546.

References

Yano T. (1996) The nonspecific immune system: Humoral defense. *In* Chapter 3: The Fish Immune System: organism, pathogen and environment. Eds. Iwama G. and Nakanishi T. (1996), Academic Press, Chapter 3 pp. 105 – 157.

Yasumoto S., Kuzuya Y., Yasuda M., Yoshimura T. & Miyazaki T. (2006) Oral immunisation of common carp with a liposome vaccine fusing Koi herpesvirus antigen. *Fish Pathology* **41**(4): 141-145.

Yewdell J.W., Gerhard W. & Bachi T. (1983) Monoclonal anti-heamagglutinin antibodies detect irreversible antigenic alterations that coincide with the acid activation of Influenza virus A/PR/834-Mediated hemolysis. *Journal of Virology* **48**(1): 239-248.

Yoshino M., Watari H., Kojima T. & Ikedo M. (2006) Sensitive and rapid detection of Koi herpesvirus by LAMP method. *Fish Pathology* **41**: 19-27.

Yu X., Trang P., Shah S., Atanasov I., Kim Y., Bai Y., Zhou Z.H. & Liu F. (2005) Dissecting human cytomegalovirus gene function and capsid maturation by ribozyme targeting and electron cryomicroscopy. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 7103-7108.

Yuasa K. & Sano M. (2009) Koi herpesvirus: status of outbreaks, diagnosis, surveillance and research. *The Israeli Journal of Aquaculture – Bamidgeh* **61**(3): 169-179.

Yuasa K., Ito T. & Sano M. (2008) Effect of water temperature on mortality and virus shedding in carp experimentally infected with koi herpesvirus. *Fish Pathology* **43**(2): 83-85.

Yuasa K., Kawana M., Ito T., Sano M. & Iida T. (2007) Fate of koi herpesvirus (KHV) in surviving fish post infection. – Is the brain the final habitat for the virus? *In* Abstracts of 13th International Conference of the EAFP, 17-22 Sept 2007, Grado.

Yuasa K., Kurita J., Kawana M., Kiryu I., Ohseko N. & Sano M. (2012a) Development of mRNA-specific RT-PCR for the detection of koi herpesvirus (KHV) replication stage. *Diseases of Aquatic Organisms* **100**: 11-18.

Yuasa K., Sano M., Kurita J., Ito T. & Lida T. (2005) Improvement of a PCR method with the Sph 1-5 primer set for the detection of koi herpesvirus (KHV). *Fish Pathology* **40**: 37-39.

Yuasa K., Sano M. & Oseko N. (2012b) Effective procedures for culture isolation of koi herpesvirus (KHV). *Fish Pathology* **47**(3): 97-99.

Zapata A.G., Chiba A. & Varas A. (1996) Cells and tissues of the immune system. *In* The fish immune system: Organism, pathogen and environment, Eds. Iwama G. and Nakanishi T. Academic Press Inc. pp. 12-62.

Zapata A., Varas A. & Torroba M. (1992) Seasonal variations in the immune system of lower vertebrates. *Immunology Today* **13**: 142-147.

Zaugg W. & McLain L. (1970) Adenosinetriphosphatase activity in gills of salmonids: seasonal variations and salt water influence in coho salmon, *Oncorhynchus kisutch*. *Comparative Biochemistry and Physiology* **35**: 587-596.

Zhang H.G. & Hanson L.A. (1996) Recombinant channel catfish virus (Ictalurid herpesvirus 1) can express foreign genes and induce antibody production against the gene product. *Journal of Fish Diseases* **19**: 121-128.

Zhao L. & Zheng C. (2012) The first identified nucleocytoplasmic shuttling herpesviral capsid protein: Herpes Simplex Virus Type 1 VP19C. *PloS One* **7**: e41825.

Zhao S., Jin M., Li H., Wang G., Zhang R. & Chen H. (2005) Detection of antibodies to the non-structural protein (NS1) of avian influenza viruses allows distinction between vaccinated and infected chickens. *Avian Diseases* **49**(4): 488-493.

Zhao X. & Xi J. (2011) The vaccines for Bovine herpesvirus type-1: A review. *African Journal of Biotechnology* **10**(50): 10072-10075.

Appendix I

Figure A. 1 Mascot search results of the gel band excised from Fig. 6.12. The red bar on the right represents KHV ORF92 (KHV major capsid protein) and is significant. Protein scores greater than 84 are significant (p<0.05).

Figure A. 2. Mascot search results of the gel band excised from Fig. 6.12 The red bar on the right represents KHV ORF84 (KHV capsid protein) and is significant. Protein scores greater than 84 are significant (p<0.05).

Figure A1

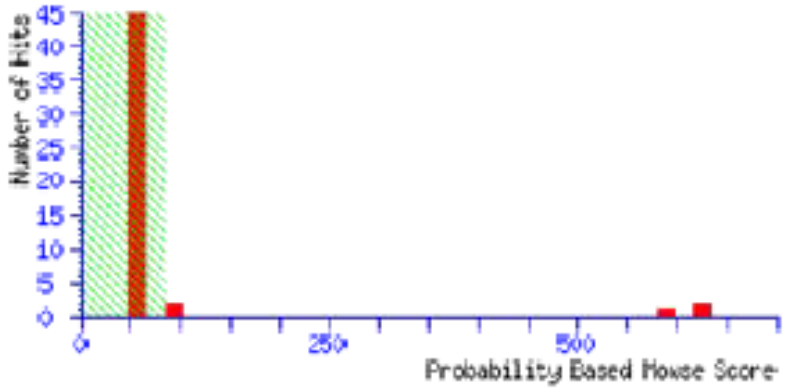


Figure A2

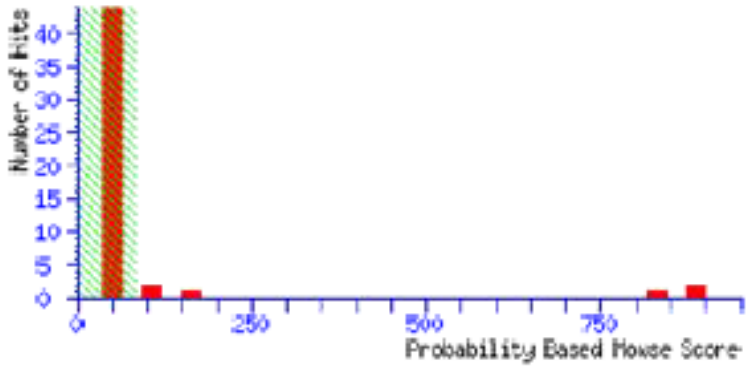


Table A1

Source country, clinical signs of koi and PCR results of cases from Singapore Koi herpesvirus (KHV) Surveillance Program used in study. Cases from Singapore surveillance programme described in Section 6.2.3.

Case ID	Clinical signs	Source country	PCR results and primers used	KHV exposure group	Fish id
Group 1					
C1	Clinically healthy koi.	Malaysia	Positive by IQ2000 test kit & Bercovier TK primers.	1	12-14
C2	Diseased koi. Reddened skin and loose scales with ulcerated body, pale gills and cloudy eyes.	Malaysia	Positive by IQ2000 test kit	1	15-18
C3	Clinically healthy koi associated with C1 and C2.	Malaysia	Negative by Bercovier TK primers.	1	19-26
C4	Diseased. Koi were thin with reddened, dry skin, cloudy and sunken eye.	Malaysia	Positive by Bercovier TK primers.	1	27-38
C5	Clinically healthy koi.	Malaysia	Positive by IQ2000 test kit and Yuasa-Grey Sph primers.	1	39-40
C6	Diseased koi with reddened skin, loose scales and pale gills.	Malaysia	Positive by IQ2000 test kit and Yuasa-Grey Sph primers.	1	41-42
C7	Diseased koi.	Unknown	Positive by Bercovier TK and Yuasa-Grey Sph primers.	1	43-47
C8	Clinically healthy when sampled but all 30 koi were dead on arrival. Pale gills and ascitic fluid in abdomen.	Malaysia	Positive by Bercovier TK and Yuasa-Grey Sph primers.	1	48-52
C9	Clinically healthy koi, 2 out of 30 koi dead on arrival.	Malaysia	Positive by Bercovier TK and Yuasa-Grey Sph primers.	1	53-57
C10	Clinically healthy koi.	Malaysia	Positive by Bercovier TK primers.	1	58-63
C11	Clinically healthy koi.	Malaysia	Positive by Bercovier TK primers.	1	64
C27	Diseased koi with abnormal swimming, showing respiratory distress and pale gills. Reddening of the body and mouth seen.	Singapore	Positive by real-time TaqMan PCR.	1	144-146
C31	Diseased koi.	Singapore	Positive by real-time TaqMan PCR.	1	156-158
2					
C12	Clinically healthy koi.	Malaysia	Negative by IQ2000 test kit and Yuasa-Grey Sph primers.	2	65-74

C13	Clinically healthy koi.	Malaysia	Negative by Bercovier TK primers.	2	75-76
C14	Clinically healthy koi.	Japan	Negative by Bercovier TK primers.	2	77-80
C15	Clinically healthy koi.	Japan	Negative by Bercovier TK primers.	2	81-90
C16	Clinically healthy koi.	China	Negative by Bercovier TK primers.	2	91
C17	Clinically healthy koi.	Japan	Negative by Bercovier TK primers.	2	92-97
C18	Clinically healthy koi with pale gills.	Malaysia	Negative by Bercovier TK primers.	2	98
C19	Clinically healthy koi.	Malaysia	Negative by Bercovier TK primers.	2	99-105
C20	Clinically healthy koi.	Singapore	Negative by Bercovier TK primers.	2	106-109
C21	Clinically healthy koi.	Japan	Negative by Bercovier TK primers.	2	110-112
C22	Clinically healthy koi.	Japan	Negative by Bercovier TK primers.	2	113-120
C23	Clinically healthy koi.	Japan	Negative by Bercovier TK primers.	2	121-129
C24	Clinically healthy koi.	Malaysia	Negative by Bercovier TK primers.	2	130-138
C25	Clinically healthy koi.	Malaysia	Negative by Bercovier TK primers.	2	139-140
C26	Clinically healthy koi.	Malaysia	Negative by Bercovier TK primers.	2	141-143
C28	Clinically healthy koi.	Singapore	Negative by Bercovier TK primers.	2	147-149
C29	Clinically healthy koi.	Singapore	Negative by Bercovier TK primers.	2	150-152
C30	Clinically healthy koi.	Singapore	Negative by Bercovier TK primers.	2	153-155
C32	Clinically healthy koi.	Malaysia	Negative by real-time TaqMan PCR.	2	159-161
C33	Clinically healthy koi.	Singapore	Negative by real-time TaqMan PCR.	2	162-163
C34	Clinically healthy koi.	Malaysia	Negative by real-time TaqMan PCR.	2	164-165
C35	Clinically healthy koi.	Japan	Negative by real-time TaqMan PCR.	2	166-173

Appendix 2

PAPERS SUBMITTED FOR PUBLICTION

Monaghan S.J., Thompson K.D., Adams A. and Bergmann S.M. 2013. Sensitivity of seven PCRs for early detection of koi herpesvirus in experimentally infected carp (*Cyprinus carpio* L.) by lethal and non-lethal sampling methods *Journal of Fish Diseases* (*Submitted*)

PAPERS PRESENTED AT CONFERENCES

Monaghan S.J., Thompson K.D., Smith P.D. and Adams A. 2009. Development of DIVA vaccines for the protection of Atlantic salmon (*Salmo salar* L.) against Infectious salmon anaemia (ISA). *Presented at* NowPas Salmonid Doctoral Research Workshop, Mar 18 – 22, Southampton, England

Monaghan S.J., Bergmann S.M., Thompson K.D., Werner I. and Adams A. 2011. Koi herpesvirus: Early stage molecular detection in carp, *Cyprinus carpio*, and a DIVA strategy. *Presented at* Institute of Aquaculture student Seminar, University of Stirling, March, Stirling, Scotland

Bergmann S.M., **Monaghan S.**, Thompson K.D., Adams A. and Fictner D. 2011 Investigation on early pathogenesis of KHVD in carp (*Cyprinus carpio* L.). *Presented by Dr. Sven Bergmann* at the International symposium on Emergence of viral diseases: Ecology and Evolution of Koi herpesvirus. Institute for Evolution and Biodiversity, Westfälische, Wilhelms-Universität, Jul 4-5, Münster, Germany

Bergmann S.M., **Monaghan S.J.**, Adams A. and Fichtner D. 2011. Investigation on Early Pathogenesis of KHVD in Carp (*Cyprinus carpio* L.). *Presented by Dr. Sven Bergmann* at European Association of Fish Pathologists 15th International Conference on Diseases of Fish and Shellfish, Sept 12 -16, Split, Croatia

Monaghan S.J., Chee D.M., Thompson K.D., Leaver M., Bergmann S.M., Auchinachie N., Muir K., Jung T.S., Aoki T. and Adams A. 2011. Applications of serological diagnostics for aquaculture: Developments and challenges of DIVA (Differentiating Infected from Vaccinated Animals) strategies. *Presented at* European Association of Fish Pathologists 15th International Conference on Diseases of Fish and Shellfish, Sept 12 -16, Split, Croatia

Bergmann S.M., **Monaghan S.**, Adams A. and Fichtner D. Pathogenesis of koi herpesvirus disease (KHVD) in carp, 2012 *Presented by Dr. Sven Bergmann* at the European Union Reference Laboratory for Fish Diseases, 16th Annual meeting of the National Reference Laboratories for Fish Diseases (National Veterinary Institute, Technical University of Denmark, May 30-31, Aarhus, Denmark

Monaghan S.J., Chee D.M., Thompson K.D., Bergmann S.M., Jung T.S., Aoki T., Leaver M., Bron J.E., Auchinachie N., Muir K., McEwan H.J., Smith P.D. and Adams A. 2012. A DIVA (Differentiating Infected from Vaccinated Animals) strategy for vaccination against Koi Herpesvirus Disease (KHVD). *Presented at* 3rd biennial Institute of Aquaculture Ph.D Research conference, University of Stirling, Oct 24, Stirling, Scotland

Thompson K.D., **Monaghan S.J.**, Adams A. and Bergmann S.M. 2012. Assessment of lethal and non-lethal sampling methods and molecular tools for early detection of koi herpesvirus in acute infected carp (*Cyprinus carpio* L.). *Presented by Dr. Kim Thompson* at International Conference on One Health and 24th VAM Congress, Sept 21-23, Marriott Putrajaya, Malaysia.

Thompson K.D., Herath T., **Monaghan S.J.** and Adams A. 2012 The use of *in vitro* assay systems for vaccine efficacy testing and marker vaccine development for fish. *Presented by Dr. Kim Thompson* at the International Vaccine Workshop, National Fisheries Research and Development Institute, Dec 11-12, Busan, Republic of Korea