STUDIES ON THE CHARACTERISATION AND DETECTION OF *Piscirickettsia salmonis*

By

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To Tayfun and my family

for their endless love and support

DECLARATION

I hereby declare that this thesis has been composed entirely by myself and is the result of my own investigations. It has not been previously submitted or accepted for any other degrees.

Sema Yuksel

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ABSTRACT

Piscirickettsia salmonis, the aetiological agent of piscirickettsiosis, has recently been responsible for significant disease outbreaks in a variety of economically important freshwater and seawater fish species cultured worldwide. The development of effective control strategies for the disease has been limited due to a lack of knowledge about the physiology, intracellular growth, transmission and pathogenesis of the organism. It is also notknown how *P. salmonis* isolates differ when isolated from different locations and from different fish species. The aim of the present study was to characterise phenotypic and serological differences between various *P. salmonis* isolates. The growth of these isolates *in vitro* was also examined together with their virulence and their pathogenesis. The antibody response of fish to live and killed *P. salmonis* was examined in an attempt to understand the antigenicity of the organism, and various diagnostic techniques were developed as a means of controlling the disease.

A simple and effective method for the purification of *P. salmonis* from fish cells to examine the antigenic properties of *P. salmonis* was developed. *P. salmonis* purified using differential pelleting and 30% Percoll (v/v) gradient gave yields with the highest purity and the highest infectivity. Fish cell lines were used to examine growth characteristics of *P. salmonis* isolates *in vitro.* It appears that *P. salmonis* has a 3-5 days (d) lag-phase and an 8 d log-phase of exponential growth in CHSE-214 cells. When the susceptibility of different fish cell lines to different *P. salmonis* isolates were compared, the highest $TCID_{50}$ ml^{-1} was obtained in CHSE-214, SHK-1 and EPC cells.

The antigenic structure of the ten *P. salmonis* isolates was analysed using SDS-PAGE. *P. salmonis* isolates shared many bands but did appear to have differences in the low molecular weight regions of their profiles. Silver staining of Proteinase-K digested *P. salmonis* isolates showed that all isolates contained carbohydrate moieties below 30 kDa. Further characterisation of these was performed using a glycoprotein determination kit and a number of different biotin-labelled lectins. The SDS-PAGE profile of isolate R-29 appeared different when passaged three times through fish cell lines. The bacteria grown in CHSE-214, EPC and SBL cells, which showed a higher susceptibility to the bacterium, had more material in the lower region of the gel than bacteria recovered from less susceptible BF-2 and RTG-2 cells where the bands in this region had disappeared. A nested polymerase chain reaction (N-PCR) was used to differentiate different *P. salmonis* isolates. Isolate R-29 was the only isolate which resulted in a PCR product different to that of the other isolates examined.

A whole cell preparation of purified *P. salmonis* (type strain LF-89) was used to prepare a rabbit polyclonal antibody and six monoclonal antibodies. These antibody probes were used to compare the antigenicity between *P. salmonis* isolates. Major antigens of *P. salmonis* were observed at 95, 72, 60, 36, 32 and 20 kDa in Western blot (WB) analysis. The antibodies were also used to develop a variety of antibody-based tests for the more specific determination of the pathogen. A number of staining methods was also tried in an attempt to establish a rapid diagnostic method for piscirickettsiosis.

The virulence of *P. salmonis* isolates was compared in an experimental infection of Atlantic salmon. It was possible to re-isolate the bacterium from infected kidney tissue during the first 4 weeks post-inoculation (wpi) and to detect by ELISA in the kidney of fish sampled 8 wpi. The histopathology observed was similar to that seen during natural outbreaks of piscirickettsiosis. No obvious difference was seen in the pathology between the different isolates used in the experimental infection. Atlantic salmon were immunised with heat–killed preparation of various *P. salmonis* isolates. The highest antibody response was obtained in sera raised against *P. salmonis* isolate R-29. This was also found to be the case in fish infected with live R-29 compared with other *P. salmonis* isolates. Sera from fish either challenged with live bacteria or immunised with heat-killed bacteria were examined in WB analysis. Proteins between 30-60 kDa appeared to be recognised with sera raised against live *P. salmonis*, unlike the sera raised against heat-killed *P. salmonis*, except for a strong reaction with the band seen at 60 kDa with sera from fish immunised with isolate R-29. Other bands at 95, 72 and 20 kDa were recognised by the fish sera raised against either heat-killed *P. salmonis* or the live bacteria.

For the first time, phage particles were observed to be associated with rickettsia infecting fish. Attempts were made to identify and characterise the phages in this study with a view to using them in the control of *P. salmonis*. The significance of the characterisation of *P. salmonis* isolates and the development of tests to detect and identify the pathogen carried out in this study is discussed within this thesis, together with further research which may lead to the development of successful control strategies for piscirickettsiosis.

ABBREVIATIONS

μg	: Microgram
μl	: Microlitre
μm	: Micrometre
μM	: Micromolar
16S rDNA	: 16 small subunit ribosomal deoxyribonucleic acid
16S rRNA	: 16 small subunit ribosomal ribonucleic acid
23S rDNA	: 23 large subunit ribosomal deoxyribonucleic acid
23S rRNA	: 23 large subunit ribosomal ribonucleic acid
ABP	: Avidin-biotin-peroxidase complex
ATCC	: American Tissue Culture Collection
bp	: Base pair
BF-2	: Bluegill, *Lepomus macrochirus* Rafinesque fry cell line
BSA	: Bovine serum albumine
BS-1	: *Bandeiraea simplicifolia*
CHSE-214	: Chinook salmon, *Oncorhynchus tshawytscha* embryo cell line
Con A	: Jack bean (*Canavalia ensiformes*)
CPE	: Cytopathic effect
cpm	: Count per minute
CSS	: Chromogenic substrate solution
CTA	: Coral tree (*Erythrina cristagalli*)
d	: Days
DBI	: Dot blot immunobinding assay
dpi	: Days post-inoculation
dpif	: Days post-infection
DMDS	: Diatrizoate meglumine and diatrizoate sodium
DMEM	: Dulbecco's Modified Eagle's Medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
EDTA	: Ethylenediaminetetraacetic acid
ELISA	: Enzym-linked immunosorbent assay
EM	: Electron microscopy
EPC	: Epithelioma papulosum cyprini *Cyprinus carpio* cell line
FAT	: Fluorescent antibody technique

FBS : Foetal bovine serum

FCS : Foetal calf serum

FITC : Fluorescein isothiocyanate

h : Hours

HAT : Hypoxanthine aminopterin thymidine media supplement

H&E : Haematoxylin and Eosin

HGA : Horse gram (*Dolichos biflorus*)

HRP : Horseradish peroxidase

HSWB : High salt wash buffer

Hsp : Heat shock protein

IFAT : Indirect fluorescent antibody technique

ICU : Infected cell counting units

IgG : Immunoglobulin G

IGS : Immuno-gold staining

IHC : Immunohistochemistry

IM : Intramuscular

IOA : Institute of Aquaculture, University of Stirling

IP : Intraperitoneal

ITS : Internal transcribed spacer

IU : International units

IV : Intravaneous

kbp : Kilo base pair

kDa : Kilodalton

kV : Kilovolt

LBI : Line blot immunoassay

LM : Light microscopy

LOS : Lipo-oligosaccharide

LPS : Lipopolysaccharide

LSD : Least significant difference

LSWB : Low salt wash buffer

min : Minutes

M : Molar

MEM : Minimal essential medium

MAb : Monoclonal antibody

MAbs : Monoclonal antibodies

MIFAT : Microimmunofluorescence test

mM	: Millimolar
mrbc	: Mouse red blood cells
MSFG	: Mediterranean Spotted Fever group (rickettsiae)
MW	: Molecular weight
ng	: Nanogram
nm	: Nanometre
NEAA	: Non-essential amino acids
NCIMB	: National Collections of Industrial and Marine Bacteria, United Kingdom
N-PCR	: Nested polymerase chain reaction
OD	: Optical density
OMPs	: Outer membrane protein
OspA	: Outer surface protein
PAb	: Polyclonal antibody
PAbs	: Polyclonal antibodies
PAS	: Periodic acid-schiff
PBS	: Phosphate buffer saline
PCR	: Polymerase chain reaction
PEG	: Polyethylene glycol
PFU	: Plaque forming units
pi	: Post-immunisation
PIPES	: Piperazine-N, N,-bis-(2-ethanolsulfonic acid)
PNA	: Peanut (*Arachis hypogaea*)
Rf	: Relative Mobility
RLO	: Rickettsia-like organism
RNA	: Ribonucleic acid
rOmpB	: Rickettsial outer membrane protein B
RTG-2	: Rainbow trout, *Oncorhynchus mykiss* gonad cell line
s	: Seconds
SBL	: Sea bass, *Cetropristis striata* larvae cell line
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELISA	: Sandwich Enzym-linked immunosorbent assay
SFG	: Spotted fever group (rickettsia)
SHK-1	: Atlantic salmon, *Salmo salar* head kidney cell line
SKDM	: Selective kidney disease medium
SPA	: Species-specific protein antigen
STG	: Scrub typhus group (rickettsiae)

TAG	: Tomato (*Lycopersicon esculentum*)
TAE	: Tris acetate EDTA buffer
TB	: Transblot buffer
TBS	: Tris buffered saline
TCEs	: T cell epitopes
$TCID_{50}$	: 50% Tissue culture infective dose
TEM	: Transmission electron microscopy
TEMED	: N, N, N', N'-tetramethyl-ethylendiamine
TG	: Typhus group (rickettsiae)
TMB	: 3, 3', 5, 5'-tetramethylbenzidine dihydrochloride
TO-2	: Nile tilapia, *Oreochromis niloticus* ovary origin cells
TS	: Tris-sucrose buffer
TSA	: Tryptone soya agar
TTBS	: Tween 20-tris buffered saline
UEA-1	: Gorse seed (*Ulex europaneus*)
UK	: United kingdom
UV	: Ultraviolet
V	: Volt
VLPs	: Virus-like particles
v/v	: Volume by volume
w/v	: Weight by volume
Wb	: Wash buffer
WB	: Western blot
WGA	: Wheat (*Triticum vulgaris*)
wpi	: Weeks post-inoculation

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CHAPTER I

LITERATURE REVIEW

1.1. Introduction

The Rickettsias constitute a relatively small, but important group of obligate, intracellular micro-organisms that cause disease in both man and other vertebrate and invertebrate hosts (Buxton & Fraser 1977; Weiss & Moulder 1984).

The Rickettsias are classified into two orders, *Rickettsiales* and *Chlamydiales* (Weiss & Moulder 1984). The *Rickettsiales* are clearly different from the *Chlamydiales,* which are a narrowly defined group of energy-parasitizing, obligately intracellular bacteria (Weisburg, Dobson, Samuel, Dasch, Mallavia, Baca, Mandelco, Sechrest, Weiss & Woese 1989). Gram negative, coccoid and non-motile microorganisms, *Chlamydiales* are characterised by their ability to change from a small, rigid-walled infectious agent (elementary body) into a larger, thin walled, non-infectious form (initial body) that divides by fission. The developmental cycle is complete when the daughter cells re-organise and condense into a new generation of elementary bodies, which survive extracellularly to infect other host cells (Page 1974; Moulder 1985). Most of the organisms which belong to the order *Rickettsiales* are pleomorphic, and may vary from coccoid, ellipsoid or coccobacillary to rod-shaped, and occasionally are found as filamentous forms with their size ranging from 0.3 to 2.0 micrometer (μm) in length and 0.3 to 0.5 μm in width (Buxton & Fraser 1977). These non-motile, Gram negative micro-organisms have typical bacterial cell walls, no flagellum and only multiply within host cells (Weiss & Moulder 1984). Although they vary widely in their

characteristics, they all undergo binary fission (Turnbull 1993). The order *Rickettsiales* consists of a group of prokaryotes that share an intimate association with eukaryotic cells. In most cases the relationship with their host is obligate intracellular parasitism (Weisburg *et al.* 1989) or mutualism. The parasitic forms are associated with reticuloendothelial and vascular endothelial cells or erythrocytes of vertebrates and arthropods, which may act as either vectors or primary hosts. The mutualistic forms of rickettsias seen in insects are considered as essential for development and reproduction of the host animal (Weiss & Moulder 1984).

Initially, only one rickettsial species, *Neorickettsia helminthoeca,* responsible for salmon poisoning disease in canines, was thought to be associated with fish. It has an extraordinary mode of transmission from salmonids through the eggs, larvae and adults of a trematode vector, *Nanophyetus salmincola,* to dogs but it is not, however, a fish pathogen (Fryer & Mauel 1997; Davies 1986). Until the end of the eighties, the role of rickettsia in fish disease was unrecognised. Prior to 1989, Wolf (1981) reported that fish as well as terrestrial vertebrates are subjected to infections caused by bacteria, fungi, parasites and viruses. Wolf (1981) also reported that although chlamydia and rickettsia had been reported to cause several diseases in mammals and birds, only one chlamydia was known to infect fish and therefore the existence of rickettsia infection in fish was open to question since rickettsial diseases of terrestrial vertebrates require parasitic arachnoids and insects as a vector or transport agent of which there were few if any in aquatic environments. However, current literature reflects a large and diverse group of Gram negative, intracellular prokaryotic organisms which make up the group of Rickettsias, that are known to infect aquatic poikilotherms. Most of these infections are in marine or anadromous hosts, but they have also been reported in freshwater

environments. In the last twenty years, at least 25 species of marine bivalves and 12 aquatic crustaceans have been reported to be infected with intracellular rickettsia-like and chlamydia-like organisms in France, Canada, Japan, Spain, Scotland, Sweden, Singapore, Malaysia and USA (Fryer & Lannan 1994). Only recently was epitheliocystis shown to be a disease caused by chlamydia-like organisms belonging to the order *Chlamydiales* (Turnbull 1993). However, none of these chlamydia-like organisms have been isolated from freshwater or seawater fish, and the description of their cellular morphology and pathology is limited (Fryer & Lannan 1994). In recent years, the importance placed on these intracellular bacteria in fish disease has changed dramatically and rickettsial agents are now known to have a significant impact on fish health (Fryer & Lannan 1994).

1.2. Historical Background

Current literature has shown that the rickettsia-like organisms (RLO) observed or isolated from a variety of fish species in different geographical locations and aquatic environments around the world, make up an important group of fish pathogens, with the potential for significant effect on the health of both freshwater and seawater species (Fryer & Lannan 1996). However, prior to 1989 there were few reports of RLO in fish.

The first report of RLO associated with fish, occurred during an examination of a dead *Tetradon fahaka* from the Nile River in Egypt in 1939 (Mohamed 1939). The organism, a round coccus structure, approximately 0.25 μm in diameter, was seen in the blood and in some of the internal organs of the fish stained with Giemsa. They were named as a new species "*Rickettsia pisces*". However, no additional cases of the disease were reported either in that particular species of fish or at the same location.

No further reports of rickettsia in fish occurred until 1975 when Ozel and Schwanz-Pfitzer (1975) first cultured an intracellular RLO from rainbow trout, *Oncorhynchus mykiss* collected from a freshwater source in Germany, while testing for Egtved (Viral Hemorrhagic Septicemia) virus. They did not, however, characterise it morphologically or establish the nature of the agent associated with these mortalities in rainbow trout (Fryer & Lannan 1994). In 1986, RLO was observed in a marine fish, dragonet *Callionymus lyra* collected from Cardigan Bay, Wales, during an examination of tissue sections for a common blood parasite of dragonets under Electron microscopy (EM) (Davis 1986).

The role of rickettsiae as emerging pathogens of fish became apparent in 1989 (Fryer & Lannan 1996). During 1989, an estimated 1.5 million coho salmon *Oncorhynchus kisutch* roughly 200 g to market size (approximately 2 kg) located in areas around Puerto Montt and the Island of Chiloe in Chile, died of a disease with an unknown etiology. These mortalities resulted in losses of more than 10 million US dollars to the Chilean fish farming industry (Cvitanich, Garate & Smith, 1990). Average mortalities which occurred in some locations were approximately 60% with losses of up to 90%. No common factors were evident among the infected fish, which occurred in a variety of stocks, hatcheries, and water supplies during the freshwater phase of production. The fish had also been fed different types of food (Branson & Nieto Diaz-Munoz 1991). The disease was only observed in coho salmon and not in chinook salmon *Oncorhynchus tshawytscha*, Atlantic salmon *Salmo salar* or rainbow trout *O. mykiss*, also present in the affected area (Fryer, Lannan, Garcés, Larenas & Smith 1990). This disease was associated with high mortalities in coho salmon as early as 1981, and was known as 'Coho Salmon Syndrome' or 'Huito Disease'. The epizootics typically

occurred and peaked in autumn with repeat outbreaks in the following spring (Bravo & Campos 1989; Cvitanich *et al.* 1990).

Bravo and Campos (1989), who first described the disease, observed an unidentified parasite in the blood and internal organs of infected fish by both light microscopy (LM) and EM, although no infectious agent was isolated from diseased fish. After the outbreaks, intensive investigations were initiated to isolate and identify the causative agent, describe the pathology caused by the agent, develop diagnostic methods, examine the fish immune response to the pathogen and conduct field trials with antibiotics (Cvitanich *et al.* 1990). The causative agent of the disease was first isolated using fish cell lines, from infected coho salmon reared in seawater in Chile. The organism fulfilled Koch's postulates by reproducing the disease experimentally in coho salmon in both fresh and seawater aquaria and confirming the presence of the agent by reisolating the organisms from infected fish (Fryer *et al.* 1990; Cvitanich, Garate & Smith 1991). Initially, the organism was thought to be a pathogen only of coho salmon, but it was then shown to cause disease and mortality in Atlantic salmon, chinook salmon and rainbow trout, leading to the name for the disease of 'Salmonid rickettsial septicaemia' (SRS) (Cvitanich *et al.* 1991). Since 1989, a number of reports have documented rickettsial disease in different salmonid species: coho salmon; rainbow trout; chinook salmon and Atlantic salmon; in both seawater and freshwater in Chile (Fryer *et al.* 1990; Cvitanich *et al.* 1991; Branson & Nieto Diaz-Munaz 1991; Garcés, Larenas, Smith, Sandino, Lannan & Fryer 1991; Fryer & Lannan 1992; Bravo 1994; Cvitanich, Garate, Silva, Andrade, Figueroa & Smith 1995; Gaggero, Castro & Sandino 1995). The previously undescribed obligate intracellular pathogen isolated from infected fish, was named *Piscirickettsia salmonis* gen.nov., sp. nov, a rickettsial

organism belonging to the order *Rickettsiales*, family *Rickettsiaceae*. This became known as the agent of the epizootic outbreaks occurring in the marine netpen-reared coho salmon in southern Chile, with the disease, referred to as piscirickettsiosis (Fryer, Lannan, Giovannoni & Wood 1992).

After isolation of the RLO from Chilean salmonids, rickettsial infections were identified in netpen-reared Atlantic salmon in the coastal waters of British Colombia in 1991. The clinical signs and post-mortem findings of the disease were similar to the disease reported in farmed Chilean coho salmon caused by RLO (Brocklebank, Speare, Armstrong & Evelyn 1992). An identical disease had been reported in pink salmon *Oncorhynchus gorbuscha* cultured in seawater tanks in British Colombia in the 1970's and in farmed coho and chinook salmon in 1989 (Evelyn 1992). Following the disease outbreak in Chile and Canada, similar disease outbreaks were observed in Atlantic salmon in Norway (Olsen, Melby, Speilberg, Evensen & Hastein 1997), Ireland (Rodger & Drinan 1993) and Scotland (Grant, Brown, Cox, Birkbeck & Griffen 1996).

Up until 1994, RLO had only been isolated from or observed in salmonids. However, after this time the disease was reported in a number of different fish species located in different geographic regions. The disease was reported to affect Mozambique tilapia *Oreochromis mossambicus*, Nile tilapia *Oreochromis niloticus*, blue tilapia *Oreochromis aureus*, redbelly tilapia *Tilapia zillii*, Wami tilapia *Tilapia hornorum* and unisexual broods, such as all male *O.aureus*, *O. mossambicus* etc. in freshwater culture ponds and seawater in southern Taiwan (Chern & Chao 1994; Chen, Tung, Chen, Tsai, Wang, Chen, Lin & Adams 1994). Khoo, Dennis and Lewbart (1995) also observed RLO in blue-eyed plecostomus *Panaque suttoni*, a tropical freshwater fish that was

imported from South America to the USA for the pet fish industry. During the winter of 1993-1994, mortalities associated with pathological changes in farmed juvenile sea bass, *Dicentrarchus labrax,* occurred on the Mediterranean coast, Southern France. Comps, Raymond and Plassiart (1996) reported that a RLO was the causative agent of this disease.

Cvitanich *et al.* (1995) observed and isolated a new RLO from Atlantic salmon during December 1994 and January 1995 in Chile. The disease outbreak was observed in net pens in farms located in Lake Llanquihue (water temperature approximately 16°C) and in sea pens (water temperature approximately 14°C). Cvitanich *et al.* (1995) named this agent UA-2 or U2 meaning "unidentified agent 2" because it was different from the previously observed RLO and was later referred to as named UA "unidentified agent" (Branson & Nieto Diaz-Munoz 1991). A disease associated with a RLO occurred on four Atlantic salmon sea farms in Ireland during 1995 and 1996. A bacterium consistent with *P. salmonis* was isolated from infected fish on fish cell lines, and the disease was experimentally reproduced with the isolated agent (Palmer, Ruttledge, Callanan & Drinan 1996).

In Eastern North America, a disease began in a Nova Scotia Atlantic salmon pen site in September 1996; the fish population had been transferred to sea pens in spring 1996. Diagnostic findings strongly suggested that the agent responsible for the disease was a RLO. This was the second report of a RLO infection in North America, although from a different part of the country (Cusack, Groman & Jones 1997). The second report in Mediterranean was reported RLO infections in sea bass *D. labrax* in Greece (Athanassopoulou, Sabatakou, Groman & Prapas 1999). Twenty days (d) after transfer

to cages, fish showed erratic swimming and abnormal behavior with high mortality up to 80% in colder months. Intravacuolar bacteria-like inclusions observed in tissue lesions showed similarities to *P. salmonis*.

In 2000, mortalities occurred among hatchery reared juvenile white seabass *Atractoscion nobilis* with infections associated with *P. salmonis*-like organisms was reported in southern California, USA. Although, the isolated bacterium induced 80% mortality in 10 d in experimentally infected coho salmon, it reacted weakly with polyclonal anti-*P. salmonis* serum (Chen, Yun, Marty, McDowell, House, Appersen, Guenther, Arkush & Hedrick 2000). Mauel and Miller (2002) reported that *P. salmonis*-like organisms were observed in tissue samples from tilapia affected epizootics similar to piscirickettsiosis, sent from Jamaica, Indonesia, Southern California and Florida during 2000 and 2001. Chen, Wang, Tung, Thompson and Adams (2000) recently reported a Piscirickettsia-like organisms in grouper *Epinephelus melanostigma,* in Taiwan. The clinical signs and histological lesions similar to piscirickettsiosis in salmonids and tilapia. The intracellular organisms similar in size and morphology to *P. salmonis* gave a positive reaction with polyclonal antibody (PAb) against *P. salmonis,* however, they did not produced cytopathic effect (CPE) in fish cell lines. In the last few years, rickettsial diseases have been observed in various locations in a variety of fish species world wide. The disease has been especially important to the Chilean salmonid fish farming industry. History of rickettsial disease in fish is reviewed in Table 1.1.

Table 1.1. History of natural outbreaks of rickettsial diseases in fish

Date of outbreaks	Host species	Location	Means of identification	Mortality	Named	Reference
1939	*T. fahaka*	Egypt (Freshwater)	LM: Small coccoid forms in monocytes leucocytes and plasma in heart-blood and liver smears of a dead fish with Giemsa staining	Not reported	*Rickettsia pisces*	Mohammed 1939
1975	Rainbow trout *O. mykiss*	Germany (Freshwater)	Isolation in fish cell line (RTG-2) and LM	Not reported	Rickettsia-like organism (RLO)	Ozel and Schwanz-Pfitzer 1975
1970, 1978 1983,1984 1984	Pink salmon *O. gorbuscha* Chinook salmon *O. tshawytscha* Coho salmon *O. kisutch*	Pacific Northwest Coast of Canada (Seawater)	LM: Gram negative, Giemsa positive, basophilic or amphophilic spheres in Haematoxylin and Eosin (H&E), best staining with Methylene blue, acid fast, Periodic acid-Schiff negative, Macchiavello negative in tissue sections from liver and kidney	Not reported	RLO/ Salmonid rickettsial sepicemia (SRS)	Evelyn 1992
1986	Dragonet *C. lyra* L.	Cardigan Bay Wales (Seawater)	EM of spleen	Not reported	RLO	Davies 1986
1989	Coho salmon *O. kisutch*	Chile (Seawater)	LM (Gram, Giemsa) and EM in blood and internal organs of infected fish	90%	Coho salmon syndrome (CSS)	Bravo and Campos 1989
1989	Coho salmon *O. kisutch*	Chile (Seawater)	Isolation using fish cell lines; LM (Gram negative, stained with Pinkerton's method and modified Gimenez. and Giemsa) and EM of isolated organisms; in vitro characterisation; no reaction with a monoclonal antibody (MAb) against the group-specific LPS chlamydial antigen by Indirect fluorescent antibody technique (IFAT)	90%	RLO/Chilean Coho salmon disease (CCSD)	Fryer *et al.* 1990
1989-1990	Chinook salmon *O. tshawytscha* Coho salmon *O. kisutch* Atlantic salmon *S. salar* Rainbow trout *O. mykiss*	Chile (Seawater)	Isolation using fish cell lines, LM [Gram negative, periodic acid-Schiff (PAS), Ziehl-Neelsen acid fast and Gimenez negative but stained with H&E, Giemsa and methylene blue on smears prepared from peripheral blood, fish tissue and infected cell culture] and EM of fish tissue and infected cell culture; fulfilled Koch's postulate; IFAT with MAb against Chlamydia and reproduced in fresh water	70%	RLO/CCSD/SRS	Cvitanich *et al.* 1990 Cvitanich *et al.* 1991
1989	Coho salmon *O. kisutch*	Chile (Seawater)	LM (Gram negative, PAS negative, not stain with Gimenez and Macchiavello) of kidney and blood smears and fish tissue; attempted to isolate with fish cell lines but with no CPE	60-90%	Unidentified agent-UA/RLO	Branson and Nieto Diaz-Munoz 1991
1989	Chinook salmon *O. tshawytscha* Coho salmon *O. kisutch* Atlantic salmon *S. salar* Rainbow trout *O. mykiss*	Chile (Seawater)	Isolation; fulfilled Koch's postulates; IFAT and Acridine orange stain	Not reported	RLO designated as strain LF-89 of SRS	Fryer and Lannan 1992

1989	Coho salmon *O. kisutch*	Chile (Seawater	EM and 16 small subunit ribosomal ribonucleic acid (16S rRNA) analysis of the designated strain LF-89	-	*Piscirickettsia salmonis* gen. nov., sp. nov./ Piscirickettsiosis	Fryer *et al.* 1992
1991	Atlantic salmon *S. salar*	British Columbia (Seawater)	LM: the organisms was Gram negative, acid fast, PAS negative, Giemsa positive, Macchiavello negative and blue with toluidine blue in infected tissue sections	0.06%/d	RLO	Brocklebank *et al.* 1992
1988-1992	Atlantic salmon *S. salar*	Norway (Seawater)	Isolation in fish cell line; LM (Gram, H&E, PAS, Ziehl-Neelsen, May-Grunwald-Giemsa, Machiavello's); Immunohistochemistry (IHC); EM; IFAT	Not reported	*P. salmonis* Piscirickettsiosis	Olsen *et al.* 1997
1991	Atlantic salmon *S. salar*	West of Ireland	LM (Gram, H&E, Giemsa and Acridine orange) and EM of tissue samples	Not reported	RLO	Rodger and Drinan 1993
1993	Coho salmon *O. kisutch* Atlantic salmon *S. salar* Rainbow trout *O. mykiss*	Chile (Freshwater)	Isolation using fish cell line; LM (Gram and Giemsa staining of infected cell culture); IFAT	Not reported	*P. salmonis*	Gaggero *et al.* 1995
1993	Rainbow trout *O. mykiss*	Llanquihue Lake in Chile (Freshwater)	LM (Giemsa) of tissue smears and Fluorescent antibody (FA)	10%	*P. salmonis* Piscirickettsiosis	Bravo 1994
1992-1993	Mozambique tilapia *O. mossambicus*, Nile tilapia *O. niloticus*, Blue tilapia *O. aureus*, Redbelly tilapia *T. zilllii* Wami tilapia *T. hornorum*	Southern Taiwan (Seawater/ Freshwater)	Isolation using fish cell lines, LM (Gram, Liu's staining, H&E, PAS, Ziehl-Neelsen acid-fast) of blood film and spleen smear and tissue sections; EM; fulfilled Koch's postulate	20-40% ; up to 95% in severe cases)	RLO	Chern and Chao 1994 Chen *et al.* 1994
1993-1994	Sea bass *D. labrax*	France	LM (H&E) and EM of affected fish tissue	20%	RLO	Comps *et al.* 1996
1995	Atlantic salmon *S. salar*	Scotland (Seawater)	Isolation, LM and EM; fulfilled Koch's postulates (With affected cell culture supernatant: no reaction to a latex agglutination test for *P. salmonis*; Enzyme-linked immunosorbent assays (ELISA)-Relisa-Rickettsia, Microtex was positive; IFAT positive for *P. salmonis*); 16S rRNA analysis	Not reported	RLO/ *P. salmonis*	Grant *et al.* 1996 Grant 1999
1995	Blue –eyed plecostomus *P. suttoni*	Colombia (Freshwater)	LM (Gram, H&E, Macchiavello's, Pinkerton's and Fite's acid fast stains) and EM	Not reported	RLO	Khoo *et al.* 1995
1994-1995	Atlantic salmon *S. salar*	Chile (Seawater/ Freshwater)	Isolation; LM (Gram, Gimenez, PAS, Ziehl-Neelsen acid-fast) and EM; IFAT negative with sera against Unidentified agent (UA)/*P. salmonis* or *Renibacterium salmoninarum*, Chlamydia and also negative in identification test for chlamydia (culture in McCoy cells, ELISA, and Gimenez stain)	4-12% /week	Unidentified agent 2 (UA2)	Cvitanich *et al.* 1995
1995-1996	Atlantic salmon *S. salar*	Ireland (Seawater)	Isolation; LM (Gram-Twort, Giemsa, H&E) and EM of tissue sections; IFAT with serum against *P. salmonis* was positive; fulfilled Koch's postulate	Not reported	Piscirickettsiosis-like disease	Palmer *et al.* 1996

1996	Atlantic salmon *S. salar*	Eastern North America (Seawater)	Isolation; LM (Gram, Giemsa, Toluidine blue) and EM; IFAT with serum against *P. salmonis* was positive; fulfilled Koch's postulate	6%	RLO	Cusack *et al.* 1997
1999	Sea bass *D. labrax*	Greece (Seawater)	LM (Giemsa) of affected fish tissue	Up to 80%	RLO	Athanassopoulou *et al.* 1999
2000	White seabass *A. nobilis*	Sothern California USA (Seawater)	Isolation; LM (white Giemsa); IFAT with serum against *P. salmonis* was reacted weakly.	Not reported	White seabass *P. salmonis-like* organism (WSPSLO)	Chen *et al.* 2000
2000	Grouper *E. melanostigma*	Taiwan	LM (H&E) and EM of affected fish tissue; IHC positive with polyclonal against *P. salmonis*; attempted to isolate with fish cell lines but with no CPE.	1% in 10 d	Piscirickettsia-like organism	Chen *et al.* 2000

1.3. The Bacterium

1.3.1. Taxonomy

Taxonomically *P. salmonis* was previously placed in the order *Rickettsiales*, the family *Rickettsiaceae*, and is both a new genus and a new species based on its unique 16S rRNA sequence, temperature requirements, host range and serological characteristics (Fryer *et al.* 1992). In the 1984 edition of Bergey's manual of Systematic Bacteriology (Weiss & Moulder 1984) the order *Rickettsiales* is divided into three families: *Rickettsiaceae, Bartonellaceae* and *Anaplasmataceae* depending upon their morphology, serology, mode of transmission and their association with the cells they infect. The family *Rickettsiaceae* includes tribes: *Rickettsieae* (genus *Rickettsia, Rochalimaea* and *Coxiella*), *Ehrlichieae* (genus *Ehrlichia* and *Cowdria*) and *Wolbachieae* (genus *Wolbachia* and *Rickettsiella*). The variation in intrinsic properties of the members of each tribe is based on the hosts which they infect. *Rickettsieae* are pathogenic for man, while *Ehrlichieae* is pathogenic for domestic animals but are possible human pathogens. On the other hand, the tribe *Wolbachieae* is a pathogen or symbiontes of arthropods (Buxton & Fraser 1977; Weiss & Moulder 1984; Drancourt & Raoult 1994). Assignment of *P. salmonis* to one of the tribes mentioned above was delayed until appropriate classification of the family has been carried out (Fryer *et al.* 1992).

The term rickettsia initially covered all intracellular bacteria, regardless of any other characteristics they exhibited. Early rickettsial taxonomy was based on a comparison of phenotypic characteristics such as the bacteria's interactions with the environment and the antigenicity of isolates. However, genomic studies including 16S rRNA sequencing, Deoxyribonucleic acid (DNA)-DNA relatedness studies, restriction profiles

and polymorphism analysis have recently been used to unravel rickettsial taxonomy (Weisburg *et al.* 1989; Drancourt & Raoult 1994). The 16S rRNA gene sequence showed *P. salmonis* to be a member of the gamma subdivision of the Proteobacteria like *Coxiella burnetti* and *Wolbachia persica*, while bacteria of the genera *Neorickettsia, Cowdria, Ehrlichia, Anaplasma* and *Rickettsia* are members of the alpha subdivision of the Proteobacteria (Mauel, Giovannoni & Fryer 1999). Therefore, the genus *Piscirickettsia* has been placed in a new class within Gammaproteobacteria and a new family *Piscirickettsiaceae* (Fryer & Hedrick 2003). Many RLO have been isolated from a variety of different fish species since 1989. Most of them are serologically identified as *P. salmonis* (Evelyn 1992; Rodger & Drinan 1993; Gaggero *et al.* 1995; Olsen *et al.* 1997, Fryer & Lannan 1996). Although the isolates react with polyclonal antibodies (PAbs) against *P. salmonis* type strain LF-89 and appear to be morphologically similar, taxonomic placement of the isolates should be based on genetic differences between the RLO isolates. Mauel *et al.* (1999) assessed the genetic variability of the 16S ribosomal DNA, the internal transcribed spacer (ITS) and the 23S ribosomal DNA of a number of different isolates from three different hosts and different locations (LF-89, coho salmon/Chile; EM-90, Atlantic salmon/Chile; ATL-4-91, Atlantic salmon/Canada; NOR-92, Atlantic salmon/ Norway; SLGO-94, rainbow trout/Chile and C1-95, coho salmon/Chile) using a polymerase chain reaction (PCR). Phylogenetic comparison of the *P. salmonis* 16S rRNA to other bacterial genes confirmed that the organism is a member of the gamma subdivison of the Proteobacteria, and closely related to the genus *Coxiella* and *Francisella.* The authors also concluded that all isolates of *P. salmonis* were from a monophyletic group within the same subdivision of Proteobacteria, and isolate EM-90 diverges genetically from other isolates based on its 16S, ITS and 23S rDNA sequence analysis (Mauel *et al.*

1999). It is agreed that the taxonomic classification of *P. salmonis* isolates needs further analysis.

1.3.2. Morphology

The size of *P. salmonis* is between 0.5-1.5 μm in diameter. It is a coccoid or ring-formed bacterium or can appear as a pair of curved rods. It is pleomorphic, non-encapsulated, non-motile and Gram negative. The bacterium usually develops within the cytoplasmic vacuoles of the host cells and can occur either singly in diffuse groups, in pairs or as dense morula-like masses. They multiply by binary fission. Individual or paired organisms enclosed in membrane bound vacuoles, are surrounded by a double membrane layer consisting of a highly rippled outer membrane and an inner membrane closely associated with the cytoplasm of the bacterium. It has a typical Gram negative cell wall and protoplasmic structure of a prokaryote (Fryer *et al.* 1990; Fryer *et al.* 1992). The cell contents are composed of numerous ribosome-like particles concentrated near the plasma membrane, a single or multiple fibrillar nucleoid(s) localized in the central region and small electron lucent vacuoles (Fryer *et al.* 1990). The vacuoles are not bound by a membrane and are variable in size and number (Cvitanich *et al.* 1991).

The RLO stain dark blue with Giemsa, methylene blue, retain basic fuchsin when stained with Pinkerton's adaptation of Gimenez, and are basophilic with H&E but they do not stain with PAS, Ziehl-Neelsen acid fast or Macchiavello's Gimenez. The organisms do not react with MAb against the group-specific chlamydial LPS antigen (Fryer *et al.* 1990; Turnbull 1993).

1.3.3. Cultural characteristics *in vitro*

P. salmonis produces a CPE in the following salmonid cell lines: chinook salmon, *O. tshawytscha* Walbaum embryo (CHSE-214); chum salmon, *Oncorhyncus keta* heart (CHH-1); coho salmon, *O. kisutch* embryo (CSE-119); rainbow trout, *O. mykiss* gonad (RTG-2); and the following non-salmonid fish cell lines: common carp, *Cyprinus carpio*, epithelioma papillosum cyprini (EPC) and fathead minnow, *Pimephales promelas* Rafinesque (FHM), but not in brown bullhead, *Ictalurus nebulosus* (Lesucur) (BB) and bluegill, *Lepomus macrochirus* Rafinesque, fry (BF-2) cell cultures. The RLO responsible for causing disease in tilapia in Taiwan was cultured in a non-salmonid fish cell line *O. niloticus*, Nile tilapia ovary origin cells (TO-2) at 25°C (Chern & Chao 1994). Attempts to culture the organism on artificial media in different culture environments have however failed (Cvitanich *et al.* 1991). A CPE in fish cell lines appears after 5-6 d and the monolayer completely lyses by 14 d at 15-18°C. The replication titer of *P. salmonis* is 10^6 to 10^7 50% Tissue Culture Infective Dose ($TCID_{50}$) ml^{-1} in fish cell lines. Several antibiotics, streptomycin, gentamycin or tetracycline, but not penicillin, inhibit replication of the organism *in vitro*. The infectivity titer is destroyed by 99% after one freeze-thaw cycle. The addition of 10% Dimethyl sulfoxide (DMSO) in the freezing medium helps act as a cryopreservative for the organism (Fryer *et al.* 1990). Although all previous attempts to obtain a CPE by *P. salmonis* in the BB cell line have failed (Fryer *et al.* 1990; Cvitanich *et al.* 1991), the bacterium produced a CPE with complete destruction of the monolayer by 78 d post-infection in BB cells (Almendras, Jones, Fuentealba & Wright 1997). Almendras *et al.* (1997) observed the organism free and in intracytoplasmic and extracellular locations under Transmission electron microscopy (TEM).

1.4. Clinical Signs

1.4.1. Gross pathology

The clinical signs of rickettsial disease differ between outbreaks and individual fish, although some fish die with very few signs of abnormality or exhibiting external signs of the disease (Turnbull 1993; Cvitanich *et al.* 1991).

Moribund fish swim near the surface or at the side of the net, with vertical or circling movement, and lethargic and anorexic behaviour (Schafer, Alvarado, Enriquez & Monras 1990; Branson & Nieto Diaz-Munoz 1991; Cvitanich *et al.* 1991; Brocklebank *et al.* 1992; Rodger & Drinan 1993; Bravo 1994; Chen *et al.* 1994; Palmer *et al.* 1996; Cusack *et al.* 1997; Olsen *et al.* 1997). Enlargement of the abdominal cavity with ascites and peritonitis is also observed (Schafer *et al.* 1990; Branson & Nieto Diaz-Munoz 1991; Chen *et al.* 1994). General internal pallor, necrotic stomatitis, a transparent seromucous liquid in the stomach and a yellowish mucous content in the intestine have also been reported (Schafer *et al.* 1990; Branson & Nieto Diaz-Munoz 1991). Petechial haemorrhages are frequently evident in the stomach, intestine, pyloric caeca, swim bladder, visceral fat and skeletal muscle and internal lesions included petechia on serosa, fibrous adhesions in the peritoneal cavity, and white nodules on the stomach, intestine and mesenteric fat (Cvitanich *et al.* 1990; Brocklebank *et al.* 1992; Chen *et al.* 1994; Palmer *et al.* 1996; Olsen *et al.* 1997).

The spleen, liver and kidney of infected fish are often swollen with pale discolouration. They often have a haemorrhaged liver with yellow multifocal subcupsular nodules scattered diffusely throughout and sometimes internal lesions. The kidney appears pale with inflammation and petechial hemorrhages. Occasionally, small white foci are seen

in the heart, kidney, spleen and skeletal musculature (Schafer *et al.* 1990; Cvitanich *et al.* 1990; Branson & Nieto Diaz-Munoz 1991; Brocklebank *et al.* 1992; Rodger & Drinan 1993; Chen *et al.* 1994; Chern & Chao 1994; Olsen *et al.* 1997). Extensive haemorrhages on the surface of the brain and softening of the brain tissue have also been reported (Chern & Chao 1994; Comps *et al.* 1996), as has the presence of a whitish pseudomembrane around the heart (Cvitanich *et al.* 1991). Many of the infected fish are dark in colour and possess skin lesions with small areas of raised scales, areas with no scales or superficial white spots, or ulcers and petechiae along the dorso-lateral surfaces or ventral surfaces (Schafer *et al.* 1990; Branson & Nieto Diaz-Munoz 1991; Cvitanich *et al.* 1991; Bravo 1994, Olsen *et al.* 1997). Other clinical signs are shallow dermal ulcers on the lateral caudal peduncle and haemorrhages in the dorsal and ventral fins, punctuate ulceration on the head, pale coloured gills, white nodules on the gills in some fish and bilateral exophthalmus (Schafer *et al.* 1990; Cvitanich *et al.* 1991; Brocklebank *et al.* 1992; Comps *et al.* 1996; Turnbull 1993; Bravo 1994, Chen *et al.* 1994; Chern & Chao 1994; Cusack *et al.* 1997; Olsen *et al.* 1997).

1.4.2. Histopathology

Although histopathological findings associated with rickettsial disease have been described in both naturally and experimentally infected fish, the sequence of histological changes and systemic dissemination of RLO has not yet been described (Almendras & Fuentealba 1997).

Rickettsia are commonly observed within macrophages, within cytoplasmic vacuoles, free in the cytoplasm or outside host cells. In some fish, darkly-stained basophilic cells,

approximately 20x10 μm in size, with large nuclei and a little cytoplasm in small or large numbers but often in close groups, appear within the haematopoietic tissue, but as yet their function is not clear (Branson & Diaz-Munoz 1991; Turnbull 1993).

The most marked pathology, necrosis and oedema with a granulomatous response is found in the haemopoietic tissue of the kidney and spleen, and in the hepatocytes in the liver. The haemopoietic tissue displays disseminated intravascular coagulation with necrotic thrombi associated with necrotic changes to vessel endothelium (Branson & Nieto Diaz-Munoz, 1991; Cvitanich *et al.* 1991; Evelyn 1992; Turnbull 1993; Rodger & Drinan 1993; Chern & Chao 1994; Chen *et al.* 1994; Palmer *et al.* 1996). Perivascular cellular inflammation and vascular lesions primarily in the liver, kidney and spleen, and in all tissue have also been reported (Branson & Nieto Diaz-Munoz 1991, Cvitanich *et al.* 1991, Chen *et al.* 1994, Olsen *et al.* 1997). Additionally, some degree of glomerular nephritis with vacuolation, oedema of the capsule and increased level of eosinophilic ground substance is also apparent in the kidney (Branson & Nieto Diaz-Munoz, 1991; Cvitanich *et al.* 1991; Rodger & Drinan 1993; Khoo *et al.* 1995). Multifocal to diffuse necrosis with oedema, invasion by inflammatory cells, and increased level of eosinophilic ground substance and some fibrosis can be seen in liver (Branson & Nieto Diaz-Munoz, 1991; Cvitanich *et al.* 1991; Rodger & Drinan 1993; Chern & Chao 1994; Olsen *et al.* 1997).

Endocarditis along with variable pericarditis which are sometimes necrotic, and focal hyaline necrosis of the myocardium are seen in the heart (Branson & Nieto Diaz-Munoz, 1991; Cvitanich *et al.* 1991; Rodger & Drinan 1993; Chen *et al.* 1994; Chern & Chao 1994; Olsen *et al.* 1997). The intestine displays necrosis and inflammation

resulting in diffuse chronic inflammatory lesions in the lamina propria and sloughing of the mucosa (Branson & Nieto Diaz-Munoz, 1991; Cvitanich *et al.* 1991; Chen *et al.* 1994; Chern & Chao 1994). A granulomatous inflammation in the meninges and mild inflammatory and thrombotic lesions in brain, pancreas, ovaries, heart, mesentery, testis, eye, muscle, pseudobranch, nasal capsule and adipose tissue are also seen (Cvitanich *et al.* 1991; Rodger & Drinan 1993; Chen *et al.* 1994; Comps *et al.* 1996; Olsen *et al.* 1997). Multifocal epithelial hyperplasia resulting in fusion of the gill lamellae, and necrotic areas and fibrin thrombi in lamellar capillaries have been observed. Varying degrees of necrosis of the epidermis, dermis and underlying musculature in the skin lesions and a spreading inflammatory response along the intra-muscular septa have also been reported (Branson & Nieto Diaz-Munoz, 1991; Cvitanich *et al.* 1991; Chen *et al.* 1994; Chern & Chao 1994; Palmer *et al.* 1996; Olsen *et al.* 1997).

1.4.3. Haematology

The pale coloration of infected fish is because of the presence of a low haematocrit during rickettsiosis. The normal haematocrit value is in the region of 35-50%, while in affected salmonids, it is often reduced to between 2 to 35% (Turnbull 1993). A similar reduction has been observed in the haematocrit values of tilapia affected by the disease (Chern & Chao 1994). Branson and Nieto-Diaz Munoz (1991) reported a simultaneous neutrophilia 10-20 times higher than normal in blood smears from affected fish. Cvitanich *et al.* (1991) demonstrated the presence of large numbers of macrophages which contained either degenerate cellular debris or the organism in peripheral blood smears, and typically normochromic–normocytic erythrocytes but rarely immature red

blood cells. These appeared to be the most striking changes observed in the hematology of moribund fish.

1.5. Transmission

Initially, rickettsial disease in fish was first associated with increased stress caused by fluctuations in water temperature, severe storms or blooms of non-toxic algae. However, significant fish losses were still observed after the disappearance of the algal bloom and stabilisation of water temperature. There has not been a strong association between disease outbreaks and environmental conditions if outbreaks worldwide are taken into consideration.

Transmission of RLO by an intermediate host has been considered. The presence of sea lice *Calligus* spp., or isopods and molluscs, which move freely through net pens, may act as possible vectors in regions of disease outbreaks. Intermediate hosts are involved in terrestrial rickettsial disease with an exception of the genus Coxielle, which forms a spore-like structure, transmitted by aerosol dust, infecting the respiratory tract of its host (Weiss & Moulder 1984). In the aquatic environment an intermediate host or a vector may not be required for delivery of rickettsia to its host since the extracellular survival of *P. salmonis* from coho salmon has been shown as 14 d at 15°C in sea water (Lannan & Fryer 1994). The survival of *P. salmonis* in salt water may provide sufficient time to allow horizontal transmission of the organism in the marine environment without the need for an intermediate host or vector. However, rapid inactivation of *P. salmonis* in freshwater may limit the transmission of the organism in this environment and may explain why the disease is rarely observed in freshwater (Lannan & Fryer 1994).

Cvitanich *et al.* (1991) reported horizontal transmission between injected and uninjected coho salmon held in static freshwater and seawater aquaria at 15°C, while Garcés *et al.* (1991) did not observe any disease transmission when uninfected coho salmon were held together with infected coho salmon at 8°C in a tank with flow-through freshwater. Chern and Chao (1994) demonstrated direct horizontal transmission of RLO in tilapia species by keeping fish intramuscularly injected with RLO together with uninfected fish in the same tank. Salinas, Contreras, Smith and Larenas (1997) demonstrated horizontal transmission of *P. salmonis* (isolate SLGO-95) experimentally in rainbow trout. They detected this agent in the bile, faeces and urine of healthy and/or moribund fish 7 d post-inoculation by using an IFAT. Although horizontal transmission of the rickettsial disease in fish has not been proved under farm conditions, granuloma lesions present on gills, or lesions on the intestines of infected fish suggest that these sites may be a possible route of entry for the RLO, or possibly that RLO can be shed from infected fish through faeces (Turnbull 1993; Chern & Chao 1994).

Almendras, Fuentealba, Jones, Markham and Spangler (1997) compared experimental routes of *P. salmonis* infection using intraperitoneal injection, and oral and gill application. They also examined the importance of physical contact in horizontal transmission of the disease in freshwater-raised Atlantic salmon. According to their study, *P. salmonis* is transmitted horizontally to fish without the need for physical contact, and the pathogen appears to enter the fish via the gills, skin and the oral route, although direct contact increased transmission of *P. salmonis* and less mortalities occurred through oral infection. Smith, Pizarro, Ojeda, Contreras, Oyanedel and Larenas (1999) investigated the portal entry of *P. salmonis* using experimental infection

of rainbow trout via intraperitoneal injection, subcutaneous injection, patch contact on the skin, patch contact on gills, intestinal intubation and gastric intubation. In this study, the cumulative mortalities were 98, 100, 52, 24, 24, and 2% at day 33 post-infection, showing that intact skin and gills may be possible routes of entry for rainbow trout.

Cvitanich *et al.* (1991) reported the existence of RLO in the ovaries and testes of immature fish, and in ovarian fluid of mature fish, suggesting the possibility of vertical transmission of the disease in Chile. Gaggero *et al.* (1995) isolated *P. salmonis* from 60-90 d old progeny of coho salmon, Atlantic salmon and rainbow trout maintained in freshwater. Whether vertical transmission of *P. salmonis* had occurred or the origin of the *P. salmonis* infection came from the freshwater environment, was not clear from this study. Larenas, Astorga, Contreras and Smith (1996) found that approximately 10% of eggs and fry of rainbow trout from experimentally infected male or female brooders were infected with *P. salmonis.* The disease agent was observed inside the yolk of the eggs, again suggesting vertical transmission of the disease. However, the source and mode of transmission of *P. salmonis* still requires further investigation.

1.6. Detection and Identification of *P. salmonis*

Diagnosis of rickettsial disease is usually based on gross and histological signs with the use of histochemical stains such as H&E, Gram, Giemsa, Acridine orange, Methylene blue, Gimenez, Macchiavello and PAS to detect the pathogen in smears or tissue sections. These techniques are fast and widely used, but they are non-specific (Fryer *et al.* 1990; Almendras & Fuentealba 1997). The most specific technique available initially was is the isolation of *P. salmonis* in cell lines. The technique is, however,

time consuming and difficult since culture has to be performed without antibiotics. This opens the cell cultures to contamination by other bacterial agents (Fryer & Lannan 1996).

Antibody-based methods are a more specific way of confirming the identity of *P. salmonis* (Fryer & Lannan 1996). IFAT and IHC using rabbit anti-*P. salmonis* PAb have been developed to detect *P. salmonis.* (Lannan, Ewing, Fryer 1991; Alday-Sanz, Rodger, Turnbull, Adams & Richards 1994). IFAT provides a sensitive and specific method for detection of the disease and can be successfully performed on blood films, tissue sections and smears. IHC is a rapid diagnostic test which can be used both on formalin-fixed and wax-embedded samples. It has the advantage that identification of the pathogen through immunodetection and examination of infected tissue and histopathological lesions can be performed at the same time. ELISA has also been used for the detection of *P. salmonis* in salmonids. It has certain advantages such as eliminating the uncertainty of microscopic interpretation, and provides an alternative to PCR from which a number of false negative results have been reported (Cassigoli 1994; Carlos, Thornton, Hackett, Valdes, Poblete, Kuzyk & Kay 1997).

Nested polymerase chain reaction is a rapid, highly sensitive and specific test and has been described for the detection of genomic DNA of *P. salmonis.* This technique is useful for the detection of the organism in the early stages of infection. It can also be used to confirm the taxonomy and ecological characteristics of the bacterium and to examine modes of transmission, range of natural hosts, and the pathogens reservoir and geographical distribution. It can also be used to characterise other RLO (Mauel, Giovannoni & Fryer 1996).

1.7. Treatment and Control of Piscirickettsiosis

1.7.1. General control

Improved management practices have been suggested to reduce the disease, including early elimination of dead and clinically diseased fish, low stocking density, high standard of the cage environment, a decreased biomass per site, separating year classes at each site to prevent transmission of the agent between year classes, appropriate disposal of blood from harvested fish, removal of infected broodstock and their eggs, and the routine disinfection of eggs (Branson & Nieto-Diaz 1991; Evelyn 1992; Rodger & Drinan 1993; Turnbull 1993; Almendras & Fuentealba 1997).

1.7.2. Chemotherapy

Antibiotic sensitivity tests with *P. salmonis in vitro* have shown them to be sensitive to streptomycin, gentamycin, chloramphenicol, erythromycin, oxytetracyclin, tetracyclin, clarithromycin, sarafloxacin and oxolonic acid and resistant to penicillin, penicillin G and spectinomycin (Fryer *et al.* 1990; Cvitanich *et al.* 1991; Grant *et al.* 1996; Palmer *et al.* 1996). Most species of the *Rickettsiales* are inhibited by penicillin (Weiss & Moulder 1984). Oral administration of oxytetracycline may have some benefit for control of rickettsial disease (Evelyn 1992; Chern & Chao 1994). However, reduced sensitivity of *P. salmonis* to oxolinic acid and oxytetracycline has been reported (Smith, Vechiola, Oyanedel, Garcés, Larenas & Contreras 1996).

1.7.3. Vaccines

Since there is no effective method of treatment for piscirickettsiosis, vaccination potentially is of great value, especially since vaccines have been used in the control of many other fish diseases. However, little information relating to vaccine trials for

piscirickettsiosis has been reported (Fryer & Lannan 1996; Almendras & Fuentealba 1997).

Smith, Lannan, Garcés, Jarpa, Larenas, Caswell-Reno, Whipple and Fryer (1995) vaccinated pre-smolt coho salmon, intraperitoneally with formalin killed preparations of *P. salmonis* at a freshwater site. Two different bacteria preparations, non-concentrated bacterin and concentrated antigen emulsified with Freund's complete adjuvant were used. After 20 weeks post-immunisation (wpi), the fish were transferred to a sea site to be naturally infected with the pathogen. A lower cumulative mortality to that of the control group was observed in fish vaccinated with the non-concentrated bacterin. However, a slightly higher mortality rate to that of the control group occurred when fish were vaccinated with a concentrated antigen emulsified in Freund's complete adjuvant. Smith *et al.* (1995) suggested that the non-concentrated bacterin caused an immunoprotective response in the fish. Although the results obtained with non-concentrated bacterin are encouraging, they must be evaluated with caution because the natural challenge may have been low and *R. salmoninarum* together with *P. salmonis* was detected in experimental fish. Thus, the trial was not long enough to determine the level of protection in larger fish in which economic losses due to the disease are more significant.

Smith, Contrearas, Larenas, Aguillon, Garcés, Perez and Fryer (1997) carried out a similar trial for coho salmon and rainbow trout. Rainbow trout showed a low level of infection when bath challenged and lower cumulative mortalities were obtained compare to the non-vaccinated control group when naturally challenged in sea water. Smith *et al.* (1997) also obtained the highest levels of antibody in the sera of fish

vaccinated with concentrated antigen contrary to the previous study by Smith *et al.* (1995). Although the results are encouraging the challenge was not strong enough to have any firm conclusion since only 20% mortalities occurred in the control fish. Kuzyk, Burian, Thorton and Kay (2001) encoded one of the immunoreactive clones identified using a rabbit polyclonal, a 17 kilodalton (kDa) antigenic outer surface protein (OspA) with 62% amino acid sequence homology to the genus common 17 kDa outer membrane lipoprotein of *Rickettsia prowazekii*, previously thought to be found in members of the genus *Rickettsia.* The OspA, recombinanatly produced in *Escherichia coli* provided a high level of protection in vaccinated coho salmon challenged with *P. salmonis* with a relative percent survival as high as 59% (Kuzyk, Burian, Machander, Dolhaine, Cameron, Thorton & Kay 2001). The authors increased the efficacy of the OspA vaccine threefold by the addition of T cell epitopes (TCEs) from tetanus toxin and measles virus fusion protein, that are universally immunogenic to mammalian immune systems. Further studies under more controlled conditions are required to develop and evaluate the response of fish to vaccine preparations.

1.8. Research Objectives

RLO is a one of the most important pathogens known to infect fish, with a significant impact on the health of various fish species worldwide. *P. salmonis* is the first rickettsial pathogen to be isolated and for which it has been demonstrated to be the etiological agent of piscirickettsiosis in salmonids.

Since the isolation of *P. salmonis* in 1989, there have been increasing numbers of reports of rickettsial disease in a diverse range of fish species cultured in a variety of geographic locations. Information in the literature relating to the pathogenicity of the

bacterium, differences between *P. salmonis* isolates, specific and sensitive detection methods and a suitable control method for the disease is still sparse. Such information is necessary to establish effective control strategies for the disease. The objectives of this study were therefore to:

- Examine the growth characteristics and morphology of *P. salmonis in vitro* and develop an effective method for the purification of *P. salmonis* from fish cell lines infected with the bacterium (Chapter III and IV)
- Investigate the antigenic structure of *P. salmonis* isolates using gel electrophoresis and the detection of genomic DNA using a polymerase chain reaction (Chapter IV)
- Produce antibody probes (polyclonal and monoclonal) against purified preparations of the bacterium to use for both developing serological diagnostic techniques and to compare various *P. salmonis* isolates (Chapter V)
- Establish an artificial challenge model to examine the virulence of different isolates and examine the immune response of fish to various *P. salmonis* isolates (Chapter VI)
- Characterisation of phage particles associated with *P. salmonis* (Chapter VII)

CHAPTER II

GENERAL MATERIALS AND METHODS

Chapter II constitutes the description of common material and methods used throughout this thesis. Any modifications or variations in particular techniques will be stated in the Materials and Methods section of the relevant chapter.

2.1. Bacteria

2.1.1 *Piscirickettsia salmonis*

2.1.1.1 *P. salmonis* isolates used in the study

The *P. salmonis* isolates used in this study, the fish species and tissue from which they were isolated and their country of origin are detailed in Table 2.1. Note that not all isolates listed in the Table 2.1 were used for every study. Those used for each study are indicated in the relevant Chapters.

2.1.1.2. Growth and isolation of *P. salmonis* isolates

(a) Cell culture

Six different cell lines were used to culture *P. salmonis.* These were derived from either salmonid fish: chinook salmon, *Oncorhynchus tshawytscha* Walbaum embryo (CHSE-214/ATCC CRL-1681); Atlantic salmon, *Salmo salar* head kidney (SHK-1); rainbow trout, *Oncorhynchus mykiss* gonad (RTG-2/ATCC CCL-55) or from warm water species: common carp, *Cyprinus carpio* epithelioma papillosum cyprini (EPC); bluegill, *Lepomus macrochirus* Rafinesque, fry (BF-2/ATCC CCL-91); and sea bass, *Cetropristis striata* larvae (SBL).

Table 2.1 *P. salmonis* isolates examined in this study

Strain	Source	Tissue	Country
LF-89 ATCC VR 1361	Coho salmon (*Oncorhynchus kisutch)*	Kidney	Chile[1]
AVG 5/268	Atlantic salmon (*Salmo salar)*	Kidney	Ireland[2]
VQ 013	Rainbow trout (*O. mykiss)*	Brain	Chile[3]
SRS-UACH	Coho salmon (*O. kisutch)*	Kidney	Chile[3]
SRS-4	Coho salmon (*O. kisutch)*	Kidney	Chile[3]
R-29	Atlantic salmon (*S. salar)*	Kidney	Chile[3]
R-61	-	-	Chile[3]
R-62	-	-	Chile[3]
SLGO-95	Coho salmon (*O. kisutch)*	Kidney	Chile[4]
R980769	Sea bass (*Dicentrarchus labrax)*	Kidney-Spleen-Brain	Greece[5]

The *P. salmonis* isolates were kindly provided by: [1] Dr John Fryer (Department of Microbiology, Oregon State University, Oregon, USA) (Fryer *et al.* 1992); [2] Dr Roy Palmer (Aquatic Veterinary Group, University College of Galway, Ireland); [3] Dr Carlos Farias (Univ. Austral de Chile, Valdivia, Chile); [4] Dr Pedro Smith (University of Chile, Santiago, Chile). [5] Infected fish tissue was supplied by Dr Athanassios Prappas (National Fish Disease Laboratory, Athens, Greece).

Cell lines CHSE-214, EPC, RTG-2 and BF-2 were maintained at 22°C in antibiotic-free Eagle's minimal essential medium (MEM) with Earle's salts (Gibco), supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), 2 millimolar (mM) L-glutamine (Gibco) and 1% (v/v) non-essential amino acids (NEAA) (100x-Sigma). This medium is referred to as MEM-10. Cell line, SBL, was maintained in Leibovitz-15 medium (Gibco) with 10% (v/v) FCS, 2 mM L-glutamine, while SHK-1 cell line was cultured in Leibovitz-15 medium supplemented with 5% (v/v) FCS, 2 mM L-glutamine and 40 micromolar (μM) 2-mercaptoethanol (Sigma) at 22°C, again without antibiotics.

A variety of different tissue culture plates (6 wells, 12 wells, 24 wells, 96 wells) or tissue culture flasks (25 cm^2, 75 cm^2, 175 cm^2 or a 500 cm^2 triple flasks) (Nunc A/S Roskilde, Denmark) were used to culture *P. salmonis*, depending on the use of the culture and the quantity of bacteria required. The fish cells lines were subcultured once and a confluent monolayer was established, as determined using a phase-contrast microscope. The medium in the flask to be subcultured was removed and the cells were washed with phosphate buffered saline (PBS-1) (Gibco) (5 ml/25 cm^2) to remove traces of serum that would inhibit the action of trypsin- Ethylenediaminetetraacetic acid (EDTA). Trypsin-EDTA (Gibco) (3 ml/25 cm^2) was added to the flask and left for 15-30 seconds (s). After withdrawing the excess Trypsin-EDTA, the flask was incubated until the cells in the monolayer detached from the flask. The cells were then resuspended in fresh MEM-10 (0.1-0.2 ml/25 cm^2), counted by haemocytometer and diluted to a seeding concentration of between 5 $x10^4$ and 5 $x10^5$ cells ml^{-1} by adding an appropriate volume of fresh medium. The resuspended cells were distributed to new culture vessels and incubated at 22°C.

(b) Isolation of *P. salmonis* from infected fish tissue

Kidney tissue was aseptically removed from the fish and placed in a sterile Petri dish containing antibiotic-free MEM with Earle's salts, supplemented with 2% (v/v) FCS, 2 mM L-glutamine and NEAA (1%). This medium is referred to as MEM-2. The kidney tissue was then macerated with a sterile scalpel blade and the resulting cell suspension was transferred to a sterile centrifuge tube and left to stand for 5 minutes (min).

A tissue culture flask (25 cm^2) was seeded with CHSE-214 cells at a concentration of 5 x 10^4 cells ml^{-1}, and incubated for 48 hours (h) at 22°C. Cells were examined to ensure that they were healthy and obtained an adequate level of confluence (50-70%). The tissue culture medium was removed from the flasks and cells were washed with PBS-1 to remove any dead cells. A 0.5 ml aliquot of the kidney homogenate was added to the flask and incubated at 15 °C for 2 h. The flask was gently shaken every 15 min to allow absorption of the rickettsia by the cells. After 2 h, the cell monolayer was carefully washed with PBS-1 to remove any unabsorbed bacteria so that only the progeny rickettsiae will be harvested and 4 ml of fresh medium MEM-2 was added to the flask. The monolayer was incubated at 15 to 17°C for 20 to 30 days (d) and monitored daily for the presence of an extensive cytopathic effect (CPE) (approximately 90%).

(c) Culture of *P. salmonis in vitro*

The *P. salmonis* isolates presented in Table 2.1 were maintained in the different cell lines indicated above, at between 15 and 17°C. Tissue culture flasks were seeded with the cells at a concentration of 5 x10^4 cells ml^{-1}, and incubated for 48 h at 22°C until 50-70% confluence was observed under the microscope. The culture medium was removed and the monolayer was washed with PBS-1 to eliminate any dead cells. The

tissue culture flasks were then inoculated with a supernatant recovered from a *P. salmonis*-infected cell culture. The inoculation volume of infected tissue culture supernatants was between 1:5 and 1:10 (v/v) of the final volume used to culture the cells in the flask. The flasks were gently shaken for 2 h, incubating at 15°C to allow adsorption of the rickettsia by the cells. The supernatant was then removed and the cell monolayer washed again with PBS-1 to remove unadsorbed rickettsia cells. Fresh medium specific for the different cell lines, supplemented as described above, was added to the different cell monolayers. Infected cells were cultured for between 10 and 15 d until an extensive CPE (approximately 90%) was observed.

2.1.2. Culture of non-*P. salmonis* bacteria

The bacteria described in Table 2.2 represent a variety of bacteria from different genera used throughout the thesis to determine the specificity of rabbit serum, monoclonal antibodies (MAbs) and primers used in the identification and differentiation of *P. salmonis* isolates. The various bacterial species used in each study are detailed in the appropriate Chapters. The bacteria were grown in tryptone soya agar (TSA, Oxoid) or TSA supplemented with 2% (w/v) NaCl at 22°C, apart from *Renibacterium salmoninarum* which was grown in selective kidney disease medium (SKDM) (Austin, Embley & Goodfellow 1983) at 15°C.

Table 2.2. The non-*P. salmonis* bacteria used in this thesis to examine the specificity of the rabbit serum, the monoclonal antibodies and the primers for PCR

Bacteria	Origin
Aeromonas hydrophila	NCIMB 1134
Aeromonas salmonicida	NCIMB 1102
Bacillus subtilis	ATCC 6633
Corynebacterium aquaticum	NCIMB 9460
Edwardsiella tarda	IOA
Escherichia coli	IOA
Listonella anguillarum	NCIMB 6
Micrococcus luteus	NCIMB 570
Mycobacterium marinum	NCIMB 1297
Nocardia asteroides	NCIMB 1290
Pseudomonas aeruginosa	ATCC 2753
Pseudomonas fluorescens	NCIMB 1953
Photobacterium damselae subsp. piscicida	IOA
Renibacterium salmoninarum	IOA
Serratia sp.	IOA
Streptococcus faecalis	IOA
Vibrio anguillarium	IOA
Vibrio ordalii	NCIMB 1953
Yersinia ruckeri	NCIMB 1316

ATCC : American Type Culture Collection
IOA : Institute of Aquaculture, University of Stirling, Stirling, UK
NCIMB : National Collection of Industrial and Marine Bacteria, Aberdeen, UK

2.1.3. Preparation of bacterial samples

2.1.3.1 *P. salmonis*

P. salmonis isolates shown in Table 2.1 were prepared either using the purification procedure described in Section 3.2.2, or by collecting them from cell culture supernatants from flasks exhibiting extensive CPE (approximately 90%) using centrifugation at 20 000 x g for 30 min at 4°C. The suspended pellet in PBS-2 (5.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 116 mM NaCl, 10 mM KCl, pH 7.0) was then homogenised, vortexed and centrifuged at low speed (210 x g) to remove the host cell debris (Section 3.2.2). The rickettsia in the supernatant were then collected and washed twice with PBS-2, centrifuging at 20 000 x g for 30 min at 4°C to eliminate FCS residues. This preparation was referred to as semi-purified rickettsia. Aliquots of the isolated rickettsia in PBS-2 were made and stored at -80°C for future analysis. The protein concentration of the rickettsial preparations were determined using a protein determination kit with bovine serum albumin (BSA) as a standard (BioRad Richmond, C, USA). Prior to protein determination, the rickettsial preparation (1ml) was lysed using 0.1-0.5 mm diameter zirconia/silica beads in a mini bead-beater (Biospec, Products. Inc., Bartlesville, USA), beating for 160 s at high speed.

2.1.3.2. Non-*P. salmonis* isolates

After sufficient growth was obtained, bacteria were washed off the agar plates with 20 ml of sterile PBS-2 and centrifuged (Mistral 3000i, MES) at 3000 x g for 20 min at 4°C. The bacterial pellets were then washed twice with PBS-2 by centrifugation as described above and resuspended in PBS-2 at a concentration of $1x10^8$ cell/ml according to their own standard curves before storing the as aliquots at -20°C.

2.1.3.3. Gram staining

Gram staining was routinely applied to all bacterial preparations (*P. salmonis* or non-*P. salmonis*) used throughout the study to confirm the purity of the samples, for which bacterial preparations were prepared on microscope slides and allowed to air dry. The bacterial smears were heat fixed and stained with crystal violet (2 min). The crystal violet (BDH ltd. Poole, Dorset, UK) was washed off with Gram's iodine (BDH) until the metallic precipitate was washed away, and the slides were flooded with the iodine for 2 min. The slides were then washed in tap water, differentiated in acetone for a few seconds, immediately washed with tap water, and then counter-stained with 1% Neutral Red (BDH) in distilled water (v/v) (1 min). The slides were washed in tap water and blotted dry on filter paper before examining under a light microscope (LM).

2.2. Electrophoresis

2.2.1. Preparation of gels and electrophoresis of samples

Precast 12.5% mini-polyacrylamide gels (8 x 10 cm) (Sigma) were used throughout the study. The sample wells of the gel were first gently rinsed with deionised water and excess water removed before mounting into the gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, USA), which was then filled with reservoir buffer (RB: 192 mM glycine, 0.1% sodium dodecyl sulphate, 25 mM Tris base, pH 8.3). Whole cell preparations of *P. salmonis* placed in electrophoresis sample buffer (SB (x 5): 60 mM Tris-HCl, 25% glycerol, 2% sodium dodecyl sulphate, 0.1% Bromophenol blue, 14.4 M 2-β-mercaptoethanol) at a ratio of 1:1, were boiled for 5 min and centrifuged at 13000 x g for 5 min in a micro-centrifuge (IEC, Micromax). Molecular weight markers were also boiled as above, but not centrifuged. Twenty μl of each sample was loaded into the wells using a micro-syringe. Electrophoresis was

performed at 125 V at 22°C until the dye contained in electrophoresis sample buffer reached the bottom of the gel.

2.2.2. Staining the gel

2.2.2.1. Coomassie blue stain

After electrophoresis, the gels were placed in 0.1% (w/v) Coomassie blue R-250 dissolved in 45% (v/v) methanol and 10% (v/v) glacial acetic acid overnight with continuous shaking at 22°C. The gels were then destained using 45% (v/v) methanol and 10% (v/v) glacial acetic acid until a good contrast was achieved between the background and the bands.

2.2.2.2. Silver stain

Silver staining was carried out using a commercially available silver staining kit (Sigma), according to manufacturer's instructions. Deionized water (dH_2O) was used throughout the procedure. The gels were placed in a fixative solution of 30% (v/v) ethanol 10% (v/v) glacial acetic acid for 30 min with 3 changes of solution, and then rinsed 3 times with dH_2O for 7.5 min with each rinse. Gels were equilibrated in the silver equilibrium solution supplied with the kit for 30 min and promptly rinsed with dH_2O for between 10 and 20 s. The gels were then placed in three changes of developer solution (10 min each) and the reaction was stopped by placing the gel into stop solution (1% (v/v) acetic acid in dH_2O). The gels were rinsed as above and placed in reducer solution for 10-30 s followed by a quick rinse with tap water for 1 min. The gels were finally rinsed three times with dH_2O.

2.2.3. Molecular weight determination

The molecular weight of bands on the gel was determined from a standard curve of known molecular weight standards (BioRad or BioLabs or Amersham Pharmacia Biotech) against the R_f (Relative Mobility) value. The R_f value of the molecular weight markers was calculated according to Equation 2.1:

Equation 2.1

$$R_f = \frac{\text{Distance of Protein Migration}}{\text{Distance of Tracking Dye Migration}}$$

The molecular weights of the protein bands in the samples were determined from a graph of the $\log_{10}$ of the molecular weights of the protein markers, of known molecular weight, as a function of their R_f values (See & Jackowski, 1989).

2.3. Immunoblot Analysis

2.3.1. Western blot (WB)

Samples were subjected to electrophoresis as described in Section 2.2.1 and resolved antigens were transferred to nitrocellulose membranes (0.45 micrometre (μm) pore size; BioRad Laboratories, Hercules,CA, USA) using a wet transblot system (Hoefer TE 22 transfer unit). The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel, nitrocellulose membrane and filter papers were first incubated in transblot buffer (TB: 192 mM glycine, 25 mM Tris base, 20 % methanol pH 8.3) for 10 min with gentle shaking.

They were then mounted in the transblotting apparatus in the following order: device envelope (-ve electrode), filter pad, three sheets of filter paper, gel, nitrocellulose membrane, three sheets of filter paper, filter pad and device envelope (+ve electrode). The envelope was put into the transblot chamber and the transfer of proteins was

performed at 50 V for 120 min at 22°C, in transblot buffer stirring continuously. After the transfer, the nitrocellulose membrane was placed in 1% (w/v) BSA in Tris-buffered saline (TBS: 10 mM Tris base, 0.5mM NaCl, pH 7.5) for 1 h at 37°C to block non-specific binding sites. The membrane was washed 3 times with TBS containing Tween-20 (0.1% v/v) (TTBS) for 5 min on each wash, and then incubated with either rabbit anti-*P .salmonis* serum, diluted 1/100 in TBS, neat Monoclonal antibody (MAb) supernatant or Atlantic salmon anti-*P. salmonis* sera diluted 1/10 in TBS. The membranes were incubated at 22°C for 2 h, before washing as described above. Membranes incubated with the rabbit serum or the hybridoma supernatant were incubated with anti-rabbit IgG- horseradish peroxidase (HRP) (Diagnostics Scotland), diluted in 1/200 in TBS, or anti-mouse IgG-HRP (Diagnostics Scotland or Sigma) diluted in 1/100 in TBS respectively, for 1 h at 22°C. Membranes incubated with fish sera, were first placed in supernatant from hybridoma cell line 4C10 containing monoclonal antibodies that recognised salmon IgM (Courtesy of Dr. Ann Thuvander, Department of Pathology, Swedish University of Agricultural Science, S-75007 Uppsala, Sweden) for 3 h at 22°C. The membranes were washed 3 times with TTBS, before incubating with the anti-mouse (IgG-HRP) for 2 h at 22°C. When avidin-biotin-peroxidase (ABP) complex was applied, the membrane was incubated with anti-rabbit or anti-mouse IgG-biotin conjugate (Diagnostics Scotland), diluted in 1/100 in TBS, instead of anti-rabbit IgG-HRP or the anti-mouse IgG-HRP conjugates respectively. After a 90 min incubation at 22°C, the membrane was washed as described above and incubated with streptavidin-HRP (Diagnostics Scotland), diluted 1/100 in TBS for 1 h at 22°C. All membranes were then washed twice with TTBS and once with TBS, 5 min per wash. The reaction was developed at 37°C using chromogenic substrate solution (CSS): 2 ml of 4-chloronaphthol solution (0.33 % (w/v) in methanol) (Sigma), 10 ml of

PBS (PBS-3: 0.02 M Phosphate, 0.15 M NaCl, pH 7.2) with 10 μl of H_2O_2 (0.01% (v/v)). Once bands had developed, the reaction was stopped with distilled water.

2.3.2. Dot blot immunobinding assay (DBI)

A Dot-blot immunobinding assay was developed by combining and modifying the methods described Koay, Tay, Cheong and Yasin (1995), and Bio-dot Microfiltration Apparatus Instruction Manual (Undated). A Bio-dot Microfiltration apparatus (BioRad) was used for the procedure. Nitrocellulose membranes (0.45 μm pore size; BioRad) were soaked with TBS for 30 min at 37°C before replacing in the apparatus. Whole cell preparations of *P. salmonis* (0.5 microgram (μg) protein in 10 μl TBS) were placed into each of the wells of the apparatus which already contained 100 μl $well^{-1}$ of TBS and incubated for 2 h at 22°C. Each well was then washed twice with TTBS and once with TBS (400 μl), and buffers were aspirated off under vacuum. Non-specific binding sites were blocked with 300 μl 3% (w/v) BSA in TBS, incubating for 2 h at 22°C. The blocking solution was then aspirated off under vacuum and the membranes washed as described above. The wells were then incubated with either 100 μl of anti *P. salmonis* rabbit serum, diluted 1/100 in TBS for 1 h or 100 μl MAb supernatant for 2 h at 22°C. The wells were washed twice with TTBS and once with TBS, and then incubated with anti-rabbit IgG-HRP or anti-mouse IgG-HRP (Diagnostics Scotland), diluted 1 in 100 in TBS accordingly for 1 h at 22°C. Afterwards, the membrane was washed as described above and removed from the dot-blot apparatus. The reaction was developed at 37°C using CSS until dots appeared. The reaction was then stopped with distilled water.

2.3.3. Line blot immunoassay (LBI)

Line-blot immunoassay was developed using the method described by Raoult and Dasch (1989) with slight modifications. Whole cell preparations of *P. salmonis* (1μg protein in 10 μl TBS) in sample buffer at a ratio of 1:1, was applied to sheets of nitrocellulose as lines using a quill. Lines of each antigen were placed 0.5 cm apart onto the membrane and the membrane was then left to air-dry at 22°C. The membrane was immersed in 3% (w/v) BSA in TBS for 2 h at 37 °C to block non-specific binding sites, and then washed once with distilled water and 3 times with TBS (5 min on each wash). The membrane was allowed to air-dry and then each line was either cut into 0.5 cm sections or a multiscreen apparatus was used to incubate each line with either anti-*P. salmonis* rabbit serum diluted 1/100 in TBS or with the different hybridoma supernatants in incubation trays. After 2 h incubation with the rabbit serum at 22°C or overnight with the MAb supernatants at 4°C, each line was washed as above. The nitrocellulose strips were placed in either anti-rabbit IgG-HRP or anti-mouse IgG-HRP, as appropriate, diluted in 1/100 in TBS, for 2 h at 22°C. The strips were finally washed with distilled water and TBS as above, and placed into CSS until a reaction was observed. The reaction was then stopped with distilled water.

2.4. Enzyme linked immunosorbent assay (ELISA)

2.4.1. Indirect-ELISA

The following protocol was used to determine the antibody titre of the anti-*P.salmonis* rabbit-serum and the anti *P. salmonis* fish sera, screening the MAb supernatants and determining the specificity and sensitivity of the rabbit serum.

Ninety-six-well ELISA plates (Immulon ™, Dynatech, USA) were coated with 50 µl well^{-1} of 0.01% poly-L-lysine (Sigma) in carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), for 1 h at 22°C. The plates were then washed 3 times with low salt wash buffer (LSWB: 0.02 M Trisma base, 0.38 M NaCl, 0.05% Tween-20, pH 7.3) and incubated with 100 µl well^{-1}of antigen suspension (1-5 µg protein) in PBS-3 overnight at 4°C. The bacteria were then fixed to the wells by adding 50 µl well^{-1} of gluteraldehyde (0.05% v/v) diluted in PBS-3 for 20 min at 22°C. The plates were washed with LSWB. Non-specific binding sites were blocked with 250 µl well^{-1} BSA, 1% (w/v) in PBS, incubating the wells for 2 h at 22°C. The plates were then washed as described above. When the ELISA was used to screen the MAbs against *P. salmonis* or determine the antibody response of the rabbit and mouse sera, plates were incubated with 100 µl well^{-1} of either neat supernatant or a ten fold dilution of the rabbit or mouse sera in PBS-3. Pre-immune rabbit or mouse sera and PBS-3 were used as negative controls, and rabbit anti *P. salmonis* sera kindly gifted by Prof. J. Fryer, Oregon State University, Corvallis, OR, USA, diluted 1/1000, was used as a positive control. After a 1 h incubation period at 22°C, the plates were washed 5 times 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 soak on the last wash. Anti rabbit IgG-HRP (Diagnostics Scotland) and anti-mouse IgG-HRP (Diagnostics Scotland or Sigma), diluted 1/1000 in PBS-3, were added to the wells at 100 µl well^{-1}, as appropriate, incubating for 1 h at 22°C. When anti *P. salmonis* fish sera was used, sera were diluted two-fold in PBS-3 and added to the wells at 100 µl well^{-1}. Two-fold dilutions of pre-immune fish sera and PBS-3 were used as negative controls. After incubating overnight at 4°C, the plates were washed 5 times with HSWB and allowed to stand for 5 min after the last wash before removing the buffer. The plates were incubated with MAb supernatant of cell

line, 4C10 (100 μl well^{-1}) for 2 h at 22°C and washed with HSWB as described above. The plates were then incubated with HRP-conjugated anti-mouse-IgG (Diagnostics Scotland) diluted 1/1000 in PBS-3. All plates were then washed with HSWB as above. Finally, chromogen (42 mM TMB, 3'3'5'5'-Tetramethylbenidine dihydrochloride) (Sigma) in substrate buffer (0.1 M Citric acid, 0.06 M Sodium acetate, 1 % H_2O_2, pH 5.4) was added at 100 μl well^{-1} and the reaction was stopped after 10 min by the addition of 50 μl well^{-1} of 2 M H_2SO_4. The reaction was read with an ELISA reader (Dynatech) at 450 nm Optical density (OD).

2.4.2. Indirect-Sandwich Enzyme linked immunosorbent assay (SELISA)

The sandwich ELISA was used for determination of the specificity and sensitivity of MAbs against *P. salmonis*. It was performed at three stages:

2.4.2.1. Sodium sulphate precipitation of antisera

Rabbit anti *P. salmonis* serum was thawed and warmed to 25°C in a water bath for 5 min. Sodium sulphate, 14% (w/v), was added to 2 ml of the antiserum and returned to the water bath for 5 min at 25°C. The solution was then centrifuged for 15 min at 15 000 rpm in a micro-centrifuge (IEC, Micromax). The supernatant was removed and the precipitate was first washed twice with 14% Na_2SO_4 solution warmed to 25°C in the water bath and then dissolved in 2 ml of PBS-3 at 25°C. The OD of the solution was read at 280 nm to determine the protein concentration using BSA (Sigma) as a standard, which was then adjusted to 1 mg ml^{-1}. This antiserum was subjected to acid treatment prior to coating the ELISA plates to open the hinge region of the polyclonal antibody (PAb) and allow better binding to antigen.

2.4.2.2. Acid-treatment of the antibody

PBS-3 was prepared and adjusted to pH 2.1 with HCl. The acidic PBS-3 was added to the sodium sulphate precipitated antisera (Section 2.4.2.1) adjusting the protein concentration to 3.6 mg/ml. The solution was left stirring very slowly for 30 min on a magnetic stirrer, after which time, the pH of the solution was adjusted to 7.0 with solid Tris.

2.4.2.3 Sandwich ELISA

Ninety-six-well ELISA plates were coated with 1-10 μg ml^{-1} acid-treated antibody prepared as above, and incubated overnight at 4°C in coating buffer. The plates were then washed 3 times with LSWB. Non-specific binding sites were blocked with 1% w/v BSA in PBS-3 (250 μl $well^{-1}$) for 60 min at 37° C. The plates were washed as above, before adding 100 μl $well^{-1}$ of bacterial solution (1-5 μg protein) in PBS-3 for 60 min at 22°C. The plates were washed 5 times with HSWB incubating for 5 min on the last wash, and then 100 μl $well^{-1}$ of neat MAb supernatant was added to the wells for 60 min at 22°C. At the end of the incubation period, the plates were washed with HSWB as described, and incubated with 100 μl $well^{-1}$ conjugate anti-mouse IgG-HRP, diluted 1/1000 in PBS-3, for 60 min at 22°C. Plates were washed again with HSWB, incubating for 5 min on the last wash and 100 μl $well^{-1}$ chromogen in substrate buffer as described in 2.4.1 was added into the wells and incubated for 10 min. The reaction was stopped by adding 50 μl $well^{-1}$ of 2 Molar (M) H_2SO_4. The resulting reaction was read at 450 nm in an ELISA reader.

2.5. Immunohistochemistry (IHC)

The IHC procedure was performed according to Alday-Sanz, Rodger, Turnbull, Adams and Richards (1994), with slight modifications. Paraffin-embedded tissue sections obtained from Atlantic salmon, either naturally affected by rickettsiae-like organisms

(RLO) in Ireland (from archival material held at the Institute of Aquaculture, University of Stirling) or experimentally affected with the bacterium as described in Section 6.2.1.2, were prepared by cutting into 5 μm sections, and placing on microscope slides. Sections were dewaxed in xylene (twice for 5 min), dehydrated in 100% alcohol for 5 min and 70% alcohol for 3 min, and then rinse in distilled water. The tissues were encircled in a wax ring using a PAP pen (BDH Ltd. Poole, Dorset, UK), and exogenous peroxidase activity was then blocked by incubating the slides at 22°C with 10% (v/v) hydrogen peroxide in methanol for 10 min. The slides were washed three times with TBS, pH 7.5 and incubated with normal donkey (for rabbit) or goat (for mouse or for fish) serum diluted 1/10 in TBS at 20°C for 10 min in a humid chamber. The serum was removed and excess liquid removed by tapping the edge of the slides onto a paper towel. Immunised rabbit or mouse sera diluted in 1/100, 1/1000 and 1/10 000 in TBS or neat MAb supernatant were placed onto the sample and slides were incubated at 22°C for 60 min in a moist chamber. Pre-immune rabbit and mouse sera and rabbit anti-*P. salmonis* sera (kindly provided by Prof. J. Fryer, Oregon State University, Corvallis, OR, USA), diluted as above, were used as negative and positive controls respectively. One slide was incubated with TBS and acted as a further control. All slides were then washed as above and incubated with anti-rabbit-HRP or anti-mouse-HRP (1/50 in TBS) (Diagnostics Scotland), as appropriate, at 22°C for 30 min in a moist chamber. The slides were again washed as above. When anti-*P. salmonis* fish sera were used as antibodies, the sera diluted 1/10 in PBS-3 was applied onto the tissues and incubated for 2 h in a humidified chamber at 22°C. One slide was incubated without additions of antisera as negative control. The slides were washed three times with TBS and incubated with MAb 4C10 for 2 h in a humidified chamber at 22°C. The slides were washed again three times with TBS. Anti-mouse-HRP (1/50 in TBS)

(Diagnostics Scotland) was applied on to the slides as secondary antibody. The slides were washed again with TBS as above.

All samples were incubated with True blue (Kirkegaard Perry Laboratories, UK) for 10 min. The reaction was stopped by immersing the slides in tap water and the sections were counter-stained with Contrast Red (Kirkegaard Perry Laboratories, UK) for 2 min. The slides were then left in tap water for 10 min, dehydrated in 70% (v/v) alcohol for 3 min and 100% (v/v) alcohol for 5 min, and rinsed twice in xylene, 5 min each rinse. Slides were mounted using Pertex and tissues observed under a light microscope for the presence of rickettsia.

2.6. Indirect fluorescent antibody technique (IFAT)

IFAT procedures were performed with slight modification and combination of the methods described by Lannan, Ewing and Fryer (1991) and Fenollar and Raoult (1999).

Microscope slides containing 21 wells, 4 mm in diameter (DYNEX) were used for the microimmunofluorescence test (MIFAT). Bacterial suspensions (7 μl) were placed onto the wells of the slide, allowed to air dry and fixed in absolute methanol for 5 mins. Paraffin-embedded tissue sections (obtained from Atlantic salmon, in Ireland affected by RLO) were prepared from archival material held at the Institute of Aquaculture, University of Stirling, cut into 5 μm sections, dewaxed in xylene twice for 5 mins, dehydrated in 100% alcohol for 5 mins and 70% alcohol for 3 mins and then rinsed in distilled water. Tissue smears from experimentally infected Atlantic salmon (Section 6.2.1.2) were also air-dried and fixed in absolute methanol for 5 mins. Tissue sections and tissue smears were encircled with a wax PAP pen (BDH).

IFATs were conducted in the following manner for all samples above. The slides were then incubated with rabbit or mouse sera diluted in 1:100, 1:1000 and 1:10 000 in PBS-3 or neat MAb supernatant for 60 min or fish sera diluted 1:10 in PBS-3 for 2 h, in a humidified chamber at 22°C. Rabbit anti-*P. salmonis* (kindly provided by Prof. J. Fryer, Oregon State University, Corvallis, OR, USA) was used as positive control and pre-immune rabbit, mouse or fish sera and PBS-3 as negative controls. Slides were rinsed thoroughly with PBS-3 and left to stand for 5 min in PBS-3, then they were rinsed once again before applying goat anti-rabbit IgG conjugated to Fluorescein isothiocyanate (FITC) (Sigma) diluted 1/80 in PBS-3, and 1:50 dilution of FITC labelled donkey anti-mouse IgG (Diagnostics Scotland) in PBS-3 to the smears for 30 min as the secondary antibody as appropriate. Slides were incubated in the dark in a humidified chamber at 22°C during this time, after which they were again washed with PBS-3 as above. When fish sera were used as primary antibody, slides were then incubated with MAb supernatant of 4C10 cell line for 2 h in a humidified chamber at 22°C and the slides were rinsed with PBS-3 as described above. The slides were then incubated with 1:50 dilution of FITC donkey anti-mouse IgG (Diagnostics Scotland) in PBS-3 for 30 min in the dark in a humidified chamber at 22°C before they were again washed with PBS-3 as above.

Samples were observed for the presence of bacteria under B excitation using an Olympus IMT-2 microscope with a reflected fluorescent attachment, and exciter and barrier filters for FITC. For MIFAT each well was incubated with 10 μl of each solution.

2.7. Immuno-gold staining (IGS)

The purified *P.salmonis* was pelleted at 20 000 x g for 30 min at 4°C. The pellet was fixed overnight at 4°C in 2.5% gluteraldehyde in PIPES buffer (0.2 M Piperazine N'N'bis [2-ethanesulphonic acid], pH 5.5) (Sigma) and then washed 3 times with the same buffer followed by a rinse in distilled water twice. The tissue was dehydrated through an alcohol series (30-70%) at 40 min intervals and then infiltrated overnight with 70% ethanol: LR white resin (London Resin Co., Reading, England) (1:1). The solution was replaced with 100% LR white resin for 8 h and then changed with fresh resin and this time incubating overnight. The tissue section was put in pointed capsules with fresh LR white and polymerised at 50°C for 24 h. The samples were removed from the capsules and cut into ultra-thin sections and mounted on nickel grids. The grids were floated up-side down on drops of filtered (0.45 μm) wash buffer (Wb: 0.2 M TBS, 1% Tween, 1% BSA) containing 10% FCS which had been spotted onto a piece of a parafilm placed in a moist container, to block non-specific binding, overnight at 4°C. The grids were then transferred to hybridoma supernatants or fish sera by incubating overnight at 22°C. The sections were washed twice with Wb by floating them in a 24-well plate with occasional agitation for 90 mins each wash. The hybridoma supernatant applied grids were then incubated up-side down on drops of 1 in 40 anti-mouse IgG gold conjugate, 5 nm (Sigma) in blocking buffer overnight at 22°C. Fish sera-treated grids were incubated with MAb 4C10 overnight at 22°C, washed as described above and then incubated in 1 in 40 dilution of anti-mouse IgG gold conjugate, 5 nm (Sigma) in blocking buffer overnight at 22°C. All the samples were washed again as described above, followed by holding under a stream of distilled water for a few seconds. The 5 nm immunogold labeling was enhanced by placing on drops of silver enhancing solution (British BioCell Int. Cardiff, UK) for 2 min and the

reaction was stopped by immersing the grids through a series of three vials containing distilled water. The sections were counter-stained using uranyl acetate and lead citrate and viewed under a Phillips 301 electron microscope at 80 kilovolt (kV).

CHAPTER III

PURIFICATION OF *PISCIRICKETTSIA SALMONIS* FROM CELL CULTURE AND ELECTRON MICROSCOPY OF PURIFIED BACTERIA

3.1. INTRODUCTION

Rickettsia are highly fastidious bacteria that share properties common with other intracellular pathogens (Palmer, Mallavia, Tzianabos & Obijeski 1974). Culture of rickettsia *in vitro* within cell lines, is necessary to study both molecular and cellular aspects of the bacterium and for vaccine development. The ability of the organism to infect host cells has also been examined through cell culture. It has been shown that rickettsia released from infected host cells are able to infect uninfected adjacent cells either through the continuous shedding of rickettsia or from the release of the rickettsia as host cells lyse (Winkler 1990). Because of its intracellular nature, the release of rickettsia from host cells is often incomplete, with more than half of the total infectious yield frequently remaining associated with intact host cells or cell debris (Moulder 1985).

For many types of analysis such as antigenic, chemical or morphological studies, it is necessary to isolate and purify the organism from the host cell in which it is grown. Several techniques have been used to purify the rickettsia from host tissue, whereby the organism is first inactivated with low concentrations of formaldehyde or by ultraviolet irradiation (Anacker, Gerloff, Thomas, Mann, Brown & Bickel 1974 and Palmer *et al.* 1974). Where viable rickettsia are required, celite, bovine serum albumin, cation ion exchange resins (Bovarnick & Synder 1949; Bovarnick & Miller 1950 and Yamamoto,

Kawamura, Hara & Aikawa 1958), and/or sucrose, Renografin, Verografin or Percoll density gradient centrifugation have been used to eliminate host cell contamination (Weiss, Coolbaugh & Williams 1975; Tamura, Urakami & Tsuruhara 1982 and Aniskovich, Eremeeva, Balaeva, Ignatovich, Artemiev, Emelyanov & Smirnova 1989).

Isopycnic centrifugation, in which particles are separated based purely on their density, has proved to be an extremely useful technique for the fractionation of biological material. Biological particles pass through a density gradient under centrifugation until their density reaches the same as that of the surrounding medium (Rickwood 1987). However, there are limitations with the method resulting from the physico-chemical properties of the substance used to form the density gradients, and with ionic strength, osmolarity, viscosity, solubility and density of each density gradient medium (Rickwood & Birnie 1975). The isotonic nature of Percoll allows successful fractionation of various cells types, organelles and viruses due to the simple feature of self-forming gradients, which result during isopycnic centrifugation.

P. salmonis can be cultured successfully within fish cell lines *in vitro* (Fryer, Lannan, Garcés, Larenas & Smith 1990), providing sufficient quantities of the organism to allow characterisation of rickettsial proteins. Techniques for purifying *P. salmonis* have been reported by a number of authors. Kuzyk, Thorton and Kay (1996) described a method of purifying *P. salmonis* from tissue culture supernatants by centrifugation using a 40% (v/v) Percoll gradient. This method was based on previous methods described by Tamura *et al.* (1982) and Weiss *et al.* (1975). Jamet, Aguayo, Miquel, Muller, Arriagada, Becker, Valenzuela and Burzio (2001) used a 45% (v/v) Percoll gradient to purify *P. salmonis* based on a modification of the protocol described by Tamura *et al.*

(1982). Barnes, Landolt, Powell and Winton (1998) used a Renografin density gradient, modified from the procedure described by Weiss *et al.* (1975).

This Chapter describes the development of an effective purification protocol for *P. salmonis* isolates, which was quick and easy to perform, but still maintained the viability and antigenicity of the bacterium. Initially, a modified version of the method described by Kuzyk *et al.* (1996) was used to purify different isolates of *P. salmonis*. A purification method was then developed using differential centrifugation together with 30, 40 or 50% (v/v) Percoll gradients. The purity of each preparation was assessed by transmission electron microscopy (TEM). The growth cycle of *P. salmonis* within fish cell lines and at different stages of the purification protocol was also examined by TEM and light microscopy (LM).

3.2. MATERIAL AND METHODS

3.2.1. Culture of *P. salmonis in vitro*

P. salmonis examined in this study was maintained in chinook salmon, *Oncorhynchus tshawytscha* Walbaum embryo cell line (CHSE-214/ATCC CRL-1681) at 15 to 17°C as described in Section 2.2.1.2 (c), using antibiotic-free Eagle's minimal essential medium (MEM) with Earle's salts, supplemented with 2% (v/v) foetal calf serum (FCS), 2 millimolar (mM) L-glutamine and 1% non-essential amino acids (NEAA) (v/v) (MEM-2).

3.2.2. Density gradient centrifugation

Six triple flasks (3000 cm^2 culture area) (Nunc A/S Roskilde Denmark) were seeded with CHSE-214 cells at a seeding concentration of 5 $x10^4$ cells ml^{-1}, and incubated for

48 hours (h) at 22°C. The culture medium was removed and each flask was then inoculated with 20 ml of supernatant recovered from a *P. salmonis*-infected cell culture. After incubation at 15°C for 2 h, the supernatant was removed and approximately 100 ml of fresh MEM, supplemented as above, was added to each flask. The cells were cultured for between 10 and 15 days (d) until an extensive cytopathic effect (CPE) (approximately 90%) was observed. The cell culture supernatant was collected and centrifuged at 20 000 x g for 30 minutes (min) at 4°C and the resulting pellet was resuspended in 12 ml Tris-sucrose buffer (TS, 33 mM Tris-hydrochloride containing 0.25 M sucrose, pH 7.4). Three ml of the supernatant was separated to use as a crude rickettsia for protein determination and viability experiments. The remainder was homogenised with a Dounce tissue homogeniser (Kinematica, Switzerland) at a moderate speed for 2 min, then gently vortexed for 1 min. Homogenates were centrifuged at 210 x g for 10 min at 4°C to remove host cell debris. Three ml aliquots of the homogenate were loaded onto 27 ml of Percoll (Pharmacia Biotech AB, Uppsala, Sweden), prepared at 30, 40 and 50% (v/v) in polycarbonate centrifuge tubes (25 by 89 mm). The tubes were centrifuged at 25 000 x g for 60 min at 4°C to allow a self-forming gradient to develop by isopycnic centrifugation. Bands which formed were harvested from the gradient, diluted 10 fold in phosphate buffered saline, pH 7.4 (PBS: 5.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 116 mM NaCl, 10 mM KCl, pH 7.0) and rickettsia pelleted by centrifuging at 20 000 x g for 30 min at 4°C. The pellet was resuspended with PBS and centrifuged as described above to eliminate residual Percoll from the preparations. Differential pelleting and density gradient centrifugation was performed using a fixed angle rotor (type TFT-70.38) in a Kontron, Centrikon T-1170 ultracentrifuge. Density marker beads (Pharmacia Biotech AB, Uppsala, Sweden) were

used to indicate the density profile of the gradients and the density at which sample bands formed.

3.2.3. Protein yields

Protein concentrations of the rickettsial preparations before and after purifications were determined using a protein determination kit (BioRad) as described Section 2.1.3.1.

3.2.4. Assessing the viability of *P. salmonis* by a plaque assay

The plaque assay was performed by combining and modifying the protocols described by Kordova (1966), Burleson, Chambers and Wiedbrauk (1992) and Barnes (1996). Tissue culture plates, with either 24 or 96-wells, were seeded with CHSE-214 cells at a concentration of 5 $x10^4$ cells ml^{-1}. The plates were incubated for between 24 and 48 h at 22°C until a cell monolayer with 50-70% confluence was established. Culture medium was removed from the wells and the monolayer was washed with PBS (Gibco) to eliminate any dead cells. The wells (four replicate wells) were then inoculated with a ten-fold dilution of the rickettsial preparations (un-purified and purified using different concentrations of Percoll) which had been diluted in PBS. A 1 ml volume of the preparations was added to 24-well plates, while 100 microlitre (μl) was added to 96-well plates. After centrifugation of the plates at 200 x g for 10 min, the fish cells were incubated for 2 h to allow rickettsial absorption. At the end of the incubation period, the supernatant was removed and a 2.5% (v/v) methylcellulose overlay (See Appendix 5.1) was added to the wells (250 μl $well^{-1}$). CHSE-214 cell culture was used as negative control. After a 17 d incubation period, the well contents were fixed with 10% (v/v) buffered formalin for 15 min, and the liquid was then removed. The wells were stained with 1% (w/v) Crystal Violet solution (Sigma) (See Appendix 5.2) for 15 min

and the reaction stopped by washing the wells with tap water. The plaques were counted under a LM. A dilution series was selected in the plaque assay, which gave between 20 and 200 plaques $well^{-1}$. The number of plaque forming units (PFU) ml^{-1} was determined by taking the mean number of plaques for four monolayers at that dilution and multiplying it by the reciprocal of the dilution and the reciprocal of the volume added.

3.2.5. Determination of the 50% tissue culture infective dose ($TCID_{50}$ ml^{-1})

Tissue culture plates with 96-wells were seeded with CHSE cells and then inoculated with ten-fold dilutions of the rickettsial preparations (un-purified and purified using different concentrations of Percoll). The bacteria were diluted in PBS and four replicate wells were set up as the plaque assay protocol described above. After centrifugation at 200 x g for 10 min, the plates were incubated for a further 2 h to allow rickettsia absorption. The supernatant was removed and fresh MEM-2 was added to the wells (250 μl $well^{-1}$). The plates were incubated at 15°C. During the incubation period, the wells were monitored microscopically on 1, 3 and 5 d and the development of the CPE recorded. At the end of the 7 d incubation period, $TCID_{50}$ ml^{-1} titers were calculated using the method of Reed and Muench (1938). The development of the CPE was recorded as follows: (-) no CPE; (+) partial CPE (around 25% or less than 50%); (++) about 50% of the cells exhibit CPE; (+++) about 75% of the cells exhibit CPE; (++++) the monolayer is totally destroyed. CPEs with a score of 1 (+)- 4 (++++) were recorded as positive when calculating the $TCID_{50}$ of the cell cultures. Uninfected CHSE-214 cells were used as a negative control.

3.2.6. Transmission electron microscopy (TEM)

Samples of *P. salmonis* were prepared from CHSE-214 cells 2 h after the initial inoculation, from different steps during the purification process, and with purified rickettsia and CHSE cells for TEM, by fixing cell pellets with 2.5% (v/v) glutaraldehyde for 2 h at 4°C. They were then placed in cacodylate buffer and rinsed overnight at 4°C. The pellets were post-fixed with 1% osmium tetroxide in the same buffer for 1 h, stained with 2% uranyl acetate in 30% acetone for 1 h in the dark, dehydrated through a series of acetone concentrations, and then embedded in Spurr's embedding medium. Before preparation of sections for TEM, initial sections with a 1-3 micrometer (μm) thickness were cut, stained with 1% True blue in distilled water (v/v) for 45 seconds (sec), washed with distilled water, air-dried on a hot-plate and examined under a LM. Sections with a 100 nanometre (nm) thickness were then cut, and stained for 2 min with uranyl acetate saturated in 50% ethanol before placing the sections in lead citrate for 2 min. They were viewed with a Philips 301 electron microscope (EM) at 80 kilovolt (kV) in transmission mode.

3.2.7. Indirect fluorescent antibody technique (IFAT) with the rabbit anti *P. salmonis* serum

The purity of the *P. salmonis* preparation resulting from the purification process was examined by IFAT according to the method outlined in Section 2.6 using rabbit anti *P. salmonis* (PAb-1) developed in Chapter V against *P. salmonis* type strain LF-89. Bacterial smears were fixed onto slides, and PAb-1 (diluted 1/1000 in PBS) was used as the primary antibody and goat anti-rabbit IgG conjugated to Fluorescein isothiocyanate (FITC) (Sigma) as the secondary antibody. One slide was incubated with PBS as a

negative control. The results were observed under an Olympus IMT-2 microscope with a reflected fluorescent attachment, and exciter and barrier filters for FITC.

3.2.8. Analysis of purified *P. salmonis* by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-Blot (WB) analysis

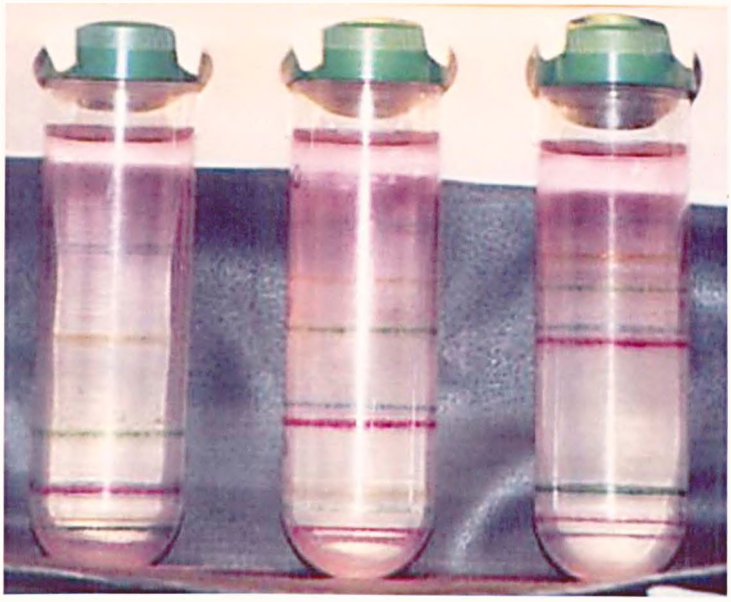
P. salmonis, purified from the different density gradients (see Section 3.2.2), were analysed by SDS-PAGE (see Section 2.2) and WB analysis (see Section 2.3.1). CHSE-214 cells, CHSE-214 cells infected with type strain LF-89 or purified type strain LF-89 (100 microgram (μg) ml^{-1}) resuspended in sample buffer were loaded onto the gel, and electrophoresis was performed at 125 Volt (V) at 22°C. The gels were then either stained with Silver stain or Coomassie blue stain to examine differences in the protein banding pattern of the samples, or antigens resolved in the gel were transferred to nitrocellulose membranes and incubated with rabbit anti *P. salmonis* serum (PAb-1) developed in Chapter V against *P. salmonis* type strain LF-89, diluted 1/100 in Tris-buffered saline (TBS). Anti-rabbit IgG- horseradish peroxidase (HRP) (Diagnostics Scotland) was used as the secondary conjugated antibody in WB analysis.

3.3. RESULTS

3.3.1 Purification of *P. salmonis* by Percoll density gradient centrifugation

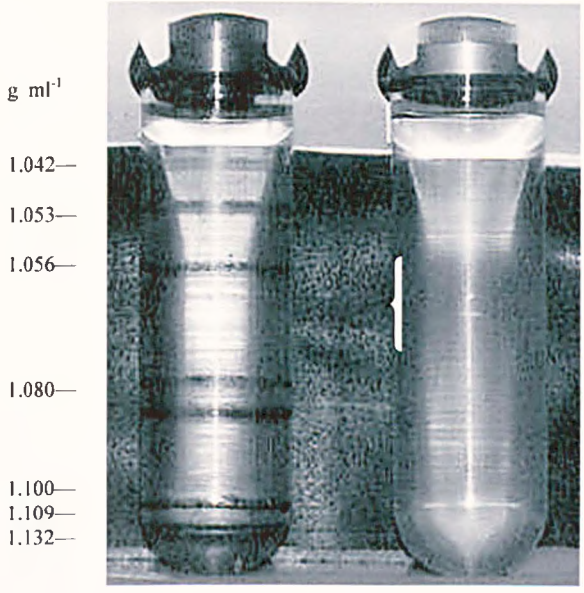
After the development of a CPE in infected CHSE-214 cells, *P. salmonis* was isolated from host cell debris by differential pelleting. The partially purified *P. salmonis* was then fractionated using either a 30, 40 or 50% (v/v) Percoll density gradient by centrifugation in a fixed-angle rotor. The density gradient which developed within each of the tubes was determined using coloured density marker beads (Figure 3.1). A wide, whitish diffuse band with a density of between 1.056 and 1.080 g ml^{-1} formed within

the gradient when homogenised infected cell culture supernatant was applied to the gradient and centrifuged. The gradient, which developed within the 40% (v/v) Percoll density gradient, is shown in Figure 3.2 together with the band of *P. salmonis* (verified by TEM, see below) which resulted during the purification process. The distance from the meniscus was calculated for each density band (Figure 3.3). The CHSE-214 cell proteins had a density of between 1.053 and 1.055 g ml^{-1}, determined from uninfected cell preparations in the gradient.



a b c

Figure 3.1. Banding of density marker beads within each Percoll gradient (a) 30% (v/v), (b) 40% (v/v) and (c) 50% (v/v), which were used to determine the different densities of the Percoll gradient



Control *P. salmonis*

Figure 3.2. *P. salmonis* band obtained during density gradient centrifugation with 40% (v/v) Percoll. The bands of density marker beads in the control tube represent different densities present in the tube

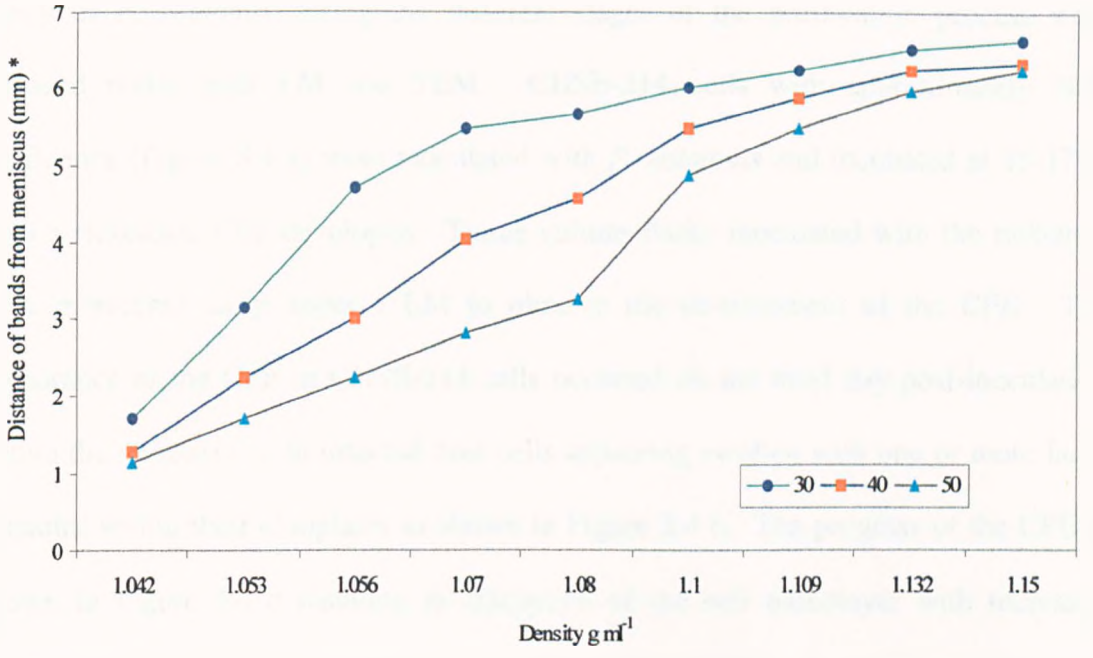


Figure 3.3. Development of density gradients with 30, 40 and 50% Percoll after centrifugation in a fixed-angle rotor at 25 000 x g for 60 min at 4°C. Gradient densities were determined using coloured density marker beads. (*) Values represent a single operation.

The progress of a CPE in CHSE-214 cells by *P. salmonis* before purification, and the purity of *P. salmonis* during the different stages of the purification process were assessed under both LM and TEM. CHSE-214 cells with approximately 50% confluence (Figure 3.4 a) were inoculated with *P. salmonis* and incubated at 15-17°C until a rickettsial CPE developed. Tissue culture flasks inoculated with the rickettsia were monitored daily under a LM to observe the development of the CPE. The appearance of the CPE in CHSE-214 cells occurred on the third day post-inoculation within the rickettsia, with infected host cells appearing swollen with one or more large vacuoles within their cytoplasm as shown in Figure 3.4 b. The progress of the CPE is shown in Figure 3.4 c resulting in disruption of the cell monolayer with increased vacuolation, both in size and number, within the host cells. An uninoculated control flask was also checked daily to be sure it remained healthy and uncontaminated (Figure 3.4 d).

The initial infection of the CHSE cells by *P. salmonis* was examined by TEM. Within the first 2 h post-inoculation *P. salmonis* were observed inside cytoplasmic vacuoles (Figure 3.5 a and b). The progress of the CPE is shown in Figure 3.5 c. A thin section of uninoculated CHSE cells under TEM is also shown in Figure 3.5 d.

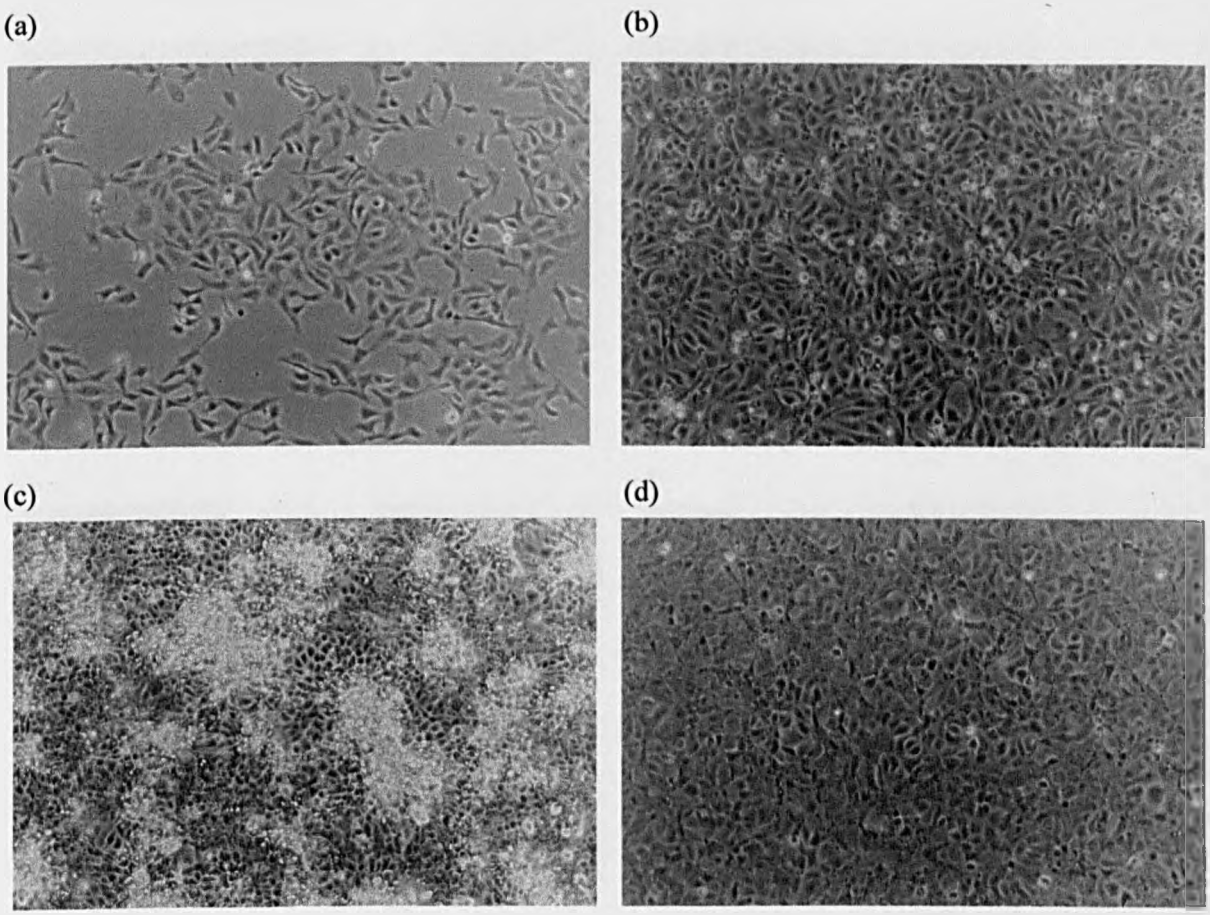


Figure 3.4. Development of CPE by *P. salmonis* in CHSE cells (a) CHSE-214 cell line (2 d old) with around 50% confluence (magnification x 100); (b) *P. salmonis* growing in CHSE-214 cell line on the 3 d post-inoculation (magnification x 100); (c) CPE produced by *P. salmonis*, 7 d post-inoculation (magnification x 100); (d) 100% confluent CHSE-214 cell line (5 d old) (magnification x 100)

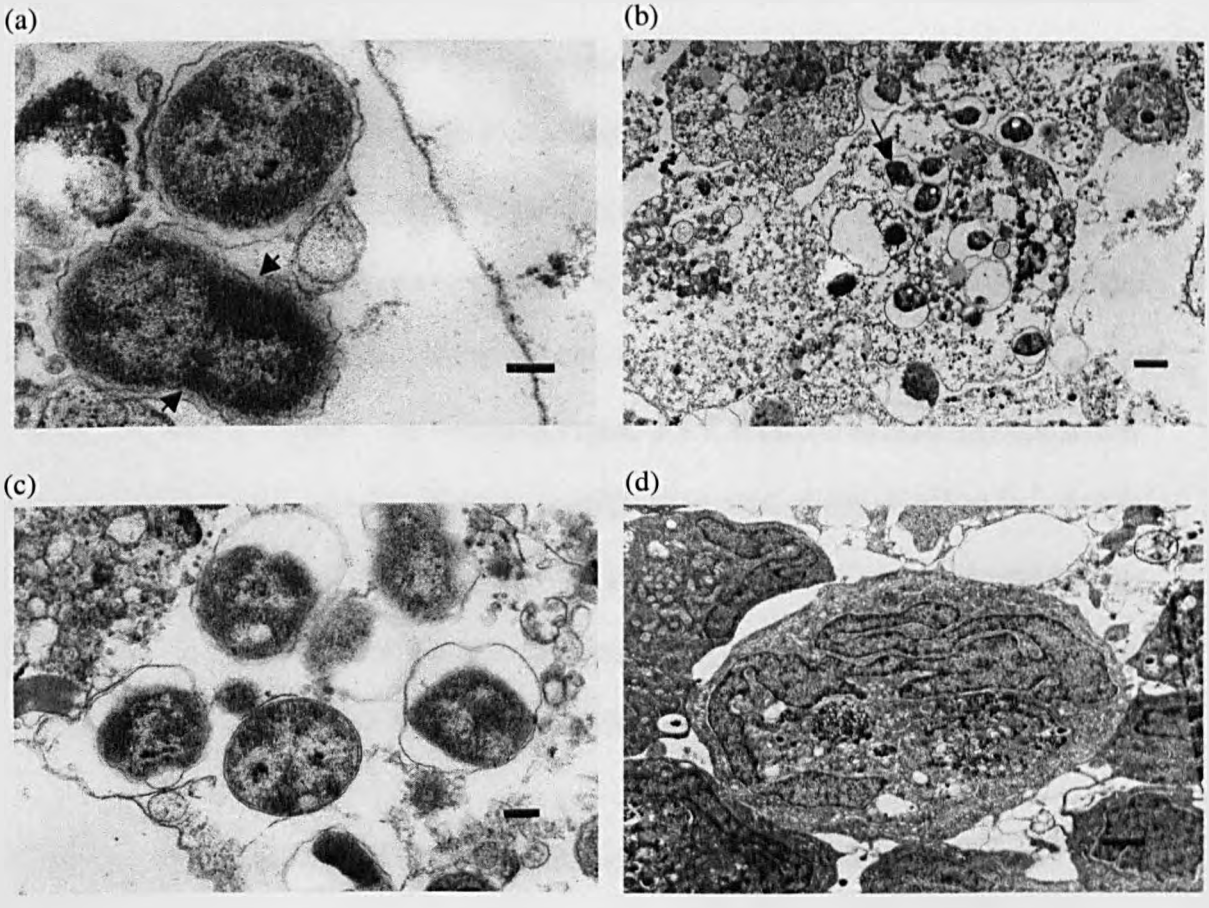


Figure 3.5. Progress of CPE by *P. salmonis* in CHSE cells examined by TEM (a) *P. salmonis* in CHSE cell (2 h post-incubation), showing the bacterium undergoing binary fission, a division into two similarly equal parts (arrows). Bar: 0.2 μm; (b) TEM of *P. salmonis* (2 h post-inoculation). Note the presence of rickettsia-like organisms inside vacuoles in the CHSE cells (arrow). Bar: 1 μm (c) Progress of the CPE (3 d post-incubation), coccoid and pleomorphic rickettsia-like bodies seen in a cell vacuole. Bar: 0.25 μm; (d) Thin section (100 nm) of uninoculated CHSE-214 cell. Bar: 1.5 μm

Each stage of the purification protocol was examined either as 5 μm sections stained with True blue under a LM, or as thin sections (100 nm) under TEM. The first step of the purification protocol consisted of harvesting the cell culture supernatant after the development of an extensive CPE in the host cell monolayer, by centrifugation at 20 000 x g for 30 min at 4°C, the LM results of which are shown in Figure 3.6 a. The pellet was then homogenised, vortexed and centrifuged at low speed (210 x g) to remove the host cell debris. As shown in Figure 3.6 b, most of the host cell debris was removed at this stage and this fraction is referred to semi-purified rickettsia. A thin section of this material is shown in Figure 3.7 a. A thin section was also prepared of the CHSE cell debris obtained by the low speed centrifugation (210 x g). This material is shown under LM in Figure 3.6 c and under TEM in Figure 3.7 b respectively.

Following fractionation on Percoll, the band with the density of 1.056-1.080 was found to be composed entirely of rickettsiae under LM and TEM examination, (Figure 3.6 d and 3.7 c, respectively). The size and morphology corresponded to that expected for *P. salmonis*, i.e. pleomorphic and ca. 0.5-1.5 μm in diameter (Fryer & Lannan 1994). The sample removed from the Percoll density gradient purification was also assessed by IFAT using PAb-1 from Chapter IV as in Figure 3.6 e, and again found to be composed of rickettsiae.

The preparations were mainly composed of whole-cell *P. salmonis,* with only very small amounts of fragmented material depending upon the gradient concentrations. *P. salmonis* purified using a 30% Percoll gradient appeared to have hardly any fragmented material and purity of the preparation was very good (Figure 3.7 f) compared with the sample of *P. salmonis* purified on the 40 and 50% Percoll gradients (Figure 3.7 d and

e). Two membrane layers were evident on each rickettsial body, an external rippled cell wall and an inner membrane. While some organisms contained one or more electron-lucent spherical structures, electron dense areas resembling ribosome-like structures were seen throughout the cell as well as Deoxyribonucleic acid (DNA)-like material presented as a filamentous network. The *P. salmonis* recovered from the 30% Percoll gradient seemed to retain their intracellular structure better than *P. salmonis* obtained from the 40 and 50% Percoll gradient. Following Percoll density gradient sedimentation and collection of the rickettsial band, the supernatant was centrifuged again at 25 000 x g for 1hour at 4°C to recover any rickettsia that was still in the supernatant. Although no band was evident, the region between 1.056 and 1.080 g ml^{-1} was collected and examined under EM (Figure 3.7 g). There was very little cellular material present in the Percoll gradient residue. Different *P. salmonis* isolates (Table 2.1) purified using differential pelleting and Percoll density gradient centrifugation, appeared similar when examined under EM (Figure 3.8 a-h). Nevertheless, *P. salmonis* isolate R980769 appeared to be highly pleomorphic while, isolate R-29 was bigger than the other isolates examined.

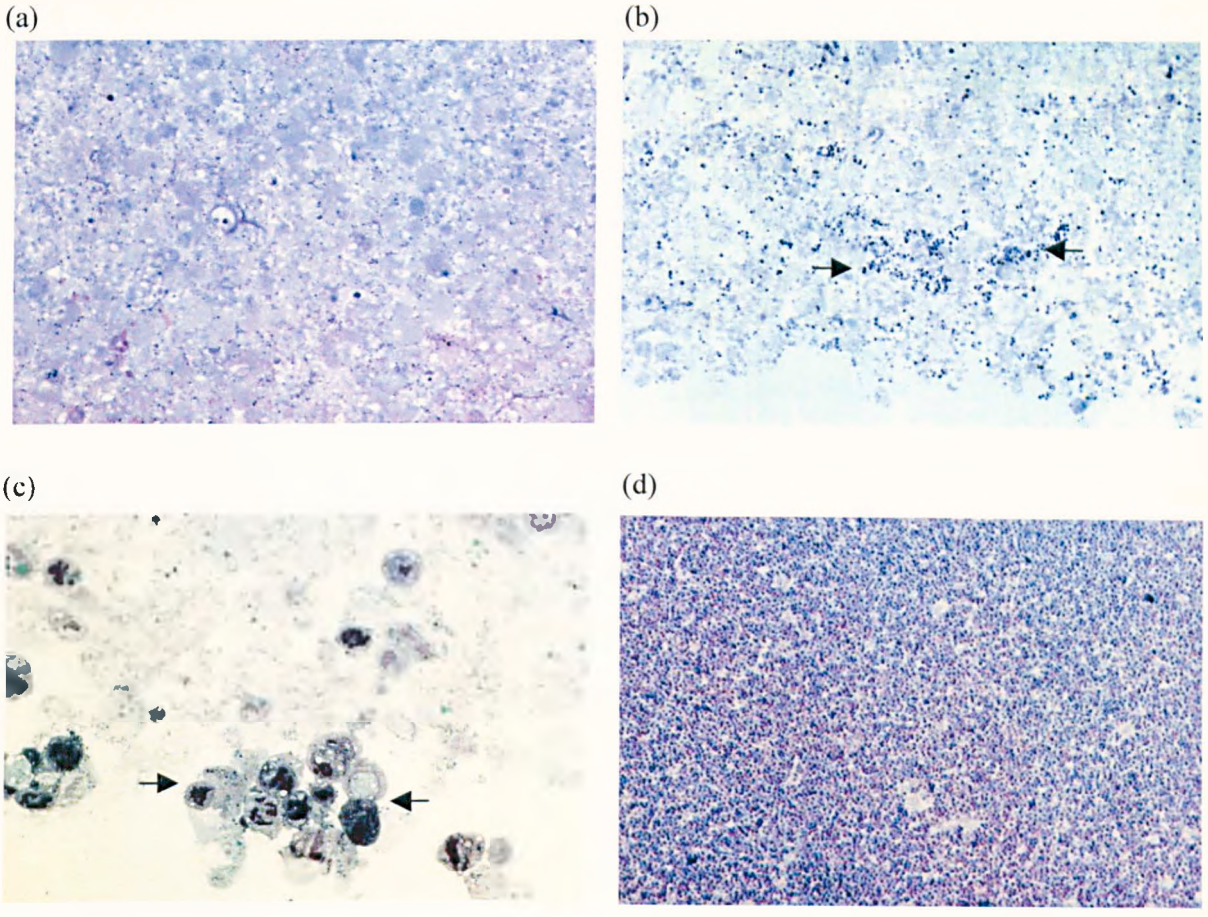


Figure 3.6. The purity of *P. salmonis* during different stages of the purification process examined under LM and by immunofluorescences; (a) The first step of the purification process in which cell culture supernatant infected with *P. salmonis* (Strain LF-89) was pelleted at 20 000 x g for 30 min at 4°C. The section was stained with True blue. (magnification x 1100); (b) Rickettsia-like bodies (Strain LF-89) (arrows) separated from cell debris by homogenisation, vortexing and low-speed centrifuge at 210 x g for 10 min at 4°C, stained with True blue (magnification x 1100); (c) CHSE-214 cell debris (arrows) removed before Percoll gradient purification stained with True blue (magnification x 1100); (d) *P. salmonis* (Strain LF-89) after density gradient purification using 30% Percoll gradient, stained with True blue (magnification x 1100); (e) IFAT, *P. salmonis* (Strain LF-89) after density gradient purification using 40% Percoll gradient stained with rabbit anti-*P. salmonis* PAb (magnification x 100)

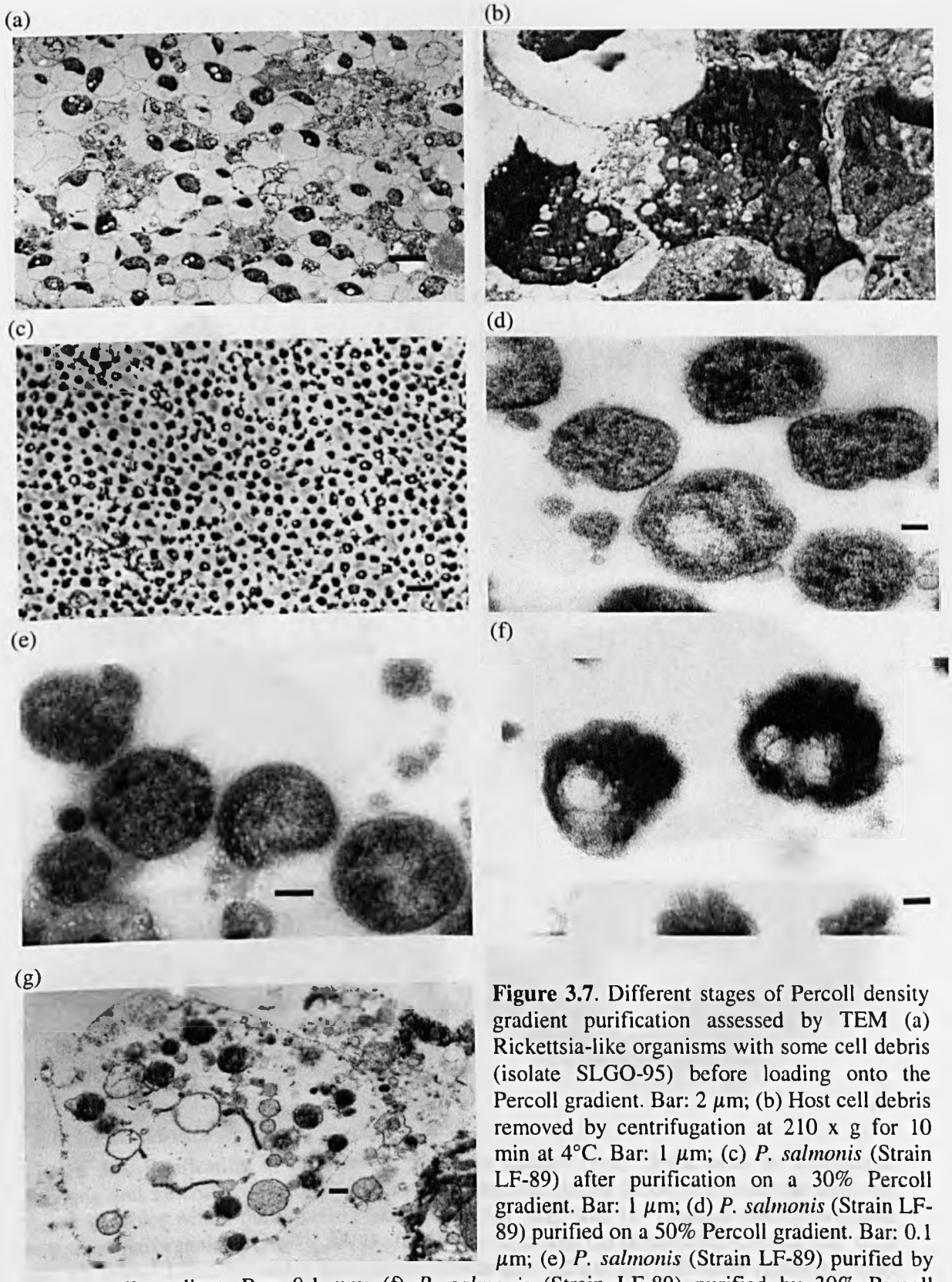


Figure 3.7. Different stages of Percoll density gradient purification assessed by TEM (a) Rickettsia-like organisms with some cell debris (isolate SLGO-95) before loading onto the Percoll gradient. Bar: 2 μm; (b) Host cell debris removed by centrifugation at 210 x g for 10 min at 4°C. Bar: 1 μm; (c) *P. salmonis* (Strain LF-89) after purification on a 30% Percoll gradient. Bar: 1 μm; (d) *P. salmonis* (Strain LF-89) purified on a 50% Percoll gradient. Bar: 0.1 μm; (e) *P. salmonis* (Strain LF-89) purified by 40% Percoll gradient. Bar: 0.1 μm; (f) *P. salmonis* (Strain LF-89) purified by 30% Percoll gradients. Bar: 0.1 μm; (g) The resulting preparation from the second Percoll density gradient sedimentation. The region between 1.056 and 1.080 g ml^{-1} was collected and examined under EM. Bar: 0.3 μm

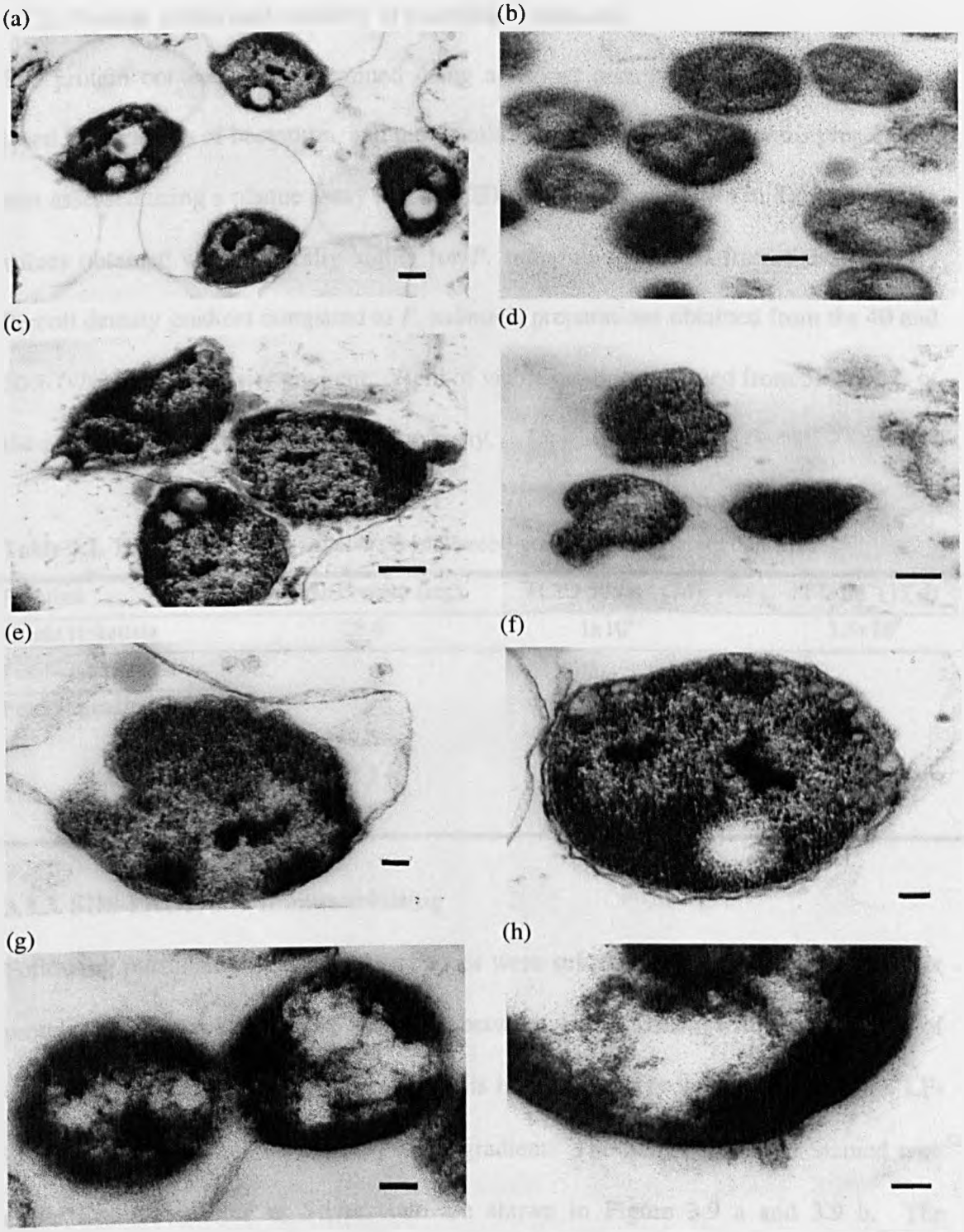


Figure 3.8. Purification of different *P. salmonis* isolates from CHSE cells by differential pelleting and Percoll density gradient centrifugation. (a) Isolate SLGO-95. Note the electron-lucent vacuoles within the rickettsia-like bodies. Bar: 0.25 μm; (b) Coccoid and pleomorphic rickettsia-like organisms (isolate AVG-5/268). Bar: 0.25 μm; (c) Isolate SRS-UACH. Bar: 0.25 μm; (d) Isolate R980769 with a high pleomorphism Bar: 0.2 μm; (e) Individual rickettsial body with high magnification after 40% Percoll density gradient (Isolate SLGO-95). Bar: 0.1 μm; (f) Isolate SLGO-95 showing the rippled cell wall. Bar: 0.1 μm; (g) Isolate R-29 after 30% Percoll density gradient apparently following the binary fission. Bar: 0.2 μm; (h) High magnification of Isolate R-29 after purification. Bar: 0.1 μm.

3.3.2. Protein yields and viability of purified *P. salmonis*

The protein content was determined using a Biorad protein determination kit using lysed preparations of bacterium, and the viability of the purified *P. salmonis* preparation was assessed using a plaque assay and a $TCID_{50}$ ml^{-1} assay as shown in Table 3.1. The values obtained were generally higher for *P. salmonis* recovered from the 30% (v/v) Percoll density gradient compared to *P. salmonis* preparations obtained from the 40 and 50% (v/v) Percoll density gradient. Yield of viable organisms varied from 55 to 65% of the original sample measured by plaque assay.

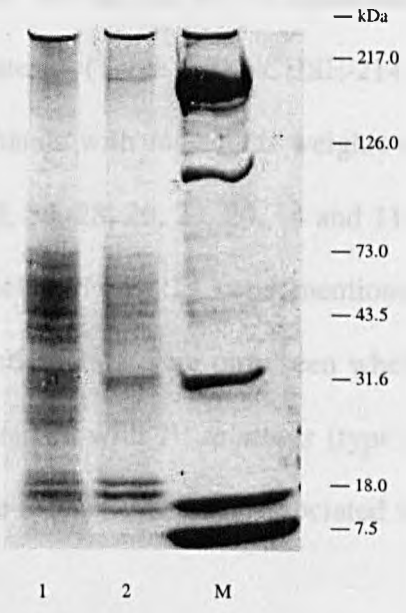
Table 3.1. The recovery of *P. salmonis* from Percoll gradients of different densities

Fraction	Protein content (mg)	TCID 50 ml^{-1} (7d)	PFU ml^{-1}(17 d)
Crude rickettsia	28.6	$1x10^{6.5}$	$3.5x10^{9}$
Purified rickettsia			
Percoll gradient (%)			
30	7.5	$1x10^{5.8}$	$2.3x10^{9}$
40	7.2	$1x10^{5.35}$	$1.94x10^{9}$
50	6.8	$1x10^{5.35}$	$2.1x10^{9}$

3.3.3. SDS-PAGE and Immunoblotting

Following purification, isolated *P. salmonis* were subjected to SDS-PAGE. Different protein banding patterns were obtained between lanes loaded with preparations of uninfected CHSE-214 cells, CHSE-214 cells infected with *P. salmonis* type strain LF-89, and LF-89 purified on a 40% Percoll gradient. The SDS-PAGE gels stained with either Coomassie blue or Silver stain are shown in Figure 3.9 a and 3.9 b. The molecular weights of the proteins bands in each sample were determined from a graph of the log of the molecular weights of the protein standards as a function of their relative mobilities run in each gel.

(a)



(b)

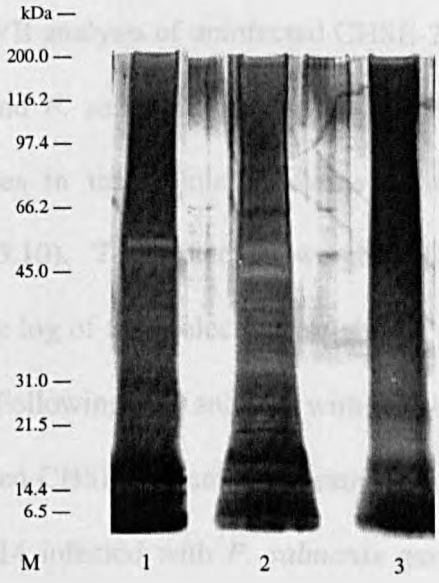


Figure 3.9. a) Coomassie blue staining of SDS-PAGE of lanes (1) *P. salmonis* (LF-89) infected cell culture and (2) uninfected CHSE-214 cell and; (M) Molecular weight marker (Bio-Rad); **b)** Silver staining of SDS-PAGE of (1) uninfected CHSE-214 cell, (2) *P. salmonis* (LF-89) infected cell culture and (3) purified *P. salmonis* (LF-89) using with 40% Percoll density gradient

SDS-PAGE profiles of uninfected CHSE-214 cells contained protein bands with molecular weights of 183, 83, 64, 54, 46, 42, 38, 31 kilodalton (kDa) together with some lower molecular weight material (Table 3.2). CHSE-214 cells infected with *P. salmonis* type strain LF-89 had bands with molecular weights estimated at 157, 140, 115, 95, 72, 60, 56, 51, 40, 36, 32, 30, 28, 26, 23, 20, 14 and 11 kDa, as well as bands associated with the uninfected cells CHSE-214 cells mentioned above. The lower molecular weight bands 11, 14 and 15 kDa were only seen when the gels here stained with Silver stain. The bands obtained with *P. salmonis* (type strain LF-89) purified using a 40% Percoll gradient were the same as these associated with the *P. salmonis* in the infected CHSE-214 cells.

Comparison of antigens by WB analysis of uninfected CHSE-214 cells, CHSE-214 cell infected with *P. salmonis* and *P. salmonis* type strain LF-89 purified using Percoll gradients revealed differences in the staining patterns between the samples when stained with PAb-1 (Figure 3.10). The molecular weights of the antigens were again estimated from a graph of the log of the molecular weights of each standard against its relative mobility in the gel. Following WB analysis with rabbit serum raised against *P. salmonis* LF-89, the uninfected CHSE-214 cell preparation gave only faint bands at 83 and 64 kDa, while CHSE-214 infected with *P. salmonis* gave bands with molecular weights 115, 95, 83, 72, 64, 60, 51, 40, 36, 32 kDa. *P. salmonis* purified using the 30, 40 and 50% Percoll gradient also gave bands at 115, 95, 83, 72, 60, 51, 40, 36 and 32 kDa in WB analysis but with different staining intensities. *P. salmonis* purified using the 40% Percoll gradient appeared to have the bands at 60, 51 and 40 and a faint band at 32 kDa while the band at 40 kDa had a stronger reactivity with *P. salmonis* purified on 50% Percoll gradient than *P. salmonis* purified on the 30 and 40% Percoll gradient.

However, *P. salmonis* purified using the 30% Percoll gradient had more intense bands at 95 and 32 kDa than seen in the other two preparations. A band was also seen at 64 kDa with *P. salmonis* purified on 50 and 40%, as also seen in uninfected CHSE-214 cell and CHSE-214 cells infected with *P. salmonis.* This band was not present with *P. salmonis* purified on the 30% Percoll gradient (Figure 3.10). The major bands reacting with PAb-1 in WB analysis are summarised in Table 3.2 (indicated by the asterisk).

Table 3.2. Molecular weights (kDa) of bands observed on a 12.5% SDS-PAGE gel containing samples of uninfected CHSE-214 cells, *P. salmonis* infected CHSE-214 cells and *P. salmonis* purified on a 40% Percoll gradient stained with Silver stain

The estimated molecular weights of bands (kDa)		
CHSE-214	*P. salmonis* (LF-89) infected CHSE-214	Purified *P. salmonis*
~200	~200	
183	183	-
-	157	157
-	140	140
-	115*	115*
-	95*	95*
83*	83*	83
-	72*	72*
64*	64*	64
-	60*	60*
-	56	56
54	54	-
-	51*	51*
46	46	-
42	42	-
-	40*	40*
38	38	-
-	36*	36*
-	32*	32*
31	31	-
-	30	30
-	28	28
-	26	26
-	23	23
-	20	20
19	19	-
18	18	18
16	16	16
15	15	-
-	14	14
-	11	11

* Major bands recognised in WB analysis

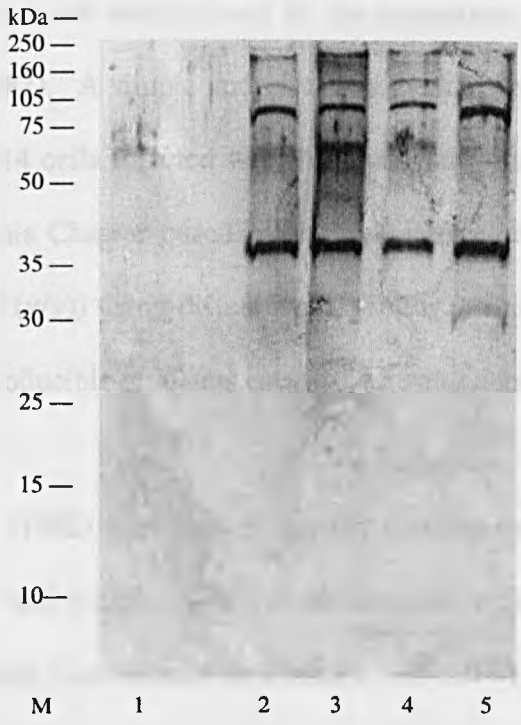


Figure 3.10. WB analysis of uninfected CHSE-214 cells, *P. salmonis* (LF-89) infected cell culture and purified *P. salmonis* recovered from different Percoll gradient concentrations and stained with anti rabbit *P. salmonis* serum. (M) Molecular weight marker Amersham Life Science; (1) Uninfected CHSE-214 cell; (2) *P. salmonis* (LF-89) infected cell culture; (3) After 50% Percoll density gradient centrifugation; (4) After 40% Percoll density gradient centrifugation; (5) After 30% Percoll density gradient centrifugation

3.4. DISCUSSION

Percoll gradients are widely used in the separation of cells, organelles and viruses (Rickwood 1987). A simple and effective method for the purification of *P. salmonis* from CHSE-214 cells infected with the bacterium after the development of a CPE, is described in this Chapter based on the use of three different Percoll gradients (30%, 40% and 50% (v/v)) using differential pelleting. This purification protocol generated linear and reproducible gradients established using density marker beads.

Tamura *et al.* (1982) used Percoll density gradient centrifugation to purify *Rickettsia tsutsugamushi* and found a 40% Percoll gradient to be optimal for the isolation of the organism, which was located at a density of 1.07-1.08 g ml^{-1}, with little host cell contamination and some degree of plasmolysis. Kuzyk *et al.* (1996) later used a similar gradient to purify *P. salmonis,* and found two bands formed in the gradient, one of a low-density, devoid of rickettsial whole-cell material, and the other a high-density band composed of organisms with the size and morphology of *P. salmonis* and some abundant vesicular material. Barnes *et al.* (1998), on the other hand, used diatrizoate meglumine and diatrizoate sodium (DMDS) density gradient centrifugation to purify the organism; however, preparations of purified *P. salmonis* were still associated with small amounts of CHSE-214 host cells. Using a modification of a method described by Tamura *et al.* (1982) in which 45% Percoll gradient was used, Jamet *et al.* (2001) obtained a major diffuse band of high density (1.070-1.083 g ml^{-1}) and a low density band near the top of the gradient, which had a large proportion of cell debris.

A modification of the method described by Kuzyk *et al.* (1996) was used in this study to purify *P. salmonis* from cell culture. A wide, whitish band with a density of between

1.056 and 1.080 g ml^{-1}, as determined from density marker beads, formed within the gradient. The normal CHSE-214 cell proteins had a density of 1.053-1.055 g ml^{-1} in the control gradient. This agrees with the report of Barnes *et al.* (1998) who demonstrated that CHSE-214 proteins were much lighter than *P. salmonis* cells.

It was shown in this study that rickettsia, both crude and purified preparations, retained their infectivity after differential pelleting and Percoll gradient centrifugation. However, the yield of protein obtained from *P. salmonis,* type species LF-89, in the purification protocol differed depending on the concentration of the Percoll used in the purification procedure. The highest protein content (7.5 mg ml^{-1}) which is 26.2% of the original sample, with the most infectious rickettsial yield recovered from preparations purified on the 30% Percoll gradient, while 40% and 50% Percoll gradients resulted in preparations of rickettsia with a lower protein content, 25.1% and 23.7% of the original sample respectively and a lower infectious dose. Recovery of purified *P. salmonis* using 30% Percoll gradient was 65% of the viable *P. salmonis* used as starting material. The amount of purified rickettsial protein obtained from total crude rickettsiae, expressed as a ratio, was similar to those previously reported for rickettsial purification from cell culture using Percoll gradient centrifugation. Tamura *et al.* (1982) reported that the amount of protein obtained from purified intracellular rickettsia (*R. tsutsugamushi*) was 6.3 mg, which represents 19.5% of the crude intracellular rickettsia (32.3 mg) before Percoll density gradient purification. They found that there was 68% of infectivity of the crude intracellular rickettsiae (2.85×10^9 infected cell counting units (ICU)) retained in the purified fractions (1.94×10^9 ICU) after Percoll density gradient (Tamura *et al.* 1982). Barnes *et al.* (1998) obtained yields of viable organisms ranging from 0.6% to 3% of the original sample after purification of *P. salmonis* by

DMDS density gradient centrifugation. The authors determined viability of gradient-purified *P. salmonis* using a plaque assay. Weiss *et al.* (1975) reported that yields of purified *Rickettsia typhi* protein after Renografin density gradient centrifugation was 16 to 18 mg obtained from 40 l cell culture flasks (approx. 475 ml each).

To assess the viability of rickettsiae purified in this study, $TCID_{50}$ and plaque assays were performed simultaneously with the same preparations; however, $TCID_{50}$ titers were calculated after 7 days post-inoculation (dpi), while plaque assay titers were calculated after formation of plaques at 17 dpi. The titre obtained in the $TCID_{50}$ assay was lower than those obtained in the plaque assay. Barnes (1996) also compared the $TCID_{50}$ assay with the plaque assay and found $TCID_{50}$ titers of infectivity were not as high as titres obtained in the plaque assay. The author also claimed that the lower titre obtained with the $TCID_{50}$ assay might have resulted because of a decreased viability or infectivity of the organism. The $TCID_{50}$ assay also seemed to have a greater degree of variation between replicates compared with the plaque assay.

Entry of rickettsia into host cells has been shown to occur both *in vivo* and *in vitro* by the interaction of viable rickettsia with host cells by a mechanism resembling parasite-directed endocytosis (Moulder 1985). *P. salmonis*, an obligate intracellular bacterium, replicates within membrane-bound vacuoles in fish cell lines (Fryer & Lannan 1996). Urakami, Tsuruhara and Tamura (1983) suggested that rickettsia, enveloped by host cell membrane material, are able to enter non-infected host cells by a phagocytic processes, and some of the rickettsia escape from the phagocytic vacuole into the host cell cytoplasm as the membranes of the phagosomal vacuole disintegrate. They also demonstrated *in vitro* that naked rickettsia purified by Percoll density gradient

centrifugation that were not surrounded by a host cell membrane, were able to penetrate into host cell cytoplasm and enter into their multiplication stage.

Propagation of rickettsia in fish cell lines *in vitro,* a necessary pre-requisite for molecular studies and vaccine development, was successfully achieved in this study. A good quality preparation of rickettsia requires a method which results in the release of the bacterium from the host cell and then prompt harvesting of the organism, before it loses its viability in the extracellular environment (Weiss 1973). In this study, a simple and effective method was used to separate rickettsia from host cell material, by first using differential pelleting, then homogenising and vortexing the sample before loading it onto the Percoll gradient.

The culture of rickettsial isolates *in vitro,* in which rickettsia enter host cells and form a CPE was assessed using LM and EM, and Immunofluorescence. The efficacy of the purification process using different Percoll densities to form the gradient was also assessed. A band with a density of 1.056-1.080 g ml^{-1} resulted following Percoll gradient centrifugation which was seen to be composed of cells of the expected size and morphology of *P. salmonis*, i.e. pleomorphic and ca. 0.5-1.5 μm in diameter (Fryer & Lannan 1994). When examined under TEM the preparations obtained were mainly composed of whole-cell *P. salmonis* with only very small amounts of fragmented material. Samples with the lowest amount of fragmented material were obtained in preparations recovered from the 30% Percoll gradient. The appearance of the rickettsia, both the whole bacterium and its cell membrane, appeared intact with very little disruption to the morphology of the cell.

SDS-PAGE analysis revealed differences in the protein profiles between *P. salmonis* infected CHSE-214 cell and uninfected CHSE-214 cells. The two preparations had many bands in common with each other as expected, but bands specific to *P. salmonis* were also identified. Molecular weight bands at 83, 64, 18 and 16 kDa were recognised both with CHSE-214 and purified preparations of *P. salmonis*. *P. salmonis* might possess the same proteins as the host cell, or some of the bands might originate from the host cell. The protein profile of the purified *P. salmonis* was similar to that observed by Barnes *et al.* (1998), with bands observed at 157, 108, 72, 60, 56, 36, 32 and 30 kDa. The profile described by Jamett *et al.* (2001) had five major protein bands at 105, 70, 58, 42 and 30 kDa.

Kuzyk *et al.* (1996) observed six immunoreactive antigens at 65, 60, 54, 51, 16 and 11 kDa in preparations of purified type strain *P. salmonis*, LF-89, by Percoll density gradient when screened with rabbit polyclonal antiserum raised against this particular isolate. Barnes *et al.* (1998) also identified antigen on purified *P. salmonis* at 108, 95, 64, 60, 56, 40, 36, 32 and 20 kDa using anti-*P. salmonis* rabbit serum when screened against *P. salmonis* purified on a DMDS gradient. In this study, common antigens were seen between preparations of CHSE-214 cells infected with *P. salmonis* and Percoll purified *P. salmonis* at 115, 95, 72, 60, 51, 40, 36 and 32 kDa as major antigens. Most of these antigens have been identified in the other studies described by Kuzyk *et al.* (1996) and Barnes *et al.* (1998). The antigen with a molecular weight of 108 kDa seen by Barnes *et al.* (1998) is close to the size to the 115 kDa antigen seen in this study. The explanations for the minor differences in the number and molecular weights of the antigens identified in the different studies is likely to be due to the use of different

gradients, difference in the purification process and differences in the rabbit antiserum used in each study.

It was found that the rabbit serum prepared against *P. salmonis* in Chapter V reacted very slightly with uninfected CHSE-214 cells in WB analysis with bands obtained at 83 and 64 kDa with the uninfected cells. The 64 kDa antigen, together with a 68 and a 70 kDa antigen, were strongly reactive in uninfected CHSE-214 cells in the study of Barnes *et al.* (1998). The 64 kDa band was also observed in uninfected CHSE-214 cells in this study, although it was only weakly stained here. If the presence of the 64 kDa antigen indicates contamination by host material in the purified *P. salmonis* preparations, yields obtained from 50% Percoll gradient density centrifugation may have more contamination from host material compared with that obtained from the 40% and 30% Percoll gradient density centrifugation. The reaction of this band in purified preparations of *P. salmonis* was slightly less with *P. salmonis* recovered from the 40% Percoll gradient, while the recovery from the 30% Percoll gradient did not appear to react implying the presence of very little host material.

In summary the method for purifying *P. salmonis* using Percoll density gradient centrifugation with differential pelleting as used in this study, is a simple method for obtaining relatively pure preparations of *P. salmonis* with the 30% Percoll (v/v) gradient giving yields with the highest purity and infectivity.

CHAPTER IV

CHARACTERISATION OF *PISCIRICKETTSIA SALMONIS* ISOLATES

4.1. INTRODUCTION

Following the original description of *P. salmonis*, the organism associated with disease outbreaks of salmon in Chile (Fryer, Lannan, Giovannoni & Wood 1992), further rickettsial organisms infecting both salmonids (Brocklebank, Speare, Armstrong & Evelyn 1992; Rodger & Drinan 1993; Olsen, Melby, Speilberg, Evensen & Hastein 1997) and non-salmonid fish (Chern & Chao 1994; Khoo, Dennis & Lewbart 1995; Comps, Raymond & Plassiart 1996) have been reported. The clinical signs and post-mortem results of diseased fish were similar to those found with piscirickettsiosis, although lower mortalities were generally observed compared with the disease outbreaks experienced in Chile. Differences seen in the levels of mortality between the different disease outbreaks may be a result of less susceptible species of fish being infected with *P. salmonis*, differences in rearing conditions or infection by different strains of the organism. While isolates of rickettsia from salmon in Chile, Canada, Norway and Ireland appear to be serologically similar (Lannan & Fryer 1993; Alday-Sanz, Rodger; Turnbull, Adams & Richards 1994; Fryer & Mauel 1997) and isolates from Norway (NOR-92), Canada (ATL-4-91) and Chile (SLGO-94 and C1-95) are genetically similar (Mauel, Giovannoni & Fryer 1996) to the original Chilean isolate LF-89, isolate EM-90 also isolated from Atlantic salmon in Chile appears genetically different (Mauel *et al.*. 1996, Mauel 1996; Fryer & Mauel 1997; Mauel, Giovannoni & Fryer 1999).

As mentioned in Chapter I, *P. salmonis* is a non-motile, Gram-negative, obligately intracellular bacterium (Fryer & Lannan 1996). It replicates within membrane-bound

vacuoles in the cytoplasm of cells in infected fish tissue, or in fish cell lines from salmonid fish such as chinook salmon (*Onchorynchus tshawytscha*) embryo, CHSE-214; chum salmon (*Onchorynchus keta*) heart, CHH-1; coho salmon (*Onchorynchus kisutch*) embryo, CSE-119; rainbow trout (*Onchorynchus mykiss*) gonad, RTG-2; and from non-salmonid fish common carp (*Cyprinus carpio*) epithelioma papillosum cyprini, EPC; and fathead minnow (*Pimephales promelas*), FHM (Fryer, Lannan, Garcès, Larenas & Smith 1990; Cvitanich, Garate & Smith 1991). Production of a cytopathic effect (CPE) within the fish cell lines is the accepted method for establishing the viability of a *P. salmonis* isolate. Infectivity, or the ability of the organism to invade a cell and replicate within it, is the most important property of an intracellular organism. Two basic types of assays are used to measure the infectivity of viable organisms, either quantal or quantitative assays (Burleson, Chambers & Wiedbrauk 1992). Quantal assays do not consider the number of infectious particles in an inoculum, but instead the virus titer is based upon an all-or-nothing principle; is there CPE or not? The 50% tissue culture infective dose assay ($TCID_{50}$) is a quantal assay used to determine the titre (given as the number of infectious virus units per unit volume), which results in the development of a CPE. The plaque assay is a quantitative assay used to quantify the number of infectious virus particles in an original suspension. Both assays are used as indicators of infection within the cell line (Burleson *et al.* 1992), and both $TCID_{50}$ and plaque assays have been used to measure levels of rickettsia growing within fish cell lines (Barnes 1996; Kordová 1966). Barnes (1996) developed a standard plaque assay for *P. salmonis* using type strain LF-89 and compared it with a $TCID_{50}$ assay using fish cell lines CHSE-214 and EPC. In this study, a $TCID_{50}$ assay was used to examine the growth of *P. salmonis* within CHSE-214

cells, or the susceptibility of different cell lines to various *P. salmonis* isolates. A plaque assay was also used to examine the rickettsia-host cell interaction.

P. salmonis is Gram-negative and also Gimenez-negative, but retains basic fuchsin when stained by Pinkerton's method for rickettsia and chlamydia. It can also be stained dark blue with Giemsa, which is used as one of the preliminary tests to identify *P. salmonis*. Acridine orange, Haematoxylin and Eosin (H&E), Machiavello and Periodic acid-schiff (PAS) are also used to detect the pathogen in smears or tissue sections, however, confirmation of the identity of the organism can be provided by serological methods such as Indirect fluorescent antibody technique (IFAT) and Immunohistochemistry (IHC) (Branson & Nieto Diaz-Munoz 1991; Cvitanich *et al.* 1991; Lannan & Fryer 1991). The cell envelope of Gram-negative bacteria contains an inner cytoplasmic membrane, a thin peptidoglycan layer and an outer membrane, which is composed of protein, phospholipid, and lipopolysaccharide (LPS). The composition and structure of the outer membrane of this bacterium has been the subject of intense research, especially since these components can be contribute to the virulence of the pathogen, its adhesion to host cells, and protection from the immunologic response of its host (Hancock & Poxton 1988; Klesius & Horst 1991). Carbohydrates, for example are known to play an important role in the pathogenesis of a number of mammalian bacterial pathogens, however, the importance of carbohydrates in the pathogenesis of bacterial fish pathogens remains to be clarified (Jung 1999).

P. salmonis has been described as a new genus and species placed taxonomically in a new family *Piscirickettsiaceae* (Fryer & Hedrick 2003). Phylogenetic comparison of the bacterial 16 small subunit ribosomal ribonucleic acid (16S rRNA) of *P. salmonis*

with other bacterial genes showed *P. salmonis* to belong to a gamma (γ) subgroup of Proteobacteria and to be closely related to the genera *Coxiella burnetti* and *Wolbachia persica* (Mauel *et al.* 1999), unlike other Rickettsial species, *Rickettsia rickettsii, Rickettsia prowazekii* and *Rickettsia typhii* from genus *Rickettsia,* which belong to the alpha (α) subdivision of Proteobacteria (Weisburg, Dobson, Samuel, Dasch, Mallavia, Baca, Mandelco, Sechrest, Weiss & Woese 1989).

Taxonomical classification based on morphological, physiological, cultural and serological characteristics of *P. salmonis* needs further verification. This is necessary for effective diagnosis and control of piscirickettsiosis. The aim of this study was to characterise and compare a number of *P. salmonis* isolates based on the growth characteristics *in vitro*, biochemical properties, protein profiles and response in a nested polymerase chain reaction (N-PCR).

4.2. MATERIALS AND METHODS

4.2.1. Susceptibility of fish cell lines to *P. salmonis* infection *in vitro*

The six different fish cell lines derived from either salmonid species: chinook salmon embryo (CHSE-214/ATCC CRL-1681); Atlantic salmon, *Salmo salar* head kidney (SHK-1); rainbow trout, *O. mykiss* gonad (RTG-2/ATCC CCL-55) or from warm water species; EPC cells derived from common carp, *C. carpio*; bluegill, *Lepomus macrochirus* Rafinesque, fry (BF-2/ATCC CCL-91); and sea bass, *Cetropristis striata* larvae (SBL) were used to culture the different *P. salmonis* isolates shown in Table 2.1, using the method described in Section 2.1.1.2.

Two different experiments, using a $TCID_{50}$ ml^{-1} for the bacterium, were performed to quantify the levels of *P. salmonis* growing within the different cell lines examined. In Experiment (a) the time taken for the development of the CPE by the different *P. salmonis* isolates within CHSE-214 cells was examined. In Experiment (b) the ability of the different *P. salmonis* isolates to produce a CPE in the different fish cell lines was examined. In each experiment the $TCID_{50}$ ml^{-1} assay was performed as described in the Section 3.2.5. Briefly each cell line was placed in a 96-well plate at a seeding concentration of $5x10^4$-$5x10^5$ cells ml^{-1}. The cells were incubated for between 24 and 48 hours (h) until they were 50-70% confluent. The supernatant was removed from the monolayers and these were carefully washed with Phosphate buffer saline (PBS) to remove dead cells. The wells were inoculated with a ten-fold dilution of each *P. salmonis* isolate in Experiment (a) and a four-fold dilution of each *P. salmonis* isolate in Experiment (b), using a rickettsia infected CHSE-214 cell culture as the source of the *P. salmonis* (approximately 10^2 $TCID_{50}$ ml^{-1}). Four replicates were prepared for each sample in both Experiments (a) and (b). Wells containing uninfected cells were used as a negative control. After centrifugation at 200 x g for 10 minutes (min) at 15°C, the plates were left for 2 h to allow absorption of the rickettsia. The supernatant was then removed and appropriate culture medium added to the different cell lines (250 microlitre (μl) $well^{-1}$). Plastic cling film was used to seal the plates to reduce dehydration during the incubation period. In Experiment (a) the wells were examined microscopically, on 1, 3, 7 and 10 days post-inoculation (dpi) and the development of a CPE monitored. In Experiment (b) the titer, expressed as $TCID_{50}$ ml^{-1} was calculated on 10 dpi for the different cell lines. The development of a CPE within the fish cell lines was recorded as follows: (-) No CPE; (+) Partial CPE (around 25% or less than 50%); (++) Approximately 50% of the cells exhibited CPE; (+++) Approximately 75% of the

cells exhibited CPE; (++++) The monolayer was totally destroyed. CPEs in all the wells with a score of 1 (+)- 4 (++++) were recorded as positive when calculating the $TCID_{50}$ of the cell cultures using the Spearman-Karber method (Kärber 1931) (Appendix II).

A plaque assay was performed as described in Section 3.2.4 to quantify the activity of *P. salmonis* isolates, LF-89, R-29 and SLGO-95 within the different cell lines. Briefly, cells were inoculated into a 96-well plate at a seeding concentration of between $5x10^4$ and $5x10^5$ cells ml^{-1} and the plates incubated for between 24 and 48 h at 22°C until a cell monolayer with a 50-70% confluence had been obtained. Ten fold dilutions of the *P. salmonis* isolates in PBS were added to the wells (100 µl/well), with three replicate wells for each dilution. Wells containing uninfected cells were used as a negative control. The plates were centrifuge at 210 x g for 10 min and incubated for 2 h at 15°C to allow rickettsial absorption. After this time, a 2.5% (v/v) methylcellulose overlay was added to the wells (250 µl $well^{-1}$) and the plates were wrapped in cling film to prevent evaporation. After 17 dpi, the contents of the wells were fixed with 10% (v/v) buffered formalin for 15 min and stained with 1% (w/v) Crystal Violet solution for 15 min. The reaction was stopped by washing the wells with tap water, and the number of resulting plaques counted under light microscopy (LM).

4.2.2. Application of different histo-staining methods

Wax embedded tissue sections (3 or 5 micrometre (µm) thick) were prepared from Atlantic salmon obtained from Ireland which were known to be infected with a rickettsia-like organisms (RLO) (provided from archival material held at the Institute of Aquaculture, University of Stirling). A variety of staining procedures were used to

stain the sections, and the sections were then compared. The sections were first dewaxed in xylene (two 5 min rinses), hydrated in 100% alcohol for 5 min, 70% alcohol for 3 min and then rinse with distilled water for each staining method, unless otherwise stated.

4.2.2.1. Immunohistochemistry (IHC)

IHC was performed as described in Section 2.5. Anti-*P. salmonis* mouse serum (M-4) (Chapter V) diluted 1/1000 in Tris buffered saline (TBS) was used as the primary antibody and Horseradish peroxidase (HRP) anti-mouse IgG (Diagnostics Scotland) as secondary antibody. The reaction was detected using True blue (Kirgegaard Perry Laboratories, UK) for 10 min and counter-stained with Contrast Red (Kirgegaard Perry Laboratories, UK) for 2 min. The slides were then dehydrated in 70% alcohol for 3 min and 100% alcohol for 5 min, and rinsed twice in xylene (5 min/rinse). Sections were mounted using Pertex and examined under a LM.

4.2.2.2. Haematoxylin and Eosin (H&E)

The sections (5 μm) were dewaxed in xylene (5 min), absolute alcohol (2 min) and methylated spirits (1.5 min), and rinsed in tap water (2 min) before staining with Haematoxylin (BDH Gurr Certistan) (5 min). The sections were then rinsed with tap water. After 3 quick dips in acid alcohol (1%), the sections were washed with tap water (1 min), Scott's tap water (1 min) and again in tap water (1 min), respectively. The sections were stained with Eosin (BDH) (5 min), washed briefly with tap water (30 s) and dipped in methylated spirits (30 s). The sections were dehydrated with 70% (2 min) and 100% alcohol (1.5 min) and placed in xylene for 5 min, after which the slides were mounted.

4.2.2.3. Giemsa staining

The tissue sections were dewaxed in xylene (5 min) and hydrated through a graded alcohol series (100-70%) to distilled water and washed with tap water (2 min) before staining with filtered Giemsa R66 (BDH) (10% in distilled water, v/v) for 20 min. The sections were then dipped in methylated spirits for a few seconds (s), differentiated in colophonium alcohol which is colophonium saturated methylated spirits, dipped again in methylated spirits (30 s) and dehydrated very rapidly in absolute alcohol, and placed in xylene (two 5 min rinses). The sections were then mounted.

4.2.2.4. Gram staining

The tissue sections were dewaxed in xylene (5 min), hydrate with absolute alcohol (2 min), dipped in methylated spirit (1.5 min) and washed with tap water (2 min) before staining with crystal violet (2 min). The crystal violet (BDH) was washed off with Gram's iodine (BDH) until metallic precipitate was washed away, and the sections were flooded the iodine for 2 min. The sections were then washed in tap water, differentiated in acetone for a few seconds, immediately washed with tap water, and then counter-stained with 1% Neutral Red (BDH) in distilled water (v/v) (1 min). The sections were washed in tap water and blotted dry on filter paper before dehydrating in absolute alcohol. The sections were thereafter dipped twice in xylene (5 min each) and mounted.

4.2.2.5. Castañeda stain

Castañeda staining was performed following the method described by Gradwohl (1956) cited in Humason (1979) with slight modifications. After dewaxing and hydrating the sections as far as 50% alcohol, they were stained with methylene blue (BDH) solution (Appendix 6.4) for 5 min. The sections were then washed with tap water (30 s) and

counterstained in either 0.25% aqueous safranin (Raymond A Lamb, London) (v/v) for 30 s followed by a brief dip in 95% alcohol, dehydration in 2 changes of absolute alcohol, placed in 2 changes of xylene (5 min each), and then mounted.

4.2.2.6. Acridine orange stain

The sections were dewaxed and hydrated to water and then stained with Acridine orange described by Lannan and Fryer (1991). Briefly, the sections were air dried and fixed in absolute methanol for 5 min, then flooded with the acridine orange (Sigma) stain (Appendix 6.5) for 2 min. The slides were then washed with tap water and air-dried. The samples were viewed under B excitation using an Olympus IMT-2 microscope with a reflected fluorescent attachment and appropriate exciter and barrier filters for Acridine orange staining.

4.2.2.7. Macchiavello's and Pinkerton's Methods for Basic fuchsin staining

Macchiavello's staining method for Rickettsia was performed with slight modifications to the method described by Culling (1974). Briefly, the sections were hydrated through graded alcohol to water and stained in Basic fuchsin (Fisons) solution (0.25% (w/v) in distilled water) for 30 min. They were then differentiated with 0.5% citric acid (BDH) (v/v) in distilled water for about 3 s and washed in tap water. The slides were counter-stained in 1% aqueous methylene blue (BDH) for 15-30 s and washed again with tap water. They were finally dehydrated, cleared with xylene twice and mounted.

Pinkerton's staining method for Rickettsiae, were performed according to the method described by Luna (1987) with slight modifications. The sections (either 3 μm or 5 μm) were dewaxed in xylene (5 min) and hydrated through graded alcohol (100-70%) to

distilled water and washed with tap water (5 min), before staining for 6 h or overnight with methylene blue (BDH) solution (1% (w/v) in distilled water). The sections were rinsed with 95% alcohol for 5 s until the blue colour faded and were then placed in distilled water for 2-3 s. They were then stained with Basic fuchsin (Fisons) solution (0.25% (w/v) in distilled water) for 30 min. The sections were decolorized by placing in the citric acid solution (0.5% (w/v) in distilled water) for 1-2 s, then placed in absolute alcohol until the nuclei stained blue and rickettsial organisms red, followed by two rinses with xylene, (5 min each rinse) before mounting.

4.2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transfer of proteins to nitrocellulose membranes and different staining methods used to screen the nitrocellulose membranes

4.2.3.1. SDS-PAGE

A number of *P. salmonis* isolates (Table 2.1) either purified from tissue culture using a 30% Percoll gradient (Section 3.2.2) or semi-purified (Section 2.1.3.1) were used in this analysis. Bacteria samples (50 microgram (μg) ml^{-1}) were subjected to SDS-PAGE according to Section 2.2 using precast 12.5% mini-polyacrylamide gels (Sigma). SDS-PAGE gels were then stained with either Coomassie blue or Silver stain or the proteins on the gel were transferred onto nitrocellulose membranes as described in Section 2.3.1. These were analysed using either a glycoprotein detection kit or lectin staining.

SDS-PAGE was also used to compare *P. salmonis* isolate R-29, passaged three times in different fish cell lines (CHSE-214, RTG-2, EPC, BF-2 and SBL). Using the CPE obtained with *P. salmonis* isolate R-29 in CHSE-214 cells as a guide line of CPE activity of the organism, bacteria were harvested from the other cell lines (RTG-2 EPC,

BF-2 and SBL cells) inoculated with isolate R-29 at the same time as the CHSE-214 cells even though a total CPE was not observed in all of these cell lines. Following brief homogenisation, the supernatant collected from the cells was used to infect fresh fish cells. The SDS-PAGE gels were stained with Coomassie blue.

A sample of each *P. salmonis* isolate shown in Table 2.1 was digested with 1 µl of stock proteinase-K solution (1 mg ml^{-1} per 10 µl of the SDS-PAGE sample) by incubating for 60 min at 60°C. The digestion was stopped by boiling the samples for 10 min and these were used in SDS-gel.

4.2.3.2. Glycoprotein detection

P. salmonis isolates (Table 2.1) subjected to SDS-PAGE were transferred onto nitrocellulose membranes as described in Section 2.3.1. The glycoproteins on the membrane were identified using a commercially available glycoprotein detection kit (BioRad) according to the manufacturer's instructions. Following transfer of the proteins, the nitrocellulose membranes were washed with 10 ml PBS (9 millimolar (mM) sodium phosphate, 27 mM NaCl, pH 7.2) for 10 min. The membranes were then immersed in 10 ml of 10 mM sodium periodate in sodium acetate/ ethylenediminetetraacetic acid (EDTA) buffer and incubated in the dark at 22°C for 20 min. They were washed 3 times with 10 ml of PBS for 10 min, using fresh buffer on each wash. The membranes were immersed in a biotinylation solution (prepared immediately before use by adding 2 µl hydrazide solution to 10 ml sodium acetate/EDTA), and incubated in this solution for 60 min, before washing them three times with 10 ml of TBS (50 mM Tris base, 27 mM NaCl, pH 7.2). All steps were performed at 22°C. The membranes were then placed in 10 ml blocking solution

overnight at 4°C. The membranes were washed three times with 10 ml TBS for 10 min before immersing them in the conjugate solution (5 µl streptavidin-alkaline phosphatase in 10 ml TBS) and incubating them for 60 min at 22°C. The membranes were washed again as described above. The developer solution was prepared by adding 50 µl nitroblue tetrazolium and 37.5 µl 5-bromo-4chloro-3-indolyl phosphate provided in the kit, to 10 ml of development buffer (1.21 g Tris, 1.01 g $MgCl_2.H_2O$, 0.58 g NaCl dissolved in 100 ml purified H_2O, pH 9.5). The membrane was incubated in this solution at 22°C until bands appeared, after which the membrane was rinsed several times with H_2O and allowed to air dry. Gentle agitation was applied to the membrane throughout the procedure, apart from during development of the reaction.

4.2.3.3. Lectin staining

A variety of different lectins (Table 4.1) were used to examine the carbohydrate moieties present on the *P. salmonis*. *P. salmonis* (Table 2.1) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane as described in Sections 2.2.1 and 2.3.1 respectively. After transfer, the nitrocellulose membrane was blocked with 1% (w/v) bovine serum albumin (BSA) dissolved in distilled water for 60 min at 37°C. The membrane was transferred to a multiscreen apparatus (BioRad) and incubated with the biotin-labelled lectins (Sigma), diluted to 20 $\mu g\ ml^{-1}$ in PBS [0.02 Molar (M) Phosphate, 0.15 M NaCl, pH 7.2], for 60 min at 22°C. The membrane was then washed 3 times with TBS (10 mM Tris base, 0.5mM NaCl, pH 7.5) containing Tween-20 (0.1% v/v) (TTBS), washing for 5 min on each wash. Finally, streptavadin-peroxidase (Diagnostics Scotland), diluted 1/100 in TBS was applied to the membrane for 60 min and the membrane was washed as above. The reaction was developed at 37°C using 2 ml of 4-chloro-1-naphthol solution [0.33% (w/v) in methanol] (Sigma), dissolved in 10

ml PBS (v/v) to which 10 µl of H_2O_2 [0.01% (v/v)] (Bio-Rad) was then added. Once bands appeared, the reaction was stopped with distilled water.

Table 4.1. Origin, carbohydrate specificity and abbreviated name of lectins used in this study

Lectin	Abbreviation	Origin	Carbohydrate specificity
Peanut agglutinin	PNA	Peanut (*Arachis hypogaea*)	D-galactose
Bandeiraea simplicifolia	BS-1	*Bandeiraea simplicifolia*	Terminal α-D-galactosyl and N-acetyl-α-D-galactosaminyl residues
Concanavalin A	ConA	Jack bean (*Canavalia ensiformes*)	Terminal α-D-mannosyl and α-D-glucosyl residues
Horse gram agglutinin	HGA	Horse gram (*Dolichos biflorus*)	Terminal N-acetyl-α-D-galactosaminyl residues
Coral tree agglutinin	CTA	Coral tree (*Erythrina cristagalli*)	D-galactose and D-galactosides
Tomato agglutinin	TAG	Tomato (*Lycopersicon esculentum*)	N-acetyl-β-D-glucosamine oligomers
Wheat germ agglutinin	WGA	Wheat (*Triticum vulgaris*)	N-acetyl-β-D-glucosaminyl residues and N-acetyl-β-D-glucosamine oligomers
Gorse seed agglutinin	UEA-1	Gorse seed (*Ulex europaneus*)	L-fucose

4.2.4. Nested Polymerase Chain Reaction (N-PCR)

4.2.4.1. Bacterial lysates

All *P. salmonis* isolates (Table 2.1) were cultured in CHSE-214 cells in 25 cm^2 culture flasks as described in Section 2.1.1.2, until an extensive CPE was established (approximately 90%). The cell culture supernatant was collected, vortexed at maximum speed and then centrifuged at 210 x g for 10 min at 4°C (Mistral 3000i, MES) to remove the host cell debris. Rickettsial cells were collected from the supernatant by centrifugation at 20 000 x g for 30 min at 4°C. The resulting pellet was resuspended in PBS (Gibco) and washed once with PBS at 20 000 x g as above.

P. salmonis isolates R-29 were passaged three times in different cell lines (CHSE-214, EPC, RTG-2, BF-2, SBL and SHK-1) in 25 cm^2 flasks and prepared as described above. Bacteria were collected from all the cell lines (CHSE-214, RTG-2, EPC, BF-2, SBL and SHK-1 cells), once extensive CPE had developed in the CHSE-214 even though a total CPE was not observed in all of the cell lines at this time. The supernatant from the cells was used for the next round of inoculation, following brief homogenising.

Non-*P. salmonis* bacteria (Table 2.2) were cultured as described in Section 2.1.3.2, and after sufficient growth, the bacteria were harvested by centrifugation (Mistral 3000i, MES) at 3000 x g for 20 min at 4°C. The bacterial pellets were re-suspended in PBS, vortexed at maximum speed and washed twice with PBS. The absorbance of each suspension was adjusted to 1.0 at 595 nanometre (nm) (Ultrospec 2000, Pharmacia).

4.2.4.2. Deoxyribonucleic acid (DNA) isolation

Bacterial DNA was extracted using a Nucleon® DNA extraction kit (Nucleon Biosciences, Strathclyde, UK). Briefly, the bacterial samples were re-suspended with Reagent B from the kit, vortexed and incubated for 10 min at 37°C before adding 100 μl of sodium perchlorate solution and 600 μl chloroform (not supplied in the kit), and mixed gently by hand, each time. The Nucleon® resin (150 μl) was then added to the extraction without mixing the organic/inorganic phases, and the samples were centrifuged at 350 x g for 1 min. The upper phase (~450 μl) was transferred to a new 1.5 ml microtube without disturbing the resin layer and 2 volumes of cold absolute ethanol were added to the solution. DNA was precipitated from the final solution by placing the tubes at - 20°C for 20 min and inverting them several times throughout the incubation period. The microtubes were then centrifuged at 5000 x g for 5 min to pellet

the DNA and supernatants were discarded, taking care not to disturb the pellet. One ml of cold 70% (v/v) ethanol was added to the tubes, which were then inverted several times. The tubes were again centrifuged as above and the supernatant discarded. The pellets were air-dried, taking care to ensure that all of the ethanol had evaporated before adding 100 μl of Tris acetate EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0), after which the tubes were incubated for 2 h.

4.2.4.3. PCR Amplification

A N-PCR reaction described by Mauel *et al.* (1996) was used in this study. Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used to amplify the DNA extracted in Section 4.2.4.2, and the sequences reported by Mauel *et al.* (1996) were used as primers (Table 4.2). In the first round of amplification, the beads were dissolved in purified distilled water (20.5 μl) and 1 μl of primers A and B were added to each tube. Extracted DNA (2.5 μl) was then added to the reaction mixture before covering it with 50 μl of mineral oil. The tubes were subjected to 94°C for 2 min, and amplification was performed with 35 cycles of 94°C for 1 min, 59°C for 2 min, and 72°C for 3 min in a thermocycler (Omnigene, Hybriad), after which the tubes were held at 4 °C. The second amplification was performed by adding 3 μl of the first round of PCR products the beads as above with 1 μl each of either primers C and D, C and E, or C and F (Table 4.2). Non-*P. salmonis* bacteria were amplified using only primers C and D for the second round of amplification. The mixtures were again covered with 50 μl of mineral oil. The reaction conditions were 35 cycles of 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min after which the samples were held at 4°C. Resulting PCR products were examined using gel electrophoresis. This was performed using a 1.5% (w/v) agarose gel, for which 0.9 g agarose (Gibco) was added to 60 ml of

TAE buffer and melted in a microwave oven for 2 min 40 s at 750 Watt. Before pouring the gel into a horizontal tray, 6 μl of ethidium bromide (EB) (10 mg ml^{-1}) was added to the dissolved gel. Appropriate combs were inserted onto the gel, which was then allowed to cool. PCR products (10 μl) were added directly to 3 μl of loading buffer [6 x loading buffer: 30% glycerol in TAE buffer (v/v), 0.25% Bromophenol blue, kept in 4°C] and the mixture was then applied to a well of the gel. Molecular weight markers (Pharmacia) were also added as a reference. Electrophoresis was performed at 60 V for 75 min using TAE buffer containing of ethidium bromide solution (EB: 1μl EB in 10 ml TAE buffer) as the running buffer. The PCR products were viewed with a Ultraviolet (UV) transilluminator (UVP, Life Sciences, Cambridge, UK) and photographed.

Table 4.2. Sequences and specificity of primers used for the identification and differentiation of *P. salmonis* isolates (*)

Primer	Abreviation	Sequence (5'-3')	Specificity
EubB1 (27F)	A	GAGTTTGATCCTGGCTCAG	Eubacterial
EubB2 (492R)	B	TACGGNWACCTTGTTACGACTT	Eubacterial
PS2A2 (223F)	C	CTAGGAGATGAGCCCGCGTTG	*P.salmonis* (All strains)
PS2A2 (690R)	D	GCTACACCTGCGAAACCACTT	*P.salmonis* (All strains)
PS3AS (1032R)	E	TCCCGAAGGCACTTCCGCATCTC	*P.salmonis* (LF-89 Type strain)
EM90AS(1032R)	F	TCCCGAAGGCACATCAATATCTCTATC	*P.salmonis* (EM-90)

(*) Mauel et al. (1996)

4.3. RESULTS

4.3.1. Susceptibility of fish cells to *P. salmonis*

The development of a CPE by the *P. salmonis* isolates shown in Table 2.1 within CHSE-214 cells was examined over a 10 dpi period using a $TCID_{50}$ assay. The susceptibility of different fish cell lines (CHSE-214, EPC, RTG-2, BF-2, SBL and SHK-1) to various *P. salmonis* isolates was also examined using $TCID_{50}$ assay. Uninfected cell lines were used as controls for both assays.

4.3.1.1. Development of a CPE in CHSE-214 cells by different *P. salmonis* isolates

All *P. salmonis* isolates produced a CPE in CHSE-214 cells. Figure 4.1 (a-f) shows the development of CPE by various *P. salmonis* isolates in different dpi. A CPE first developed with isolates VQ013, AVG5/268, SRS-UACH and R-29 on the first dpi with a $TCID_{50}$ of $10^{1.5}$ ml^{-1}. The CPE was evident as an enlargement of infected cells with cytoplasmic inclusions.

At three dpi, isolates VQ013 and SRS-UACH yielded $10^{3.5\ \pm0.28}$ $TCID_{50}ml^{-1}$, whereas isolates LF-89 and SRS-4 gave $10^{1.5\pm0.28}$ $TCID_{50}ml^{-1}$ with a gradual development of a CPE within the cell monolayers. Although isolates R980769, SLGO-95, R-61 and R-62 gave yields of $10^{2.5\pm0.28}$ of $TCID_{50}$ ml^{-1} between 3 and 7 dpi, isolates R980769 and R-61 had $10^{5.5\pm0.28}$ $TCID_{50}ml^{-1}$, at 10 d post-inoculation indicating slightly higher level of infection than isolates SLGO-95 and R-62 ($10^{5.25\pm0.5}$ $TCID_{50}$ ml^{-1}). All of the isolates showed a sharp increase in $TCID_{50}$ values after 7 dpi. At the end of the incubation period (10 dpi), VQ013, SRS-UACH and R-29 had higher $TCID_{50}$ values than the other *P. salmonis* isolates used in the experiment (Table 4.3 and Figure 4.2).

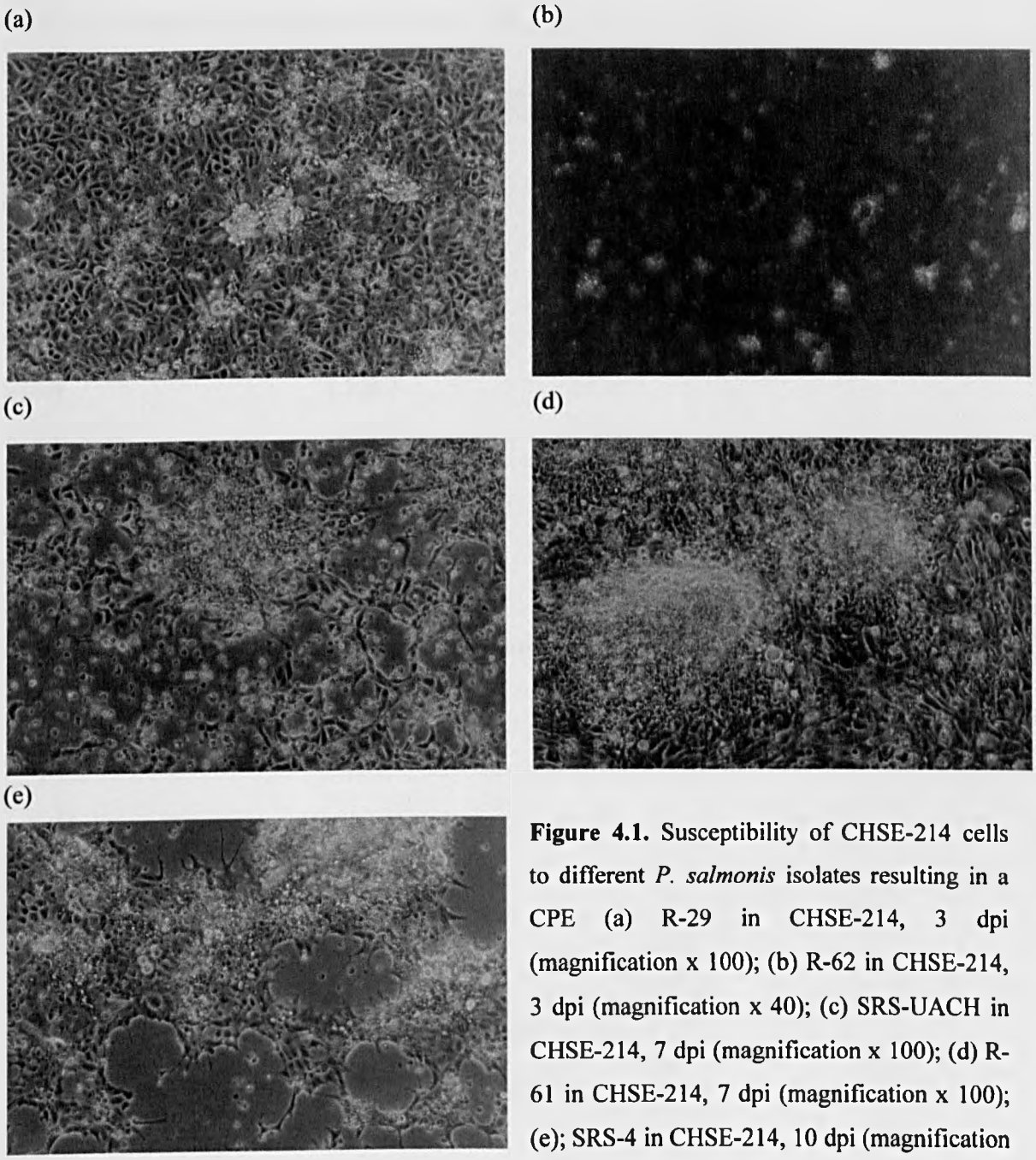


Figure 4.1. Susceptibility of CHSE-214 cells to different *P. salmonis* isolates resulting in a CPE (a) R-29 in CHSE-214, 3 dpi (magnification x 100); (b) R-62 in CHSE-214, 3 dpi (magnification x 40); (c) SRS-UACH in CHSE-214, 7 dpi (magnification x 100); (d) R-61 in CHSE-214, 7 dpi (magnification x 100); (e); SRS-4 in CHSE-214, 10 dpi (magnification x 100)

Table 4.3. The end-point dilution of different *P. salmonis* isolates when grown in CHSE-214 cells over a 10 days (d) period

	Log_{10} $TCID_{50}$ ml^{-1} of CHSE-214 cells (±95% confidence limit)		
Isolates	**3 dpi(*)**	**6-7 dpi**	**10 dpi**
LF-89	1.5±0.3	2.5±0.3	6.5±0.8
AVG5/268	2.75±0.8	3.5±0.3	6.5±0.8
R980769	2.5±0.3	2.5±0.3	5.5±0.3
VQ013	3.5±0.3	3.5±0.3	7±0.6
SRS-4	1.5±0.3	2.5±0.3	5.25±0.5
SRS-UACH	3.5±0.3	3.5±0.3	7±0.6
SLGO-95	2.5±0.3	2.5±0.3	5.25±0.5
R-29	2.5±0.3	3.5±0.3	7±0.6
R-61	2.5±0.3	2.5±0.3	5.5±0.3
R-62	2.5±0.3	2.5±0.3	5.25±0.5

(*) dpi: days post inoculation

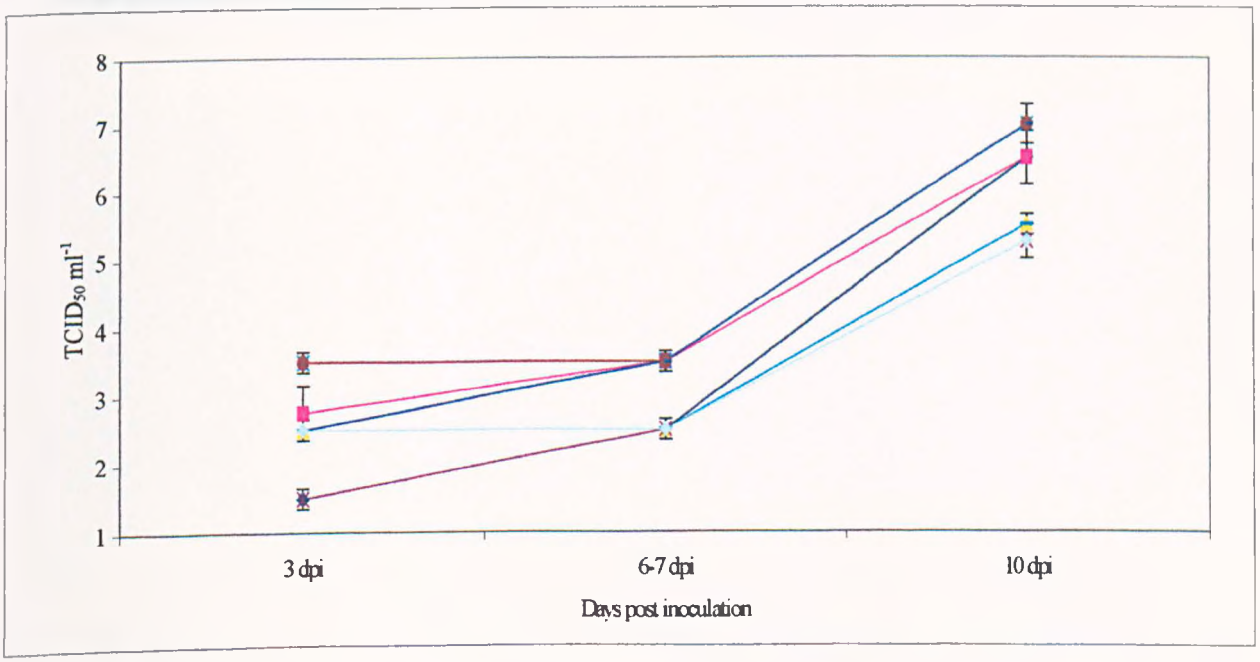


Figure 4.2. The end-point dilution of various *P. salmonis* (±SEm) isolates when grown in CHSE-214 cells in 3, 6-7 and 10 days post inoculation (dpi). *P. salmonis* isolates: (—♦—) LF-89; (—■—) AVG5/268; (—▲—) R980769; (—x—) VQ013; (—*—) SRS-4; (—●—) SRS-UACH; (—|—) SLGO-95; (—•—) R-29; (——) R-61; (—◆—) R-62.

4.3.1.2. Development of a CPE in different cell lines by different *P. salmonis* isolates

The susceptibility of fish cell lines to various *P. salmonis* isolates was calculated as a $TCID_{50}$ ml^{-1} value using the method of Spearman-Karber (Kärber 1931) (Appendix II). Uninfected cell lines used as controls for the assay are shown in Figure 4.3 a-f. The cell lines which appeared most susceptible to *P. salmonis* appeared to be CHSE-214, SHK-1 and EPC (Figure 4.4 a-f and Table 4.4). EPC cells showed a variable susceptibility to the different isolates with low $TCID_{50}$ ml^{-1} values obtained with isolates R980769 and R-61 ($10^{3.2\ \pm 0.3}$ $TCID_{50}$ ml^{-1}). However, RTG-2 cells and SBL cells had a low susceptible to all *P. salmonis* isolates examined. The level of CPE produced in BF-2 cells was not calculated since the yield obtained varied between *P. salmonis* isolates but was generally around 10 $TCID_{50}$ ml^{-1} isolate.

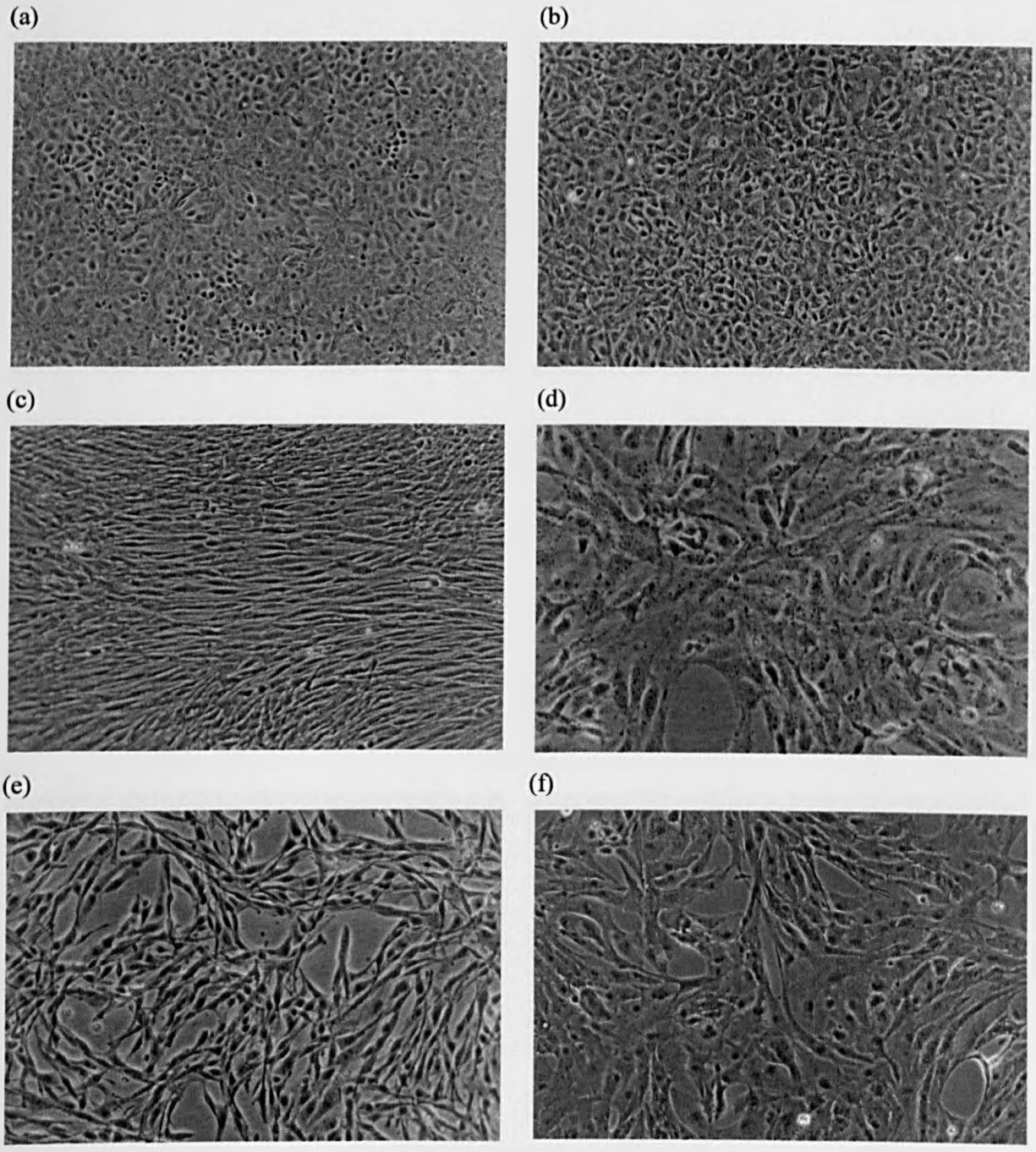


Figure 4.3. Uninfected cell lines as control in the $TCID_{50}$ assay.

Confluent cell lines (a) CHSE-214, 7 d (magnification x 100); (b) EPC, 7 d (magnification x 100); (c) RTG-2, 7 d (magnification x 100); (d) BF-2, 5 d (magnification x 200); Subconfluent cell lines (e) SBL, 5 d (magnification x 200); (f) SHK-1, 7 d (magnification x 100)

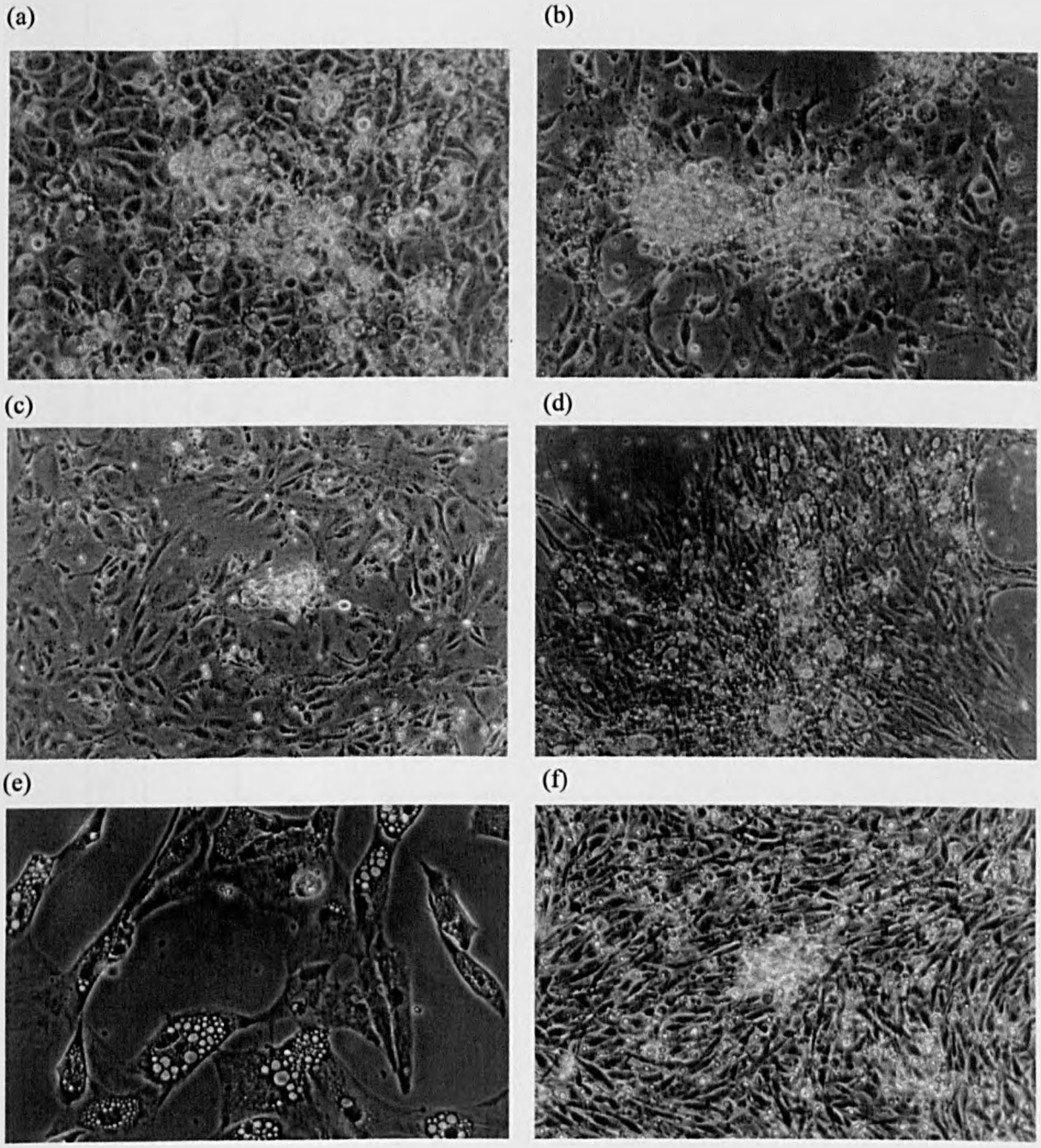


Figure 4.4. Susceptibility of various fish cell lines to different *P. salmonis* isolates (a) SLGO-95 in CHSE-214, 7 dpi (magnification x 200); (b) R-29 in EPC, 7 dpi (magnification x 200); (c) SLGO-95 in BF-2, 7 dpi (magnification x 100); (d) R-29 in RTG-2, 7 dpi (magnification x 100); (e) SLGO-95 in SHK-1, 7 dpi (magnification x 200); (f) R-29 in SBL, 7 dpi (magnification x 100).

Table 4.4. Susceptibility of fish cell lines to *P. salmonis* isolates (10 dpi)

Estimation of 50% End-Points of *P. salmonis* Calculated in Cell lines												
	CHSE-214				EPC				RTG-2			
Isolates	**m**	**V_m**	**SE_m**	**mt**	**m**	**V_m**	**SE_m**	**mt**	**m**	**V_m**	**SE_m**	**mt**
LF-89	4.5	.0075	.0866	5.5±0.17	4.5	.0075	.0866	5.5±0.17	1.5	.0075	.0866	2.5±0.17
AVG5/268	4.5	.0075	.0866	5.5±0.17	4.5	.0075	.0866	5.5±0.17	2.1	.0075	.0866	3.1±0.17
R980769	4.5	.0075	.0866	5.5±0.17	2.2	.0025	.15	3.2±0.3	2.1	.0075	.0866	3.1±0.17
VQ013	4.5	.0075	.0866	5.5±0.17	4.5	.0075	.0866	5.5±0.17	2.1	.0075	.0866	3.1±0.17
SRS-4	4.3	.0225	.15	5.3±0.3	4.5	.0075	.0866	5.5±0.17	1.5	.0075	.0866	2.5±0.17
SRS-UACH	4.5	.0075	.0866	5.5±0.17	3.4	.0025	.15	4.4±0.3	2.1	.0075	.0866	3.1±0.17
SLGO-95	4.3	.0025	.15	5.3±0.3	3.9	.0075	.0866	4.9±0.17	2.4	.015	.1224	3.4±0.24
R-29	4.5	.0075	.0866	5.5±0.17	4.5	.0075	.0866	5.5±0.17	2.1	.0075	.0866	3.1±0.17
R-61	4.5	.0075	.0866	5.5±0.17	2.2	.0025	.15	3.2±0.3	2.1	.0075	.0866	3.1±0.17
R-62	4.3	.0075	.0866	5.3±0.3	4.0	.0025	.15	5.0±0.3	2.4	.0075	.0866	3.4±0.24

m: Mean $\log_{10}$ $TCID_{50}$
V_m: Variance of mean
SE_m: Standart error of mean
mt: Mean titer ($\log_{10}$ $TCID_{50}$/ml) ± 95% confidence limit

Table 4.4. (Cont.)

Estimation of 50% End-Points of *P. salmonis* Calculated in Cell lines												
	BF-2				SBL				SHK-1			
Isolates	**m**	**V_m**	**SE_m**	**mt**	**m**	**V_m**	**SE_m**	**mt**	**m**	**V_m**	**SE_m**	**mt**
LF-89	-	-	-	-	2.7	.0075	.0866	3.7±0.17	3.9	.0075	.0866	4.9±0.17
AVG5/268	-	-	-	-	2.7	.0075	.0866	3.7±0.17	3.9	.0075	.0866	4.9±0.17
R980769	-	-	-	-	2.7	.0075	.0866	3.7±0.17	3.9	.0075	.0866	4.9±0.17
VQ013	-	-	-	-	2.7	.0075	.0866	3.7±0.17	3.3	.0075	.0866	4.3±0.17
SRS-4	-	-	-	-	2.7	.0075	.0866	3.7±0.17	3.9	.0075	.0866	4.9±0.17
SRS-UACH	-	-	-	-	2.4	.015	.1224	3.4±0.24	3.9	.0075	.0866	4.9±0.17
SLGO-95	-	-	-	-	0.9	.0075	.0866	1.9±0.17	3.9	.0075	.0866	4.9±0.17
R-29	-	-	-	-	1.5	.0075	.0866	2.5±0.17	3.9	.0075	.0866	4.9±0.17
R-61	-	-	-	-	1.2	.03	.1732	2.2±0.34	3.9	.0075	.0866	4.9±0.17
R-62	-	-	-	-	0.9	.0075	.0866	1.9±0.17	3.3	.0075	.0866	4.3±0.17

m: Mean $\log_{10}$ $TCID_{50}$
V_m: Variance of mean
SE_m: Standart error of mean
mt: Mean titer ($\log_{10}$ $TCID_{50}$/ml) ± 95% confidence limit

A plaque assay was also used to test the susceptibility of the fish cell lines to *P. salmonis*. Small plaques caused by isolates LF-89, R-29 and SLGO-95 within the different cell lines tested were observed (Figures 4.5 a-h). A dilution series was chosen which gave between 20 and 200 plaques/well in duplicate wells to determine the plaque forming unit (PFU) per ml of original supernatant. The number of PFU ml^{-1} was determined from the mean number of plaques for four monolayers at the same dilution, and multiplying it by the reciprocal of the dilution and the reciprocal of the volume added to the well. LF-89 and R-29 gave a PFU of 2.3×10^7 ml^{-1} with CHSE-214 cells and 2×10^7 ml^{-1} with EPC cells, whereas isolate SLGO-95 had a PFU value of 2×10^7 ml^{-1} and 1.8×10^7 ml^{-1} in CHSE-214 and EPC cell line respectively. Plaques of different shape were observed in cell lines CHSE-214 and EPC (Figures 4.5 c-m). The shape of the plaques varied from round to irregular and also some comet-tail forms. The diameter of the plaques sometimes extended up to 2.4 mm for irregular shapes (Figures 4.5 c, e and g), but were generally between 0.10-1.20 mm in size (Figures 4.5 d, f, h, i, j and l) or the plaques had a comet-tail shape (Figures 4.5 k) in CHSE-214 cells. However, it was not possible to calculate the PFU in the remaining cell lines (RTG-2, BF-2, SBL and SHK-1) since similar structures were also observed in the control wells, as shown for BF-2 cells with isolates R-29 and SLGO-95 in Figure 4.5 n-p.

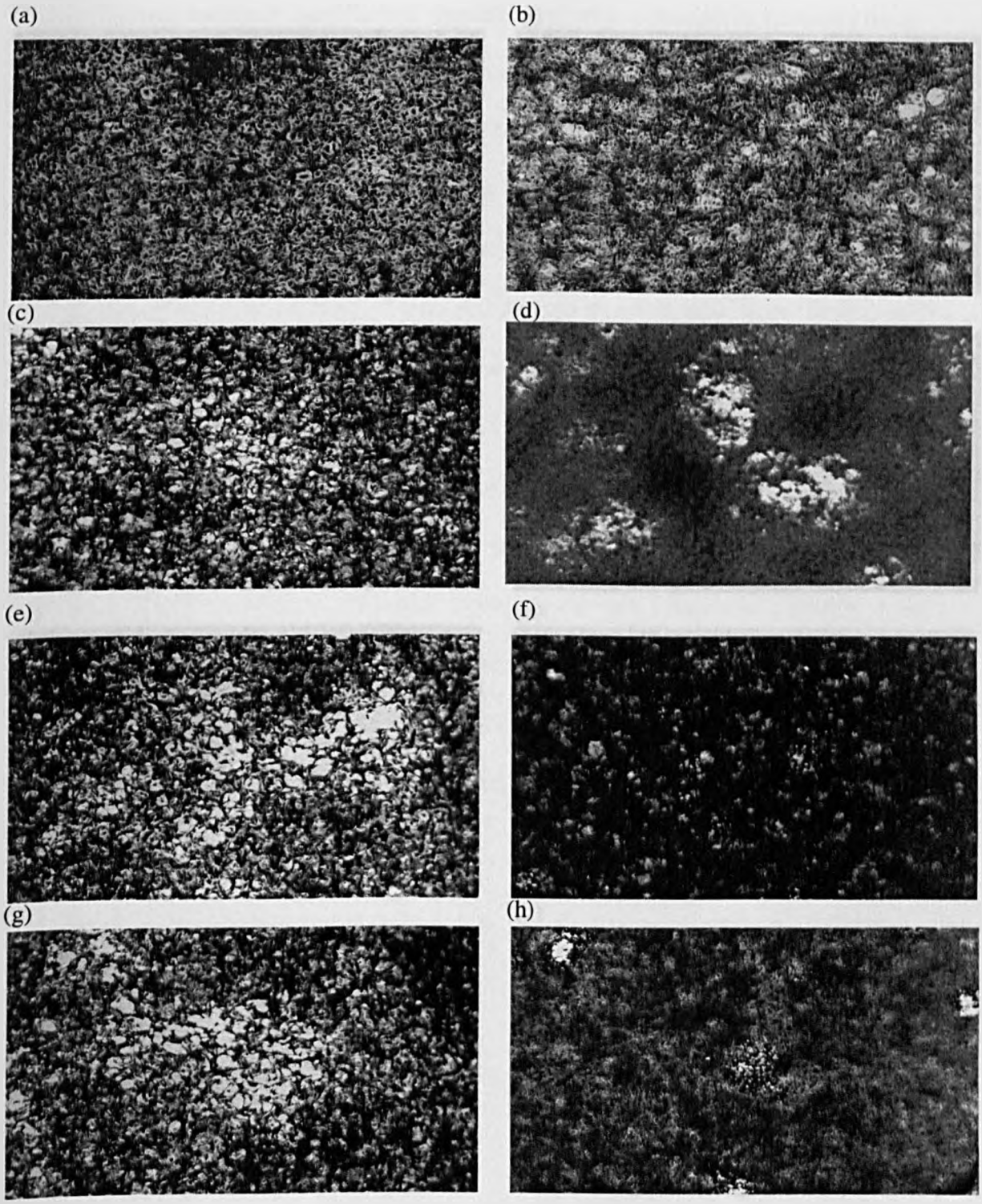


Figure 4.5. Development of plaques by *P. salmonis* in various fish cell lines (a) Uninfected CHSE-214 cells as control wells (magnification x 50); (b) Uninfected EPC cells as control wells (magnification x 50); (c) LF-89 in CHSE-214 (magnification x 100); (d) LF-89 in EPC (magnification x 100) ; (e) SLGO-95 in CHSE-214 (magnification x 50); (f) SLGO-95 in EPC (magnification x 50); (g) R-29 (magnification x 50) in CHSE-214 (magnification x 100); (h) R-29 in EPC (magnification x 50)

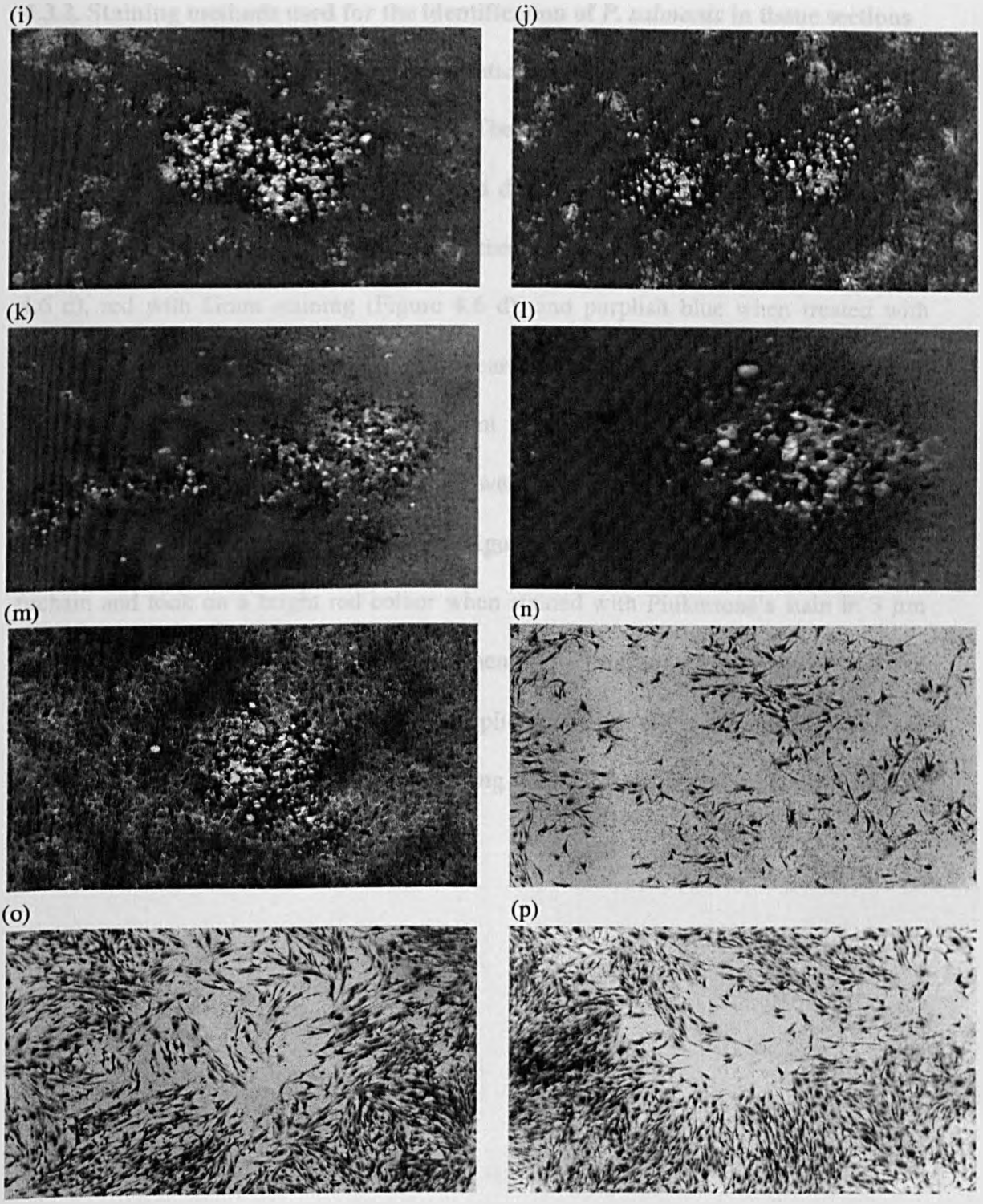


Figure 4.5 (Cont.) (i) Irregular shaped plaques: SLGO-95 in CHSE-214 (magnification x 100); (j) Two round plaques: SLFO-95 in CHSE-214 (magnification x 100); (k) Comet-tail shaped plaque: LF-89 in CHSE-214 (magnification x 100); (l) A round shaped-plaque: LF-89 in CHSE-214 (magnification x 200); (m) An irregular shaped-plaque: R-29 in EPC (magnification x 100); (n) Uninfected BF-2 cells as control wells (magnification x 50); (o) SLGO-95 in BF-2 (magnification x 50); (p) R-29 in BF-2 (magnification x 50)

4.3.2. Staining methods used for the identification of *P. salmonis* in tissue sections

Tissue samples from naturally infected Atlantic salmon were sectioned and stained with a variety of different staining methods. The RLO in infected tissue sections were stained light blue in IHC (Figure 4.6 a) and dark blue to blackish with H&E staining (Figure 4.6 b). They appeared dark blue in tissue sections stained with Giemsa (Figure 4.6 c), red with Gram staining (Figure 4.6 d), and purplish blue when treated with Castañeda stain (Figure 4.6 e). They appeared orange in colour when stained with Acridine orange, although a small amount of background fluorescence was also observed (Figure 4.6 f). When the sections were stained with Macchiavello's stain, the RLO appeared dark blue-violet in colour (Figure 4.6 g). However, they retained basic fuchsin and took on a bright red colour when stained with Pinkertons's stain in 3 μm thick tissue sections (Figure 4.6 h). When 5 μm tissue sections were used for Pinkertons's method, methylene blue was applied either for 6 h or overnight. The RLO slightly retained basic fuchsin, but the staining was better when stained for 6 h (Figure 4.6 i) rather than overnight (Figure 4.6 j).

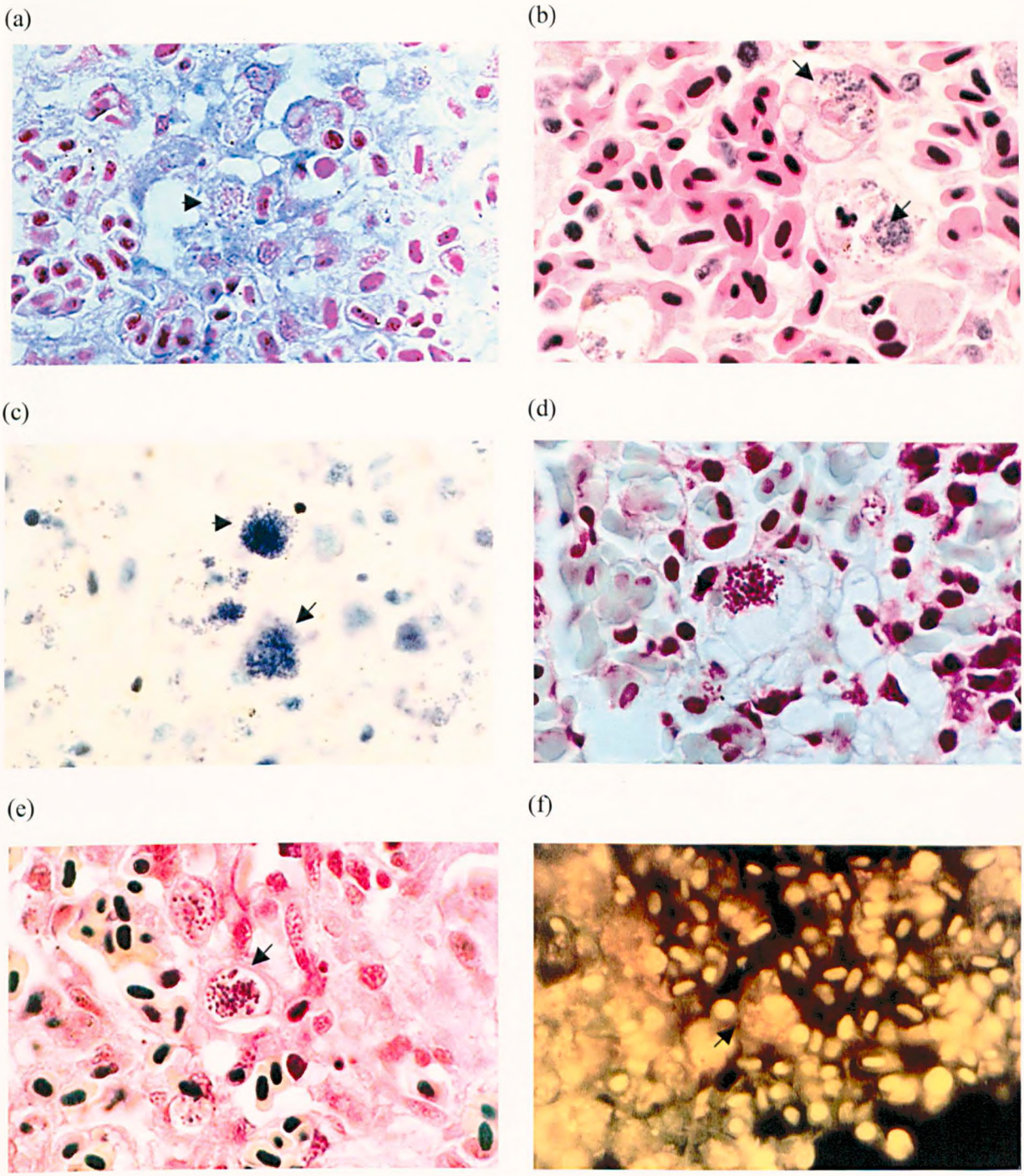


Figure 4.6. Detection of *P. salmonis* in tissue samples from RLO infected Atlantic salmon using a variety of different staining methods. All figures show the staining of RLO present on the splenic parenchyma of the fish (see arrows). (a) Immunohistochemistry (magnification x 1100); (b) H&E (magnification x 1400); (c) Giemsa (magnification x 1400); (d) Gram (magnification x 1400); (e) Castañeda (magnification x 1400); (f) Acridine orange (magnification x 1000)

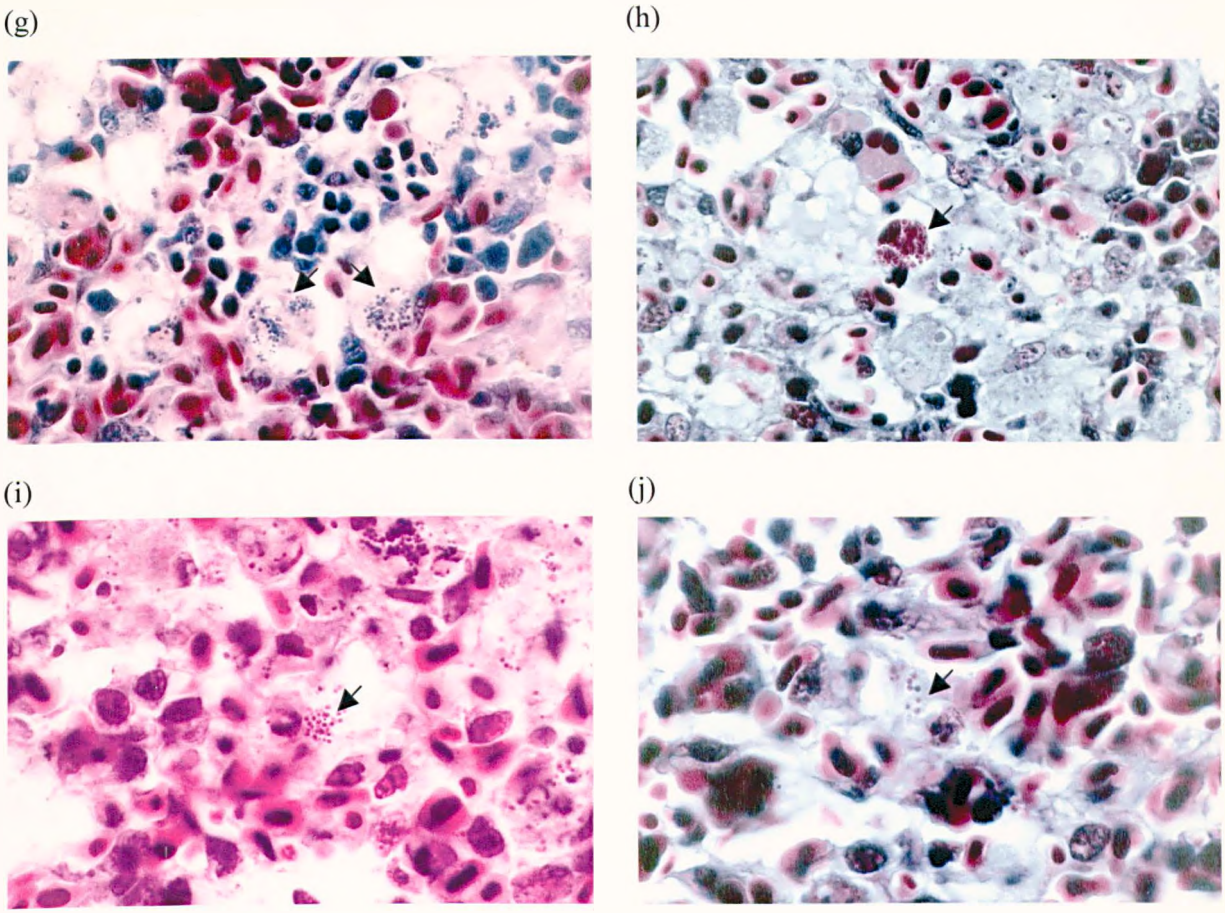


Figure 4.6 (Cont.). (g) Macchiavello's stain (magnification x 1100); (h) Pinkerton's stain, 3 µm section stained overnight with 1% Methylene blue (magnification x 1100); (i) Pinkerton's stain, 5 µm section stained for 6 hours with 1% Methylene blue (magnification x 1400); (j) Pinkerton's stain, 5 µm section stained overnight with 1% Methylene blue (magnification x 1400)

4.3.3. Characterisation of *P. salmonis* using SDS-polyacrylamide gel electrophoresis and different staining methods for the bacterial antigens captured onto nitrocellulose membranes

4.3.3.1. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was used to compare the different *P. salmonis* isolates shown in Table 2.1. The gels were stained either with Silver stain (Figure 4.7 a) or Coomassie blue (Figure 4.7 b) to compare the protein profiles between the different *P. salmonis* isolates. The silver stained banding profile of the various *P. salmonis* isolates appeared very similar, as shown in Table 4.5. Bands at 157, 140, 115, 95, 72, 60, 56, 51, 46, 40, 36, 32, 30, 28, 26, 23, 20, 16, 14 and 11 kilodalton (kDa) were observed with all *P. salmonis* isolates examined. A band was observed at 18 kDa with Silver stain with all isolates except isolates R980769, VQ013, R-29 and SLGO-95 and this band was particularly strong with SRS-UACH, R-61 and R-62. On the other hand a band of 23 kDa appeared more prominent with isolates SLGO-95 and R-29 compare to the other isolates (Figure 4.7 a and b). Gels stained with Coomassie blue also demonstrated a similar banding pattern between the different *P. salmonis* isolates, but the staining was paler than observed with silver staining, particularly with bands below 26 kDa. It was also difficult to see the bands at 83, 64 and 46 kDa which were evident with silver stain. A band observed at 64 kDa may have resulted from the CHSE-214 cells used to culture the bacterium since it was observed in the SDS-PAGE profile of CHSE-214 shown in Section 3.3.3. The 64 kDa band appeared in the profile of all isolates apart from SLGO-95 and R-29 in Silver staining.

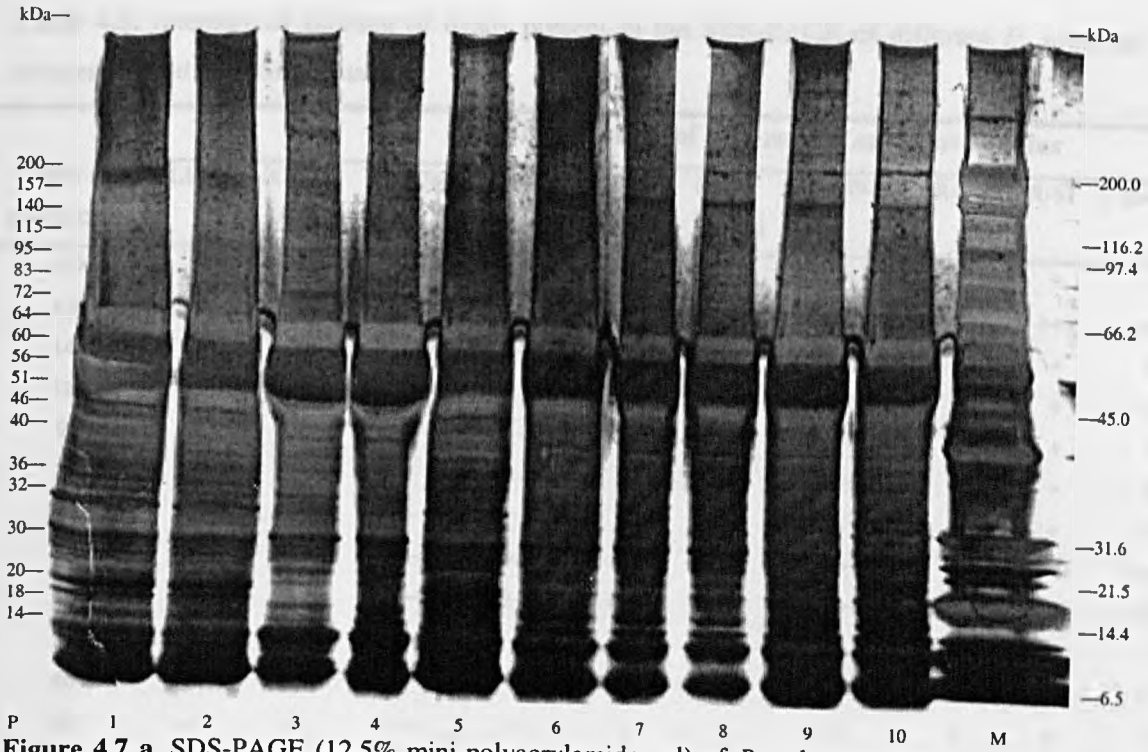


Figure 4.7 a. SDS-PAGE (12.5% mini-polyacrylamide gel) of *P. salmonis* isolates stained with Silver staining. Lanes: (P) Location of protein profiles; (1) R-62; (2) R-61; (3) R-29; (4) SLGO-95; (5) SRS-UACH; (6) SRS-4; (7) VQ013; (8) R980769; (9) AVG5/268; (10) LF-89; M: Marker (Bio-Rad); (P) Location of protein in *P. salmonis* profiles.

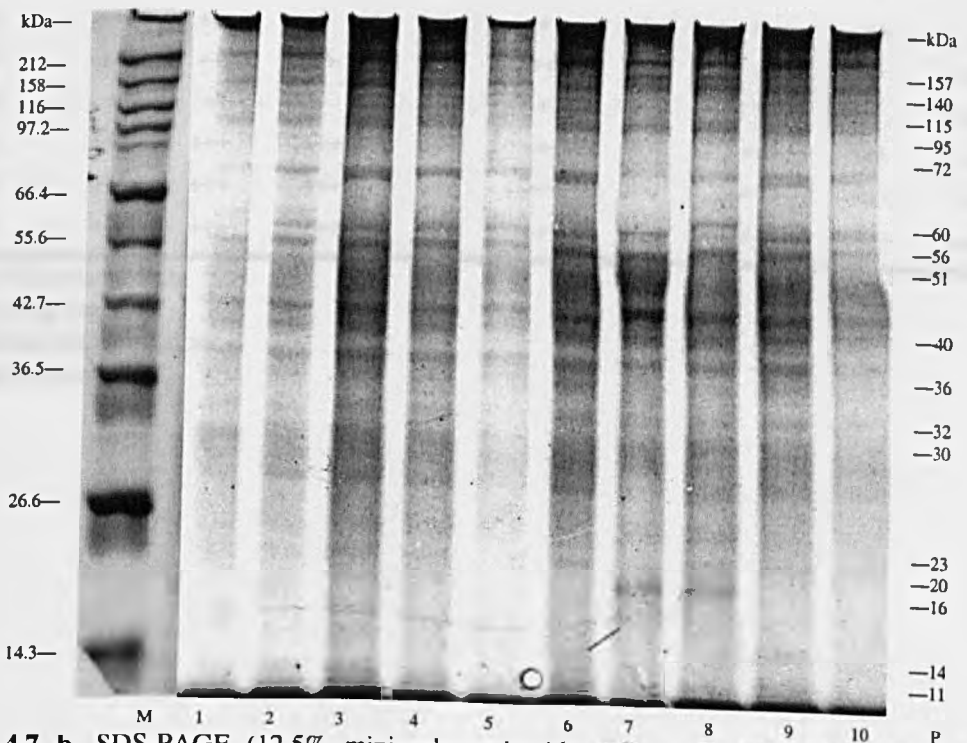


Figure 4.7 b. SDS-PAGE (12.5% mini-polyacrylamide gel) of *P. salmonis* isolates stained with Coomassie blue. Lanes: (1) LF-89; (2) AVG5/268; (3) R980769; (4) VQ013; (5) SRS-4; (6) SRS-UACH; (7) SLGO-95; (8) R-29; (9) R-61; (10) R-62; M: Marker (BioLabs); (P) Location of protein profiles.

Table 4.5. Intensity of staining of bands present in the SDS-PAGE of different *P. salmonis* isolates stained with Silver stain

MW of bands (kDa)	Staining intensity of different *P. salmonis* isolates									
	LF-89	AVG 5/268	R980769	VQ013	SRS-4	SRS-UACII	SLGO-95	R-29	R-61	R-62
~200	+	+	+	+	+	+	+	+	+	+
157	++	++	++	++	+++	+	+	+++	+++	+++
140	+	++	+	+	+	++	+	+	+	+
115	+	+	+	+	+	+	+	+	+	+
95	+	+	+	+	+	+	+	+	+	+
72	+	+	+	+	+	+	+	+	+	+
64	+	+	+	+	+	+	-	-	+	+
60	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
56	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
51	+	+	+	+	+	+	+	+	+	+
46	+	+	+	+	+	+	+	+	+	+
40	+	+	++	+	+	+	+	+	+	+
36	+	+	+	+	+	++	+	+	++	++
32	+	+	+	+	+	++	+	+	++	++
30	++	++	+++	+++	+++	+++	+++	+++	+++	+++
28	+	+	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	+	+	+
23	++	++	+	+	+	+	++	++	+	+
20	++	++	++	++	++	+++	+	+	+++	+++
18	+	+	-	-	+	++	-	-	++	++
16	+++	+++	+++	+++	+++	++	+++	++	+	+
14	+++	+++	+++	+++	+++	++	+++	+++	++	++
11	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

MW : Molecular weight
+++ : Strong reaction
++ : Moderately reaction
+ : Faint reaction

4.3.3.2. SDS-PAGE profiles of *P. salmonis* grown in different fish cell lines

P. salmonis isolate R-29 was used to establish if there was any difference in the protein profiles of the bacterium when it was grown in different fish cell lines. Isolate R-29 was passaged three times through cell line CHSE-214, EPC, RTG-2, BF-2 and SBL, and isolated bacteria were subsequently subjected to SDS-PAGE and the gel was stained with Coomassie blue (Figure 4.8). Differences were observed in the protein profile of the bacterium within the different cell lines, mostly in the lower region of the protein profile (Table 4.6). A band with a molecular weight of 32 kDa disappeared in the profile of R-29 when it was grown in RTG-2 or BF-2 cells. A slight band was also evident at 30 kDa when it was grown in RTG-2 cells, while samples of the bacterium recovered from RTG-2 and BF-2 cells had faint bands at 28 and 26 kDa. The SDS-PAGE of the bacterium had strong bands at 30, 28 and 26 kDa when it was grown in CHSE-214, EPC and SBL cells. A band at 23 kDa appeared stronger when the bacterium was cultured in CHSE-214 cells compared to bacteria recovered from the other fish cell lines. Bands at 115 and some lower bands between 14-20 kDa seemed to be missing when isolate R-29 was grown in BF-2 cells while the band at 115 kDa was only faintly evident when it was cultured in RTG-2 cells. SHK-1 cells were not analysed in this study.

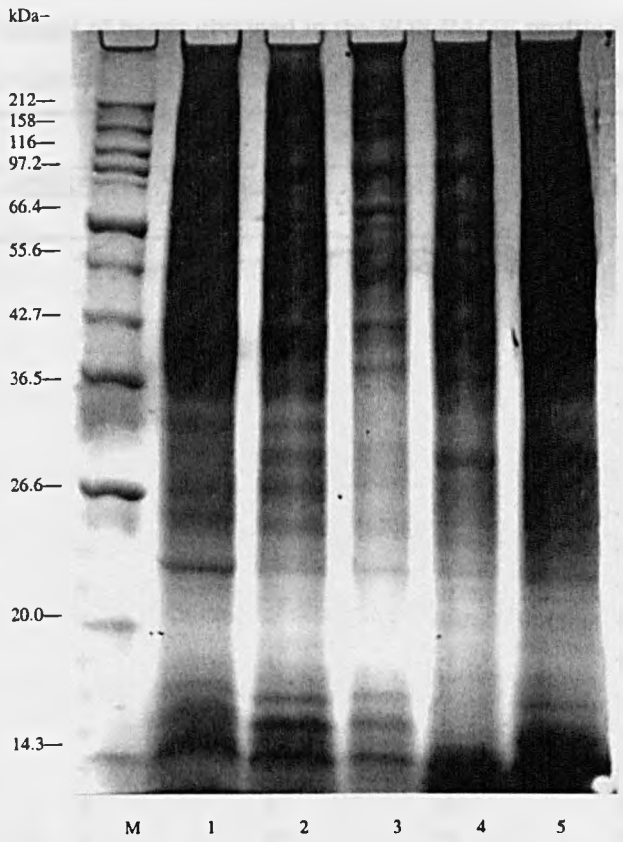


Figure 4.8. SDS-PAGE profiles of *P. salmonis* R-29 cultured in different fish cell lines stained with Coomassie blue. Lanes: (M) Marker (BioLabs); (1) CHSE-214 cells; (2) EPC cells; (3) RTG-2 cells; (4) BF-2 cells and (5) SBL cells

Table 4.6. Molecular weight of bands obtained in the SDS-PAGE profile of *P. salmonis*, isolate R-29, when cultured in different fish cell lines. Gel was stained with Coomassie blue

MW of bands (kDa)	Fish cell lines				
	CHSE-214	EPC	RTG-2	BF-2	SBL
157	+	+	+	+	+
140	+	+	+	+	+
115	+	+	±	-	+
95	+	+	+	+	+
72	+	+	+	+	+
64	+	+	+	+	+
60	+	+	+	+	+
56	+	+	+	+	+
51	+	+	+	+	+
46	+	+	+	+	+
42	+	+	+	+	+
40	+	+	+	+	+
36	+	+	+	+	+
32	+	+	-	-	+
30	+	+	±	+	+
28	+	+	±	±	+
26	+	+	±	±	+
23	+	+	+	+	+
14-20	+	+	+	-	+

MW: Molecular weight
+ : Strong band
± : Weak band
- : No band

4.3.3.3. Proteinase-K digest of *P. salmonis*

SDS-PAGE analysis was performed following Proteinase-K digest of different *P. salmonis* isolates and the gel was stained with Silver stain. Several bands were evident below 30 kDa indicating the presence of carbohydrate moieties present on the bacterium. The proteinase-K treated samples are shown altogether in Figure 4.9.

4.3.3.4. Gycoprotein detection on *P. salmonis*

The presence of glycoproteins on *P. salmonis* was examined using a commercially available glycoprotein determination kit (Figure 4.10). Several bands were present at 115, 72, 60, 56, 51, 40 and 36 kDa, while very slightly stained bands could be seen at 95, 32 and 30 kDa. Bands were also evident between 10-16 kDa.

4.3.3.5. Lectin staining

The lectins shown in Table 4.1 were used to examine carbohydrate profiles of the *P. salmonis*. Lectin ConA gave strongly stained pattern, while staining lectin TAG was fainter, but both lectins resulted in continuous staining pattern on the membrane. The reaction shown in Figure 4.11 with the lectins is hard to see in the photographs so it has also been summarised in Table 4.7.

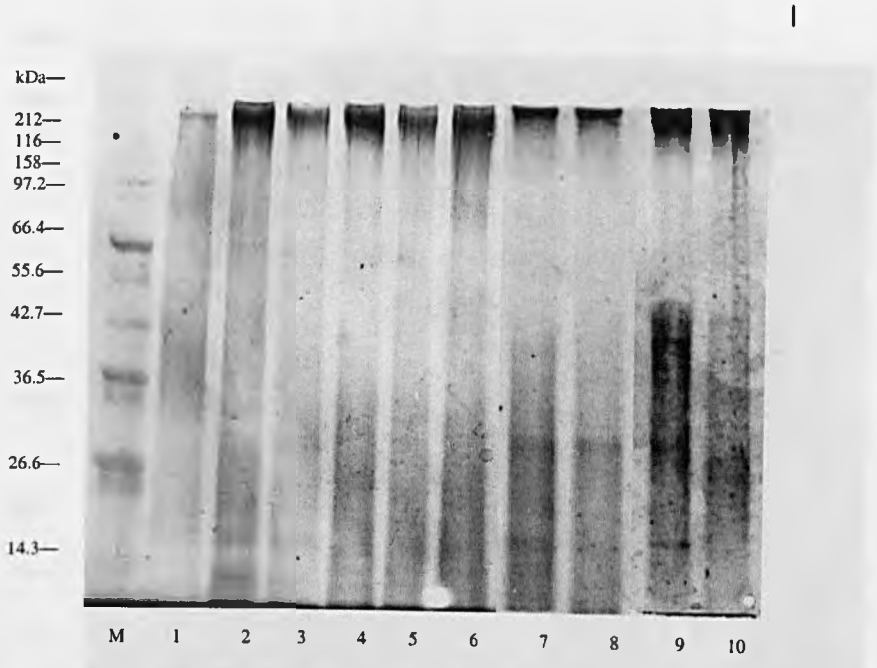


Figure 4.9. SDS-PAGE profiles of Proteinase-K digested *P. salmonis* isolates stained with Silver staining. M: Marker (BioLabs) (1) LF-89; (2) AVG5/268; (3) R980769; (4) VQ013; (5) SRS-4; (6) SRS-UACH; (7) SLGO-95; (8) R-29; (9) R-61(4); (10) R-62

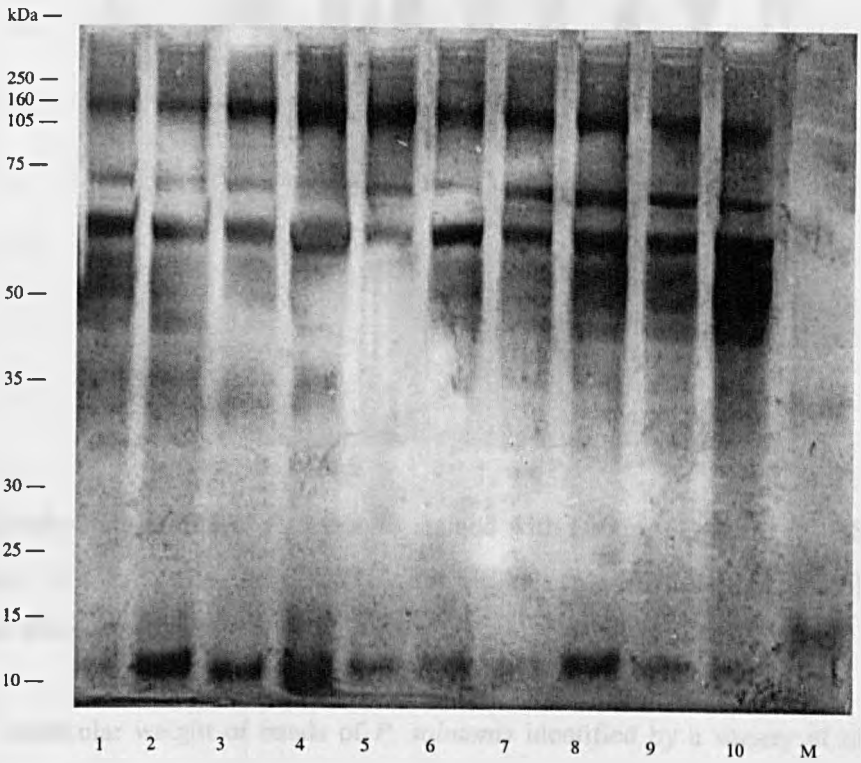


Figure 4.10. Analysis of different *P. salmonis* isolates using with a commercially available Glycoprotein Detection Kit. Lanes: (1) R-62; (2) R-61; (3) R-29; (4) SLGO-95; (5) SRS-UACH; (6) SRS-4; (7) VQ013; (8) R980769; (9) AVG5/268 (4); (10) LF-89; M:Marker (Amersham).

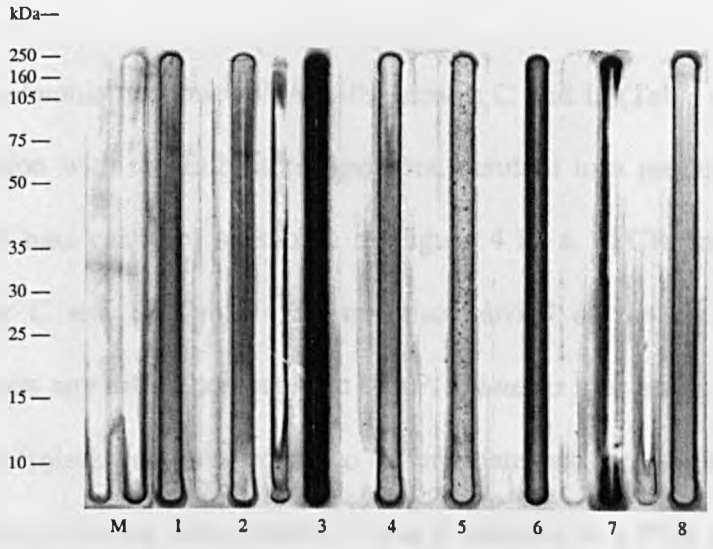


Figure 4.11. Carbohydrate profile of *P. salmonis* stained with Lectins (1) PNA; (2) BS-1; (3) Con A; (4) HGA; (5) CTA; (6) TAG; (7) WGA; (8) UEA-1; (M) Marker (Amersham). (See Figure below for abbreviations)

Table 4.7. The molecular weight of bands of *P. salmonis* identified by a variety of different lectins, when captured onto nitrocellulose membranes

	Lectins[1,2]					
Bands (kDa)	**PNA**	**BS-1**	**HGA**	**CTA**	**WGA**	**UEA-1**
115	-	+	-	-	-	-
95	+	±	-	-	-	-
72	+	+	+	±	-	-
60	-	+	+	-	-	-
56	+	+	±	-	-	-
51	+	+	-	-	-	-
40	-	+	-	-	-	-
36	-	+	-	-	-	-
32	-	+	-	-	-	-
30	-	+	-	-	-	-
26	-	+	±	-	-	-
23	-	+	±	-	-	-
20	-	±	±	-	-	-
Bands between 10-16	±	+	+	+	+	-

[1]+: Strong band; ±: Weak band; -: No band.

[2]**Abbreviations:** PNA: Peanut agglutinin (*Arachis hypogaea*); BS-1: *Bandeiraea simplicifolia*; Con A: Concanavalin A (*Canavalia ensiformes)*; HGA: Horse gram agglutinin (*Dolichos biflorus*); CTA: Coral tree agglutinin (*Erythrina cristagalli*); TAG: Tomato agglutinin (*Lycopersicon esculentum*); WGA: Wheat germ agglutinin (*Triticum vulgaris*); UEA-1: Gorse seed agglutinin (*Ulex europaneus*). Banding profile for ConA and TAG are not presented because of the continuous staining pattern obtained with these lectins.

4.3.4. N-PCR

Amplification of *P. salmonis* genomic DNA with primers C and D (Table 4.2) after a first round amplification with the Eubacterial primers, resulted in a product with the predicted size of 467 base pair (bp) as shown in Figure 4.12 a. PCR analysis with primers C and E, or C and F (Table 4.2) was also carried out in an attempt to determinate if there was any difference between the *P. salmonis* type strain LF-89 and the other *P. salmonis* isolates, and also to try to differentiate between the different *P. salmonis* isolates. Amplification with primers C and E resulted in a PCR products of 816 bp (Figure 4.12 b). However, apart from the Chilean isolate R-29 isolated from Atlantic salmon, none of the other *P. salmonis* isolates produced a 816 bp product with the primer set, C and F (Figure 4.12 c).

The *P. salmonis* isolates were amplified with sets of primers C and D, C and E, or C and F, without using the first set of universal primers (A and B). Only isolate R-29 produced the predicted product at 816 bp with primers C and F, without the Eubacterial primers.

The specificity of the N-PCR was confirmed using 15 different non-*P. salmonis* bacteria shown in Table 2.2, using the primers pair A and B and primers pair C and D. No PCR products were amplified with these bacteria (Figure 4. 13).

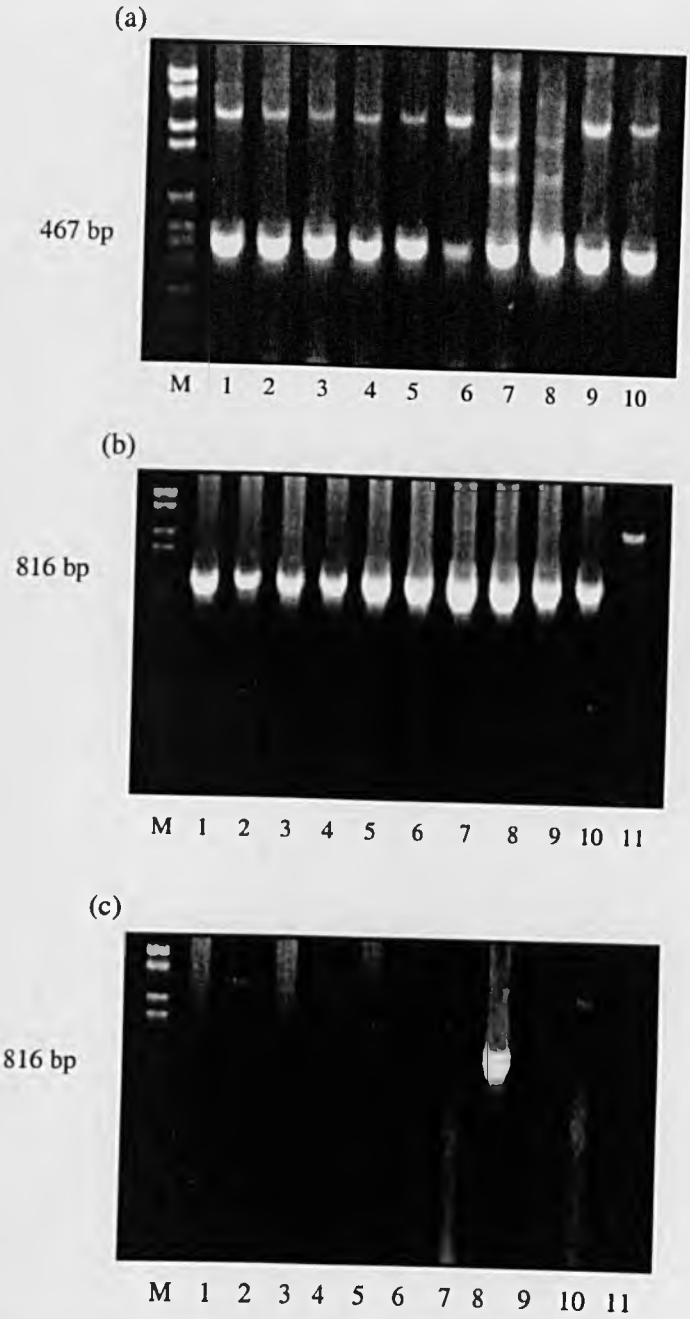


Figure 4.12. **(a)** Nested PCR with a number of *P. salmonis* isolates using primers for Eubacteria (A and B) and *P. salmonis* all strains (C and D); **(b)** *P. salmonis* isolates detected with the primers for Eubacterial (A and B) and *P. salmonis* all strains (C) and *P. salmonis* LF-89 type strain (E); **(c)** *P. salmonis* isolates detected with the primers for Eubacterial (A and B) and *P. salmonis* all strains (C) and *P. salmonis* EM-90 (F). Lanes: (M: DNA markers); (1) LF-89; (2) AVG5/268; (3) R980769; (4) VQO13; (5) SRS-4; (6) SRS-UACH; (7) SLGO-95; (8) R29; (9) R61; (10) R62; and (11) Negative control.

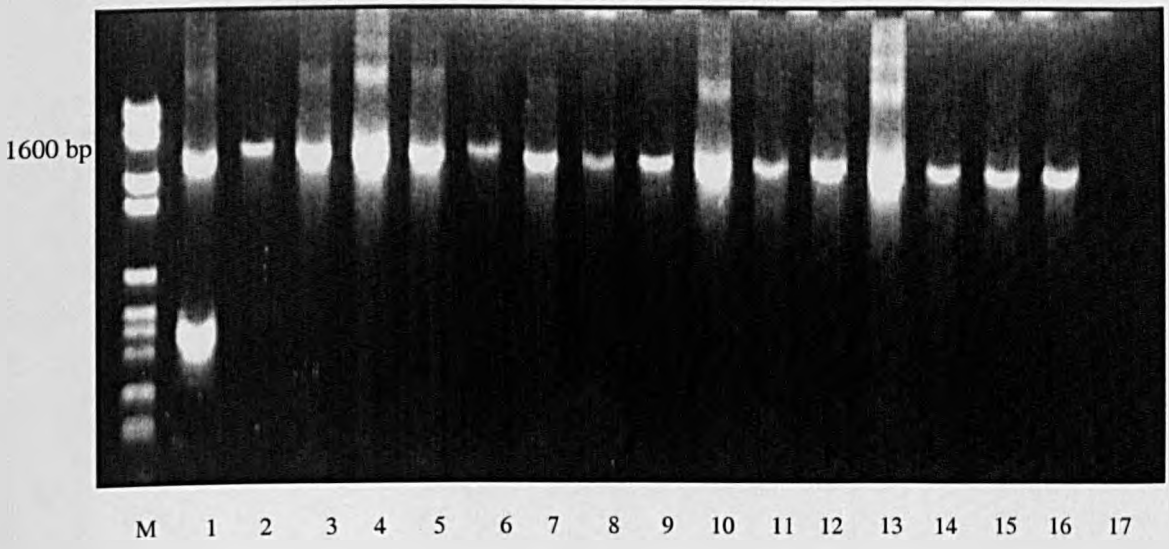


Figure 4.13. Specificity of *P. salmonis* PCR using primer pairs Eurobacterial (A and B) and *P. salmonis* all strains (C and D). Lanes: (M: DNA marker); (1) Positive control-Isolate R-29; (2) *Pseudomonas aeroginosa;* (3) *Yersinia ruckeri*; (4) *Nocardia asteroides*; (5) *Vibrio anguillarum*; (6) *Echerichia coli*; (7) *Pseudomonas fluorescence*; (8) *Micrococcus luteus*; (9) *Aeromonas salmonicida*; (10) *Vibrio ordalii;* (11) *Bacillus subtilis*; (12) *Corynabacterium aquaticum*; (13) *Mycobacterium marinum*; (14) *Edwardsiella tarda*; (15) *Streptococcus faecalis*; (16) *Renibacterium salmoniarum*; (17) Negative control.

P. salmonis isolate R-29 was tested by N-PCR to establish if there was any difference in the PCR products after the bacterium had been passaged three times in the different cell lines (CHSE-214, EPC, RTG-2, BF-2, SBL). After the first amplifying with Eubacterial primers A and B, the bacterium was re-amplified with primer set C and D (against all *P. salmonis* isolates). The product of the reaction was a band at 467 bp. On the other hand, when the samples were re-amplified with the primer pair C and F (*P. salmonis* EM-90), products with of 816 bp resulted regardless of the cell line. However, when primer pair C and E (*P. salmonis* type strain LF-89) was used as the second set of primers DNA extracted from R-29 grown in CHSE-214, RTG-2 and SBL cells gave stronger band at 816 bp compared to bacteria grown in EPC and BF-2 cells (Figure 4.14).

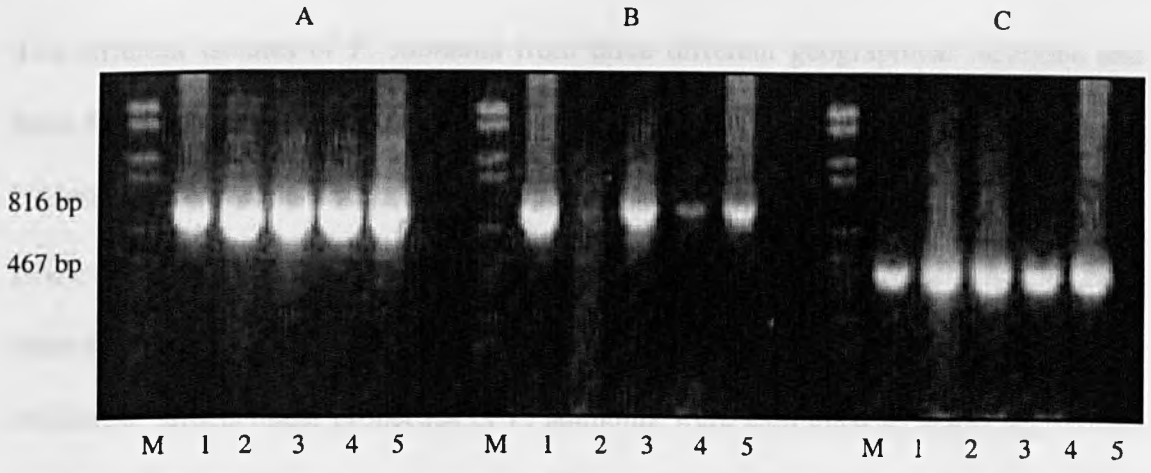


Figure 4.14. *P. salmonis* R-29 cultured in different cell lines tested by Nested PCR using different primer pairs for the second amplifications. (A) *P. salmonis* all strains (C and D); (B) *P. salmonis* LF-89 (C and E) and (C) *P. salmonis* EM-90 (C and F). Lanes: (1) CHSE-214; (2) EPC; (3) RTG-2; (4) BF-2; (5) SBL; (M) DNA marker

4.4. DISCUSSION

Ten different isolates of *P. salmonis* from three different geographical locations and from four different fish species were used in this study. Growth characteristics of the bacterium *in vitro*, its biochemical properties, protein compositions and its genomic DNA characteristics were examined. Growth characteristics of the bacterium *in vitro* were established using a $TCID_{50}$ and plaque assay, when cultured in six different fish cell lines. Biochemical properties of *P. salmonis* were examined by applying various histo-staining methods to sections of naturally affected fish tissue. SDS-polyacrylamide gel electrophoresis was performed and proteins transferred to nitrocellulose membranes. A number of different staining methods were used to stain the gels or the nitrocellulose membranes to examine the protein and carbohydrate profiles of the bacterium. A nested polymerase chain reaction was used to detect genomic DNA of the bacterium.

The growth characteristics of *P. salmonis in vitro* has been described by Fryer *et al.* (1990). It is possible to culture the bacterium in fish cell lines, and the optimal culture temperature is between 15 and 18°C, during which it produces a CPE with a replication titer of between 10^6 and 10^7 $TCID_{50}$ ml^{-1}. However, it is not possible to culture the bacteria on cell-free medium. Bacterial replication is significantly reduced above 20°C and below 10°C, and does not occur above 25°C (Fryer & Lannan 1996).

The pathology of rickettsial infections results from host-cell destruction since to survive and reproduce, the rickettsia must first enter the eucaryotic host cell, grow within the cell, and then be released from the cell to infect neighbouring cells. The rickettsiae do not however, produce exotoxins (Winkler 1990).

Six different fish cell lines (CHSE-214, EPC, RTG-2, BF-2, SBL and SHK-1) were used to culture *P. salmonis in vitro* and to examine the susceptibility of the cell lines to the different *P. salmonis* isolates used in this study. The susceptibility of CHSE-214 cell to the *P. salmonis* isolates was first determined by calculating the $TCID_{50}$ for each isolate examined in this study. All the *P. salmonis* isolates established a CPE 3 dpi, although a slight CPE could be seen with isolates VQ013, AVG5/268, SRS-UACH and R-29 as soon as 1 day post-incubation. However, no further progress in the CPE was observed by any of the isolates until 10 dpi when there was sharp increase in the $TCID_{50}$ values obtained with these isolates. Barnes (1996) produced a growth curve for the *P. salmonis* type strain LF-89, showing a 4 d lag-phase and an 8 d log-phase of exponential growth, followed by a second-log phase of growth where the remaining monolayer cells were infected by second generation *P. salmonis.* The growth curve is typical of any bacterial growth (Nicklin, Graeme-Cook, Paget & Killington 1999; Barnes 1996) and explains the increase in the $TCID_{50}$ after 7 dpi in CHSE-214 cells seen in this study. This was confirmed by Fryer *et al.* (1990) with electron-micrographs where RLO were observed being released from ruptured host cells (CHSE-214) after 8 dpi at 15°C.

The susceptibility of six different fish cell lines to ten different *P. salmonis* isolates was examined also based on the $TCID_{50}$ ml^{-1} of each isolate. The highest $TCID_{50}$ value was seen in fish cell lines CHSE-214, SHK-1 and EPC, while BF-2 cells gave the lowest $TCID_{50}$ ml^{-1} titer. Fryer *et al.* (1990) observed a CPE within all salmonid cell lines, they tested (CHSE-214, CSE-119, CHH-1, RTG-2) as well as in cell lines from warm-water fish (FHM and EPC), but no CPE was observed in BB and BF-2 cells inoculated with type strain LF-89. Cvitanich *et al.* (1991) also reported a CPE by RLO isolated

from infected coho salmon kidney 17 d after inoculation onto CHSE-214, CSE-119, CHH-1, RTG-2, FHM and EPC monolayer, but no CPE was observed in BF-2 or BB cells. However, Cvitanich, Garate, Silva, Andrade, Figueroa and Smith (1995) reported a RLO observed and isolated from Atlantic salmon in Chile and then called as an unidentified agent grew in 7 fish cell lines (EPC, CHH-1, CHSE-214, RTG-2, FHM, BB, and BF-2) at temperatures between 15 and 27°C. However, Almendras, Jones, Fuentealba and Wright (1997) first observed a CPE 45 dpi with *P. salmonis,* and, a completed CPE by 78 dpi in BB cell line, while a complete CPE was observed between 6 d and 20 dpi in EPC and CHSE-214 cells in the same experiment. Almendras *et al.* (1997) observed the bacterium free in the intracytoplasm or located outside the fish cell at 78 dpi under electron microscopy (EM). Chen, Tung, Chen, Tsai, Wang, Chen, Lin and Adams (1994) reported a RLO isolated from Nile tilapia, *Oreochronuis niloticus* from southern Taiwan. The organism appeared in cytoplasmic vacuoles of CHSE-214 cells 2 dpi, but not in FHM, BF-2 and Nile tilapia, *O. niloticus* ovary origin cells (TO-2) and no CPE was observed in CHSE-214 cells. Chern and Chao (1994) observed replication of a RLO within TO-2 and EPC cell lines at 25°C which had been inoculated with a spleen homogenate obtained the naturally infected tilapia. Results either from the previous studies or from this study indicates that the susceptibility of different fish cell lines to RLO vary and this may reflect the nature of the host cell-rickettsia interaction.

There are major differences among rickettsial species such as the host cell-bacteria relationship, but in some cases cell cultures reflected strain variations (Weiss 1973). The EPC or SBL fish cell lines used in this study showed variations in their sensitivity to the different *P. salmonis* isolates tested. Although it is difficult to extrapolate the

findings seen *in vitro* to host cell susceptibility *in vivo*, the findings of this study suggest that the different *P. salmonis* isolates used can grown both in salmonid and non-salmonid cell lines, and this may reflect the wide distributions of hosts found with this bacterium. It may also explain the difference in mortalities seen in the different disease outbreaks observed in various geographical regions.

A plaque assay was also used to examine the growth of *P. salmonis* isolates LF-89, SLGO-95 and R-29 with fish cell line. Plaques were obtained within CHSE-214 and EPC cells and they differed in shape. However, no definite plaques were obtained in the other cell lines used. A higher PFU was obtained in CHSE-214 cells than seen in EPC cells. This corresponds with the findings of Barnes (1996) who observed higher plaque numbers in CHSE-214 cell monolayers than in EPC cell monolayers. This may be related to the host cell structure or the interaction between the host cell and the rickettsia. Different plaques have been reported with different rickettsia when inoculated into different cells (Kordová 1966).

Rickettsia are barely visible under the LM, although they are morphologically typical of Gram-negative bacteria, they stain poorly with Gram stain (Levinson & Jawet 1992; Winkler 1990). Staining methods have been previously used to diagnose of piscirickettsiosis by a number of authors (Brocklebank *et al.* 1992; Fryer & Lannan 1992; Khoo *et al.* 1995; Olsen *et al.* 1997). The staining methods tried here were also used in an attempt to establish a rapid diagnostic method for piscirickettsiosis.

Bacteria are differentiated microscopically by examining their shape, grouping of bacterial cells, presence or absence of characteristic structures, and their reaction to

differential stains. Bacteria can be stained with different dyes depending on the presence or absence of certain components (Humason 1979) e.g. such as a layer of LPS external to the peptidoglycan wall found on Gram-negative bacteria, unlike Gram-positive bacteria. Selective staining can provide useful information which may allow identification of individual organisms (Horobin 1982). The Gram stain is one of the most commonly used stains to help to distinguish between two groups of bacteria based on their cell-wall composition. Gram-negative bacteria have only a thin layer of peptidoglycan unlike Gram-positive bacteria, and stain pink since they lose the crystal violet and iodine complex on washing with aseton, which makes Gram-positive bacteria appear blue. The Gram stain may also show other morphological structures of bacteria such as a thin, cross-linked peptidoglycan layer (murein) in the outer membrane of the cell wall of Gram-negative bacteria. The outer membrane in Gram-negative bacteria contains some unique features such as LPS which helps to protect to the bacterium from its host (Nicklin *et al.* 1999).

Tribe Rickettsiae, which belongs to family *Rickettsiaceae* are Gram-negative and are generally poorly stained with aqueous solutions of aniline dyes. They stain well with Giemsa, Leishman, Castañeda and Macchiavello. Some rickettsia, e.g. *R. prowazekii* and *C. burnetti* retain basic fuchsin, but others, such as *Cowdria ruminantium* and *Cytoecetes phagocytophilia* do not (Buxton & Fraser 1977). When a dye molecule moves from solution into a solid substrate, for example in a tissue section, differential staining rates occur caused by factors such as the molecular weight of dye, the staining time, pre-staining treatment, dyebath additives, various tissue substrates or substrate geometry. The surface area and section thickness of the substrate are important geometrical factors influence the staining rate. Other things being equal, the greater

ratio of surface area to volume, the faster the staining will occur, while the thinner the section the faster and more effective the staining, particularly for small structures within in a tissue sections (Horobin 1982). The thinner sections (0.3 μm) used in this study for the Pinkerton method provided more effective for staining with Basic fuchsin.

Rickettsia-like organisms in the tissue sections examined here appeared coccoid or pleomorphic, and were present either singly or in groups in cytoplasmic vacuoles. They were Gram-negative, basophilic with H&E, acid-fast, red when stained with Basic fuchsin by Pinkerton's method, but Macchiavello negative (particle stained dark blue-violet), Giemsa positive, blue with Methylene blue for Castañeda staining, bright red-orange with Acridine dye which stained the nucleic acid of the bacterium. These staining characteristics of *P. salmonis* have been previously reported by both Fryer and Lannan (1996) and Almendras and Fuentealba (1997). The staining methods tried here, such as Pinkerton's staining using with 3 μm sections, H&E, Gram, Giemsa, Castañeda or Acridine orange can be used for the rapid diagnosis of piscirickettsiosis and combined with a confirmation of the pathogen in fish tissue using IHC.

The antigenic structure of the ten *P. salmonis* isolates was analysed using SDS-PAGE in this study. SDS-PAGE profiles of all *P. salmonis* isolates shared bands in common at 157, 140, 115, 95, 72, 60, 56, 51, 46, 40, 36, 32, 30, 28, 26, 23, 20, 16, 14 and 11 kDa, and most of the bands had been previously identified by Barnes, Landolt, Powell and Winton (1998). They observed protein bands with molecular weight of 157, 108, 72, 60, 56, 36, 32 and 30 kDa with purified *P. salmonis* (LF-89) and 157, 108, 89, 83, 72, 70, 68, 64, 60, 56, 54, 36, 32, and 30 kDa with CHSE-214 cells infected with LF-89. Jamet, Aguayo, Miquel, Muller, Arriagada, Becker, Valenzuela and Burzio (2001),

on the other hand described five major bands for *P. salmonis* LF-89 with molecular weights of 105, 70, 58, 42 and 30 kDa. Any difference seen between the isolates appeared to be located in the low molecular region of the profiles. Isolates SRS-UACH, R-61 and R-62 from Chile were similar in appearance with stronger bands at 36, 32 and 20 kDa than seen with the other *P. salmonis* isolates. Isolates R980769, VQ013, SLGO-95 and R-29 had no band at 18 kDa, which was evident with isolates, SRS-UACH, R-61 and R-62. A band at 23 kDa was seen with *P. salmonis* type strain LF-89, and isolates SLGO-95 and R-29 and also on the only isolate from Ireland, AVG5/268. Isolate R980769 from sea bass obtained from Greece and VQ013 isolated from rainbow trout in Chile had a similar banding pattern to each other except for an apparent band at 40 kDa with the Greek isolate.

The bands at 56 and 60 kDa identified in this study were also seen in the study by Barnes *et al.* (1998). These proteins also appear similar to the 56 kDa major outer membrane protein found on *Orientia tsutsugamushi,* (formerly *Rickettsia tsutsugamushi*) causing human scrub typhus infections. This is the immunodominant type-specific antigen (Ching, Wang, Eamsila, Kelly & Dasch 1998; Tamura 1999), and is similar to the 60 kDa protein identified on *Rickettsia conarii,* the etiological agent of Mediterranean Spotted Fever group (MSFG) rickettsia (Teysseire & Roult 1992). These antigens appear to be homologous to the highly conserved family of heat shock proteins (Hsp) at 60 kDa, known as the Hsp 60, which are essential for bacterial growth and viability. Many of these proteins are expressed at low levels under normal physiological conditions, but their rate of synthesis is dramatically increased at elevated temperatures or under a variety of stressors (Eremeeva, Ching, Wu, Silverman & Dasch 1998). The role of the Hsp 60 is also well described for MSFG rickettsia and induces

the translocation, folding, and assembly of proteins (Teysseire & Roult 1992). The Hsp response is highly conserved among prokaryotic and eukaryotic cells and the Hsp in the Hsp 60 and Hsp 70 families in particular have been shown to be immunogenic and responsible for delayed type hypersensitivity responses and autoimmune responses. Hsp are also thought to provide effective protection for intracellular pathogens against the hostile environment of host phagocytic cells (Teysseire & Roult 1992; Eremeeva *et al.* 1998; Voet, Voet & Pratt 1999), and may be able to activate cell-mediated immunity.

Protein analysis of various *Rickettsiaceae* shows approximately 10 antigens between 20 to 120 kDa size range (Barnes 1996). The function and location of the proteins on the bacterium are unknown. However, the 120 kDa band is known to be the rickettsial outer membrane protein B (rOmpB) and the 32 kDa band is a possible membrane anchor protein. The 120 kDa rOmpB protein is strongly immunogenic and is believed to be a candidate for developing a subunit vaccine against rickettsial infections (Hackstadt, Messer, Cieplak & Peacock 1992; Barnes 1996). When an avirulant mutant of *R. rickettsii* was analysed, reduced amounts of the 120 and 32 kDa fragments were observed together with a loss in the ability to lyse infected cells (Hackstadt *et al.* 1992).

The SDS-page profile of isolate R-29 stained with Coomassie blue appeared different when passaged three times through different cell lines, (CHSE-214, EPC, RTG-2, BF-2 and SBL). The differences were observed mostly in the lower region of the gel. The CHSE-214, EPC and SBL cells which showed a higher susceptibility to the bacterium had more material in this region than bacteria recovered from less susceptible BF-2 and RTG-2 cells where the bands in this region had disappeared. A band at 115 kDa and

bands between 14-20 kDa seemed to disappear when the bacterium was grown in BF-2 cells. Isolate R-29 passaged through BF-2 cells and RTG-2 cells did not appear to have band at 32 kDa.

The 56 kDa protein found on the *P. salmonis* isolates is a major outer membrane protein of *O. tsutsugamushi*, as mentioned above. The *O. tsutsugamushi* is a Gram-negative bacterium, but in contrast to other Gram-negative bacteria, it has neither LPS nor a peptidoglycan layer. The ultrastructure of its cell wall differs significantly to that of it closest relatives belonging to the typhus group (TG) and spotted fever group (SFG) of *Rickettsia.* The former has a thicker outer leaflet compared to its inner leaflet, while the latter bacteria are surrounded by a slime layer, presumably composed of carbohydrates. Although *Orientia* isolates are highly variable in their antigenic properties, the major outer membrane protein of *O. tsutsugamushi* at 56 kDa accounts for 10 to 15% total protein content of bacterium, suggesting that it is a good candidate for use as a diagnostic antigen, as this protein is recognised by sera from most patients infected with scrub typhus (Weiss & Moulder 1984; Ching *et al.* 1998).

It has been demonstrated that the species-specific protein antigens (SPAs) found in crude typhus vaccines are protective immunogen(s). They are species-specific, have similar heat labilities and constitute 10 to 15% of the total rickettsial cellular protein. The SPAs may be an easy target for the immune system of the host since they are associated with the rickettsial cell wall (Dasch 1981). Some antigens on the SFG of rickettsia appear to be similar to the TG of SPAs. The heat stable protein antigen of *R. conarii* which belong to the SFG, found at 115 kDa, is thought to be a surface antigen

because it appears to be involved in the adhesion of rickettsia to the host cell surface (Winkler 1990).

However, high molecular weight rickettsial proteins which are SPAs, do not appear to cross-react with each other (Teysseire & Roult 1992), suggesting that the cross-reactivity which does occur is due to a LPS antigen common between the different Rickettsial species (Raoult & Dasch 1995). Bacterial cell surface polysaccharides i.e. capsular polysaccharides (such as the K-antigen) or LPS have been accepted as being among the important virulence factors of bacteria (Arakawa, Wacharotayankun, Nagatsuka, Ito, Kato & Ohta 1995). The serological specificity of Gram-negative bacteria is determined by the structure of different bacterial cell surface polysaccharides such as the O-specific polysaccharide chain (OPS, O-antigen) of the outer-membrane LPS or the K-antigen (Ziolkowski, Shashkov, Swierzko, Senchenkova, Toukach, Cedzynski, Amano, Kaca & Knirel 1997; Arakawa *et al.* 1995).

LPSs, analysed by SDS-PAGE, can be visualised with either silver stain or PAS stain. The LPSs stain dark brown by the Silver stain, which is 500 times more sensitive than PAS. The polysaccharide portion of the LPS is the reactive component in the Silver stain, however, the exact mechanism of the silver stain is not clear (Tsai & Frasch 1982). Most of the *P. salmonis* isolates examined here showed considerable dark brown staining in the lower region of gel when stained with Silver stain.

To confirm that the bands stained brown by the Silver stain were carbohydrate, the *P. salmonis* isolates were digested with Proteinase-K. Proteinaceous glycoconjugates are broken down by this enzyme. Silver stain analysis of the Proteinase-K digested *P.*

salmonis isolates showed that all isolates of the bacterium contained carbohydrate bands below 30 kDa. Kuzyk, Thorton and Kay (1996) also performed Silver staining on Proteinase-K digested *P. salmonis* and found a very faint, ladder-like banding pattern of carbohydrates ranging from 16 to 35 kDa, with a particularly clear band evident at around 20 kDa and an intense band at around 11 kDa. Tsai and Frasch (1982) cited that Palva and Mäkelä (1980) and Goldman and Leive (1980) had proposed that the LPS molecule on the bacterium have a different numbers of repeating units in their O side chains. When analysed, the fastest moving band was the core molecule of LPS; the second fastest band was the core plus one repeating unit of LPS; and so forth (Tsai & Frasch (1982), this resulting in the laddering effect. A carbohydrate banding pattern was also observed in the silver stain profile of Proteinase-K digested *P. salmonis* isolates in this study.

The LPSs isolated from TG rickettsia, *R. typhi* and *R. prowazekii,* were characterised by both chemical analysis and SDS-PAGE stained with Silver stain. LPSs from two species of TG rickettsiae contained glucose, 3-deoxy-D-manno-octulosonic acid (KDO), glucosamine, quinovosamine, phosphate, and fatty acids (β-hydroxylmyristic acid and heneicosanoic acid), but not heptose, and the O-polysaccharides of these LPSs consisted of glucose, glucosamine, quinovosamine and phosphorylated hexosamine (Amona, Williams & Dasch 1998). LPS from SFG was composed of KDO, glucosamine, quinovosamine, ribose, phosphate, and palmitic acid, but not heptose or β–hdroxy fatty acid. On the other hand, *O. tsutsugamushi* appears to have no LPS (Amona *et al.* 1998) as mentioned above.

To further characterise the carbohydrate moieties of the *P. salmonis* isolates used in this study, a commercially available glycoprotein determination kit and a number of different biotin-labelled lectins were used to identify carbohydrate moieties. The principle of the glycoprotein kit relies on a specific carbohydrate oxidation reaction to label the carbohydrate with biotin, and subsequently streptavidin-alkaline phosphates conjugate are used to detect it.

Glycoprotein bands were identified on the *P.* salmonis isolates at 115, 72, 60, 56, 51, 40 and 36 kDa with the kit, and faint bands were also seen at 95, 32 and 30 kDa. A lower band between 10-16 kDa was also present on all isolates. Glycoproteins, with a high carbohydrate content, are poorly stained by Coomassie blue, whereas both carbohydrate and protein are stained by the Silver stain, so glycoproteins would be expected to stain with the highly sensitivity Silver stain (Tsai & Frasch 1982).

The banding pattern showed that carbohydrate moieties of *P. salmonis* were composed mostly of α-D-galactosyl and N-acetyl-α-D-galactosaminyl residues, but also with D-galactose and α-D-mannosyl and α-D-glucosyl residues. Some bands were recognised by most of the lectins, possibly because they contained complex polysaccharides. The Lipo-oligosaccharide (LOS) and LPS core regions contained mostly α-D-galactosyl and N-acetyl-α-D-galactosaminyl residues and D-galactose.

P. salmonis appeared to have a similar antigenic pattern to the tribe Ehrlichia, and Fryer *et al.* (1990) initially reported that *P. salmonis* might be a member of the tribe Ehrlichieae based upon morphology and site of replication. Phylogenetic placement of *P. salmonis* based on the 16S rRNA gene sequence of a single isolate, LF-89 type strain

showed that *P. salmonis* is a member of gamma subdivision of the Proteobacteria, as are the genera *Coxiella* and *Francisella.* Thus *Piscirickettsia* strains are expected to share more characteristics with these two genera than genera *Neorickettsia, Rickettsia,* Cowdria, *Anaplasma,* and *Ehrlichia* (Fryer *et al.* 1992; Mauel *et al.* 1999).

Genomic DNA of *P. salmonis* isolates tested was detected using a nested polymerase chain reaction (N-PCR). N-PCR is more sensitive and reliable than 2-sequential amplification for *P. salmonis* using specific primers as reported by Mauel *et al.* (1996), and also confirmed in this study. *P. salmonis* isolates were differentiated with the N-PCR using different primer pairs. The first round amplification was performed with Eubacterial primer pair (A-B). A primer which is specific for all *P. salmonis* strains (C) was used as the forward primer for the second round of the N-PCR and a variety of reverse primers were also used: primer D which was specific for all *P. salmonis* strains; primer E which detected only *P.salmonis* LF-89 type strain and primer F which detected *P. salmonis* isolate EM-90, all of which described by Mauel *et al.* (1996). All *P. salmonis* isolates used in this study produced predicted products at 467 bp and 816 bp with the first two primer sets C-D and C-E respectively. The EM-90 primer set (C-F) resulted in a PCR product with a predicted size of 816 bp with only isolate R-29, and no PCR product of this size was observed with the other isolates examined. *P. salmonis* isolate EM-90 from Atlantic salmon obtained from Marine Harvest, Chile was similar to isolate R-29 on the basis of the reaction of the same primer pair (C-F) unlike other isolates used. However, R-29 produced a 816 bp product with the *P. salmonis* LF-89 type strain primer set (C-E) while EM-90 did not produce any product with this primer set when tested by Mauel *et al.* (1996). Mauel *et al.* (1999) reported that there was little nucleotide sequence variation in 16S rRNA gene sequences (98.5% similarities)

between the strains LF-89 and EM-90. Isolates SLGO-94 and CI-95 used in the study had 99.5-99.6% similarities to LF-89 type strain respectively when 1902 nucleotides of the 23 large subunit ribosomal deoxyribonucleic acid (23S rDNA) were analysed. The isolates used were supplied by P. Smith (University of Chile, Santiago, Chile), who also supplied isolate SLGO-95 (Coho salmon, Chile) used in this study. The authors used five different *P. salmonis* isolates obtained from different sources and geographical origins (LF-89 and ATL-4-91 from Atlantic salmon, British Columbia; NOR-92 from Atlantic salmon, Norway; SLGO-94 from Rainbow trout, Chile and C1-95 from Coho salmon, Chile). Comparison of the 16S gene sequences of the five isolates showed high levels of similarity (98.5%) within the group, while comparison of their internal transcribed spacer (ITS) as well as isolate EM-90 varied between isolates (95.2 to 99.7% similarity). Two-thirds of the 23 large subunit ribosomal ribonucleic acid (23S rRNA) gene was sequenced for the five isolates showing similarities of 97.9 to 99.8%. However, Mauel *et al.* (1999) also reported that the diversity within the 16S, ITS and 23S rDNA was not sufficient to divide the genus *Piscirickettsia* into several species. Systematic studies linked to determine DNA re-association, virulence, phenotypic, biochemical and antigenic characteristics are needed to have taxonomic diversity of the species. It is suggested that, serotype rather than species appears to be a more appropriate description for SFG rickettsiae isolates, since they show little base-pair sequence heterology with limited evidence from DNA sequencing studies (Kelly, Beati, Matthewman, Mason, Dasch & Raoult 1994). The specificity of N-PCR and the primer pair for *P. salmonis* was tested against 15 non-*P. salmonis* bacteria which did not produce any genomic product in the N-PCR.

N-PCR was also used to detect *P. salmonis* isolate R-29 with primer pairs (C-D; C-E and C-F) after they were passaged three times in different cell lines in this study. Each sample produced products 467 bp and 816 bp with the primer sets C-D and C-F respectively. An unexpected result was obtained in the N-PCR when isolate R-29, passaged in different fish cell lines, was tested using the primer set specific for all strain-*P. salmonis* - *P. salmonis* LF-89 (C and E) for the second round of amplification. R-29 recovered from EPC and BF-2 cells produced less intensive product bands at 816 bp than these obtained with R29 recovered from CHSE-214, RTG-2 and SBL cells. The reason for this remains unclear.

In this chapter, ten *P. salmonis* isolates were compared using their growth characteristics in fish cells, protein compositions and genomic DNA characteristics. The *P. salmonis* isolates analysed were found to be antigenically homologous, with only one isolate, R-29, appearing to differ when examined by N-PCR. Susceptibility of the fish cell lines to *P. salmonis* isolates varied slightly between isolates. However, the banding profile on SDS-PAGE gels and the intensity of product in the N-PCR appeared to be affected when *P. salmonis* isolate R-29 was cultured in different fish cell lines. The differences in the banding profile of isolate R-29 when passaged through different fish cell lines, need to be examined to identify and characterise the proteins of *P. salmonis* expressed intracellularly. This may be useful in understanding the pathogenesis of the bacterium *in vivo*. Further studies related to this work are therefore needed to help in the identification of candidate antigens and development of an effective vaccine against *P. salmonis* in different hosts from different geographical regions.

CHAPTER V

PRODUCTION OF POLYCLONAL AND MONOCLONAL ANTIBODIES AGAINST *PISCIRICKETTSIA SALMONIS* AND THEIR APPLICATION IN THE ANTIGENIC ANALYSIS AND IDENTIFICATION OF THE BACTERIUM

5.1. INTRODUCTION

Economically important fish species, cultured commercially, suffer from a wide range of different diseases. As a result of the intensification they experience in aquaculture systems, disease episodes in stocks appear to be on the increase. Specific determination of fish pathogens and the development of vaccines are necessary to provide effective control of these diseases, especially if the industry is to expand. Antibody probes are useful tools for accurate detection of pathogens, and can also be used in the development of vaccines (Klontz 1985; Adams, Thompson, Morris, Farias & Chen 1995; Austin and Austin, 1999).

Isolation of rickettsia from an affected organism with typical signs of piscirickettsiosis is the best way of proving that the organism is the causative pathogen of the disease, but isolation of rickettsia from diseased tissue can be problematic. The causative agent of rickettsiosis in fish, *P. salmonis*, can be isolated in fish cell lines (Lannan & Fryer 1991), but this is a lengthy technique and is susceptible to contamination. Diagnosis of rickettsial illness in man is most often confirmed by antibody-based tests (La Scola & Raoult 1999). Immunofluorescent antibody techniques (IFAT) and immunohistochemistry (IHC) using rabbit anti- *P. salmonis* polyclonal antibodies (PAbs) have been used successfully to detect rickettsia in fish (Lannan, Ewing & Fryer

1991; Alday Sanz, Rodger, Turnbull, Adams & Richards 1994; Almendras & Fuentealba 1997).

PAbs or monoclonal antibodies (MAbs) with their potential for greater specificity, used either individually or in combination, are useful reagents for the detection and serological characterisation of the pathogens. In spite of the problems associated with cross-reactivity with other related pathogenic species, antibody probes have made a significant impact on rapid diagnosis of fish diseases (Johnson, Wobeser & Rouse 1974; Busch & Lingg 1975; Adams *et al.* 1995; Austin and Austin 1999). MAbs have been used for the detection and characterisation of a variety of fish pathogens such as *Mycobacterium* spp (Adams, Thompson, Ewan, Chen & Richards 1996; Puttinaowarat 1999), *Renibacterium salmoniarum* (Adams *et al.* 1995), *Aeromonas* spp (Adams & Thompson 1990; Cartwright, Chen, Hanna, Gudkovs & Tajima 1994; Neelam, Thompson, Price, Tatner, Adams, Ellis & Stevens 1995), *Vibrio* spp (Espelid, Holm, Hjelmeland & Jørgensen 1988; Chen, Hanna, Altmann, Smith, Moon & Hammond 1992; Miyamoto & Eguchi 1997); *Photobacterium damselae* subsp. *piscicida* (Bakopoulos, Adams & Richards 1997); *Edwardsiella ictaluri* (Plumb & Klesius 1988), *Tetracapsula biyosalmonae* (Adams, Richards & Mateo 1992; Saulnier & De Kinkelin 1996; Morris, Adams & Richards 1997) and yellowtail ascites virus (Nakajima & Sorimachi 1996), for example.

Each species of rickettsia possesses its own unique set of antigens, which result in the production of a set of homologous antibodies within their host. The presence of these antigens can be demonstrated by various serological tests (Buxton & Fraser 1977). Determining the location and the number of specific antigens present on the bacterium

is important for vaccine development. A number of different polyclonal antisera have been used to characterise the antigens of *P. salmonis* by Kuzyk, Thornton and Kay (1996) and Barnes, Landolt, Powel and Winton (1998). In the former study, four major protein bands were found to be located at 65, 60, 54 and 51 kilodalton (kDa) on *P. salmonis,* as well as two carbohydrate antigens located at 16 and 11 kDa, while three major antigens at 56, 36 and 20 kDa were seen in the latter study. Jamet, Aguayo, Miguel, Muller, Arrigada, Becker, Valenzuella and Burzio (2001) observed several bands on *P. salmonis* using four MAbs they produced against the organism with bands recognised ranging from 100, 90, 85, 60 to 42 kDa.

The aim of this study was to produce polyclonal antibody (PAb) and monoclonal antibody (MAb) probes against whole cell preparations of *P. salmonis,* type strain LF-89, and to examine the potential of these probes for developing more specific immunodiagnostic techniques and for the characterisation of different *P. salmonis* antigens. A variety of different antibody-based tests were developed in this study to assess the yields of *P. salmonis* recovered from Percoll density gradient purification, to characterise the antigenic profiles of the bacterium and to detect the pathogen in infected fish tissue.

5.2. MATERIALS AND METHODS

5.2.1. Preparation of bacterial samples

P. salmonis, type stain LF-89, was used for the production of PAbs and MAbs. The bacteria were cultured and purified according to Section 2.1.1.2(c) and 3.2.2 respectively, and were then heat killed by placing them in a water bath at 58°C for 60

minutes (min). Protein concentrations of the rickettsial preparations were determined using a protein determination kit (BioRad) (Section 2.1.3.1).

5.2.2. Production of rabbit anti *P. salmonis* serum (PAb-1)

Polyclonal antiserum against *P. salmonis*, type-strain LF-89, was prepared in a female New Zealand White rabbit. Pre-immune serum was collected prior to the primary immunisation. Heat-killed bacteria diluted in Phosphate buffer saline (PBS: 0.02 Molar (M) Phosphate, 0.15 M NaCl, pH 7.2), (250 microgram (μg) protein) was mixed 1:1 (v/v) with Freund's complete adjuvant (Sigma) and this was administered subcutaneously into two sites of the rabbit (125 μg in 0.3 ml site^{-1}). Four weeks after the first injection, the rabbit was immunised with a further subcutaneous boost of the bacterial suspension (250 μg protein) into two sites (0.4 ml site^{-1}). Blood was collected from the marginal ear vein 11 days (d) after the each injection. This was incubated at 22°C for 2 hours (h), before the serum was separated by centrifugation at 300 x g for 5 min at 4°C. The antibody response of the rabbit was evaluated both by IHC according to Section 2.5 and the antibody titers of the serum was measured using a Microimmunofluorescence test (MIFAT) as described Section 2.6 and by Enzym-linked immunosorbent assay (ELISA), as described in Section 2.4.1. A final injection containing 1 ml of heat-killed bacteria (250 μg) in PBS was delivered intravenously (IV) into the marginal ear veins (0.5 ml ear^{-1}) 12 weeks later. The rabbit was bled out by cardiac puncture one week later.

5.2.3. Production of hybridoma cells

5.2.3.1. Immunisation of mice

Four eight weeks old balb/c female mice were used in the production of the hybridoma cells against heat-killed *P. salmonis* type-strain LF-89. Pre-immune serum was collected from the tail vein prior to the primary immunisation. Bacteria were grown and purified according to Sections 2.1.1.2 (c) and 3.2.2, and prepared for immunisation as described in Section 5.2.1. The mice were immunised and boosted intraperitoneally (IP) at one month intervals using 100 microlitre (μl) of bacterial suspension (80 μg protein), which had been diluted in PBS and emulsified 1:1 (v/v) with Freund's complete adjuvant. The antibody response of the mice was monitored using the sera collected from the tail vein of the animals 11 d later after each injection. The sera were collected as described above in Section 5.2.2 and the antibody responses of the mice examined by IHC (Section 2.5), MIFAT (Section 2.6) and ELISA (Section 2.4.1). The mouse with the highest response was injected IV with 100 μl of bacteria (50 μg protein) in PBS four days before performing the fusion.

5.2.3.2. Myeloma cell culture

Myeloma cell line, SP2/0-Ag14 (Imperial Laboratories), was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 20% (v/v) heat inactivated Foetal calf serum (FCS), 200 millimolar (mM) L-glutamine, 5000 International Units (IU) penicillin/streptomycin and 100 mM sodium pyruvate at 37°C in a 5% CO_2 atmosphere. Cells were sub-cultured with 1 in 10 fresh medium every 2 to 3 d in order to maintain good growth in the log phase for the fusion.

5.2.3.3. Fusion

The fusion of the myeloma cells with spleen cells was performed following the method of Adams *et al.* (1992) with modifications. Four days after the final booster injection, the mouse was killed by exposure to CO_2 and then "bled out" by cardiac puncture. The animal was swabbed with 70% ethanol and the spleen carefully removed. This was placed into a Petri dish containing 20 ml of DMEM (unsupplemented and warmed to 37°C). Excess fat was trimmed before washing the tissue 3 times with unsupplemented-DMEM, warmed to 37°C. Both ends of the organ were removed and cells were "blown out" using a syringe and needle containing 5 ml of warmed unsupplemented-DMEM. The cell suspension was placed in a universal and allowed to stand for 1 minute to allow large tissue particles to settle. The supernatant was collected, the volume of which was adjusted to 50 ml with unsupplemented-DMEM and centrifuged at 150 x g for 10 min at 22°C.

Myeloma cells, in mid-log phase, were removed from the tissue culture flask and centrifuged at 150 x g for 10 min. Both sets of cell pellets (spleen and myeloma) were re-suspended to 50 ml with warmed and unsupplemented DMEM and centrifuged again for 10 min at 150 x g. Cells were counted and mixed in a ratio of one myeloma cell to ten spleen cells. The cell mixture was again centrifuged at 100 x g for 10 min and the supernatant carefully removed. Two ml of polyethylene glycol (PEG 1500, Boehringer Mannheim) 50% (w/v) in DMEM warmed to 37°C) was gently added to the pellet over 1 minute and allowed to stand for 1.5 min, occasionally gently swirling. One ml and 3 ml of warmed unsupplemented-DMEM were then sequentially added over 30 seconds (s) periods. Finally, 16 ml of warmed unsupplemented-DMEM was added over 1 min and the cells were allowed to stand for 5 min. Cells were pelleted at 100 x g for 5 mins

and resuspended in 10 mls of DMEM with additives (0.1 mM hypoxanthine, 0.016 mM thymidine, 2 mM glutamine, 0.5 mM sodium pyruvate, 50 IU ml^{-1} penicillin/streptomycin and 20% heat inactivated-FCS). They were then incubated at 37°C in a CO_2 incubator for 2 to 3 h. The cell suspension was centrifuged at 100 x g for 5 min. The cell pellet was carefully resuspended in 200 ml of above DMEM with the additives and mouse red blood cells (mrbc) (3×10^7 cells ml^{-1}). The mrbc, which served as a feeder layer for the fused cells, were obtained from a non-immunised mouse. The cell suspension was placed into 96 well tissue culture plates (Sero-wel, Bibby Sterilin Ltd., UK) at 180 µl $well^{-1}$. Controls of myeloma and non-fused spleen cells were also included. The plates were incubated at 37°C in a 5% CO_2 incubator and left undisturbed for 48 h. After this time, the fusion was monitored daily and the resulting clones were ready to screen around 13 d.

5.2.3.4. Cloning and Expanding

Thirteen days after the fusion, hybridomas were screened using the dot blot immunobinding assay (DBI) as described in Section 2.3.2, and from which positive wells were selected. The selected wells were then expanded and re-screened by DBI. Positive hybridomas were re-expanded, cloned, re-cloned and re-screened using both DBI and ELISA (detailed in Section 2.4.1) until they were considered to be MAb cell lines.

5.2.3.5. Harvesting of MAb

The hybridoma cells were incubated for around 10 d until they started to die. The cell debris was then removed by centrifugation at 1000 x g for 10 min and supernatants containing high levels of MAbs collected and stored at -20°C until required.

5.2.3.6. Freeze/Thawing of Hybridomas

Healthy hybridoma cells, at mid-log phase, were counted and the number of viable cells determined. Cells were collected by centrifugation at 150 x g for 7 min. The resulting cell pellet was resuspended in a small amount of chilled freezing medium [DMEM with 20% FCS (v/v) and 10% Dimethyl sulfoxide (DMSO) (v/v)] by gently flicking the side of the test tube. The cell concentration was adjusted to 2-3 x 10^6 cells ml^{-1} with the freezing medium. The cells were then placed in a cryopreservation vial and immediately transferred to –70°C freezer in a cell freezer container. The cryovials were transferred to liquid nitrogen the following day.

Frozen vials of cells were removed from liquid nitrogen and immediately placed into a 37°C water bath to thaw the cells. After nearly completely thawing, the cells were pipetted onto 9 ml of DMEM layered upon 1 ml of FCS. The suspension was centrifuged at 150 x g for 7 min, and the pellet gently resuspended in 5 ml of DMEM and additives with 20% FCS and place in a 25 cm^2 tissue culture flask. The flask was incubated at 37°C in a CO_2 incubator and the cells were checked daily until antibody production was determined.

5.2.3.7. Isotyping of MAbs

Isotyping of MAbs was performed using Sigma mouse MAb isotyping reagents (ELISA/Ouchterlony Double Diffusion), following the manufacturers instructions.

5.2.4. Classification of PAb-1 and MAbs

5.2.4.1. Analysis of PAbs and MAbs by ELISA

(a) Sensitivity of PAbs using an indirect ELISA

The sensitivity of the rabbit PAb against *P. salmonis* type species LF-89 (PAb-1) was tested by the indirect ELISA described Section 2.4.1. An alternative anti-*P. salmonis* rabbit PAb, kindly provided by Dr John Fryer (Department of Microbiology, Oregon State University, Oregon, USA) (PAb-2) was also analysed as a comparison to the rabbit serum prepared here. *P. salmonis* type species LF-89, purified using a 40% Percoll gradient (1 $\mu g\ ml^{-1}$) was applied as antigen to duplicate wells of the ELISA plate to test the sensitivity of the PAbs (both PAb-1 and PAb-2) in the ELISA. Ten-fold dilutions of the PAbs were prepared in PBS starting at 10^{-4}. PBS was used in place of the antibodies as a negative control. Conjugated anti-rabbit IgG Horseradish peroxidase (HRP) was used as the secondary antibody, diluted 1/1000 in PBS.

(b) Specificity of PAbs in the indirect ELISA

Preparations of heat-killed whole cell suspensions of the different *P. salmonis* isolates (Table 2.1) and non-rickettsial bacteria (Table 2.2) were applied in duplicate to wells of the ELISA plate at (2 $\mu g\ ml^{-1}$) to establish the specificity of PAb-1 and PAb-2. Both PAb were diluted 1/10000 in PBS. PBS was used as negative control while chinook salmon, *Oncorhynchus tshawytscha* embryo cell (CHSE-214) monolayer was used as further control. The assay was performed according to Section 2.4.1.

(c) Optimisation of a Sandwich ELISA for *P. salmonis* using MAbs

A sandwich ELISA (SELISA) was developed in this study to detect *P. salmonis* using the method described by Hsu, Bowser & Schachte (1991) (Section 2.4.2). Two

different SELISA were performed to standardise the assay. In the first assay, concentrations of *P. salmonis* were established, while specificity of the MAbs was determined in the second assay.

(i) Sensitivity of the SELISA was tested, using whole cell or sonicated (at 25 micrometre (μm) amplitude for 6 s) LF-89 type strain purified using a 40% Percoll density gradient. Ten fold dilutions of the antigen with a starting concentration of 5 μg ml^{-1} were prepared in PBS. The dilutions were added to the ELISA plates coated with the acid-treated rabbit PAb-1 (10 μg /ml). MAbs were applied as the primary antibody and HRP anti-mouse IgG, diluted 1 in 1000 in PBS, was used as the second antibody. PBS was used in place of the antigen as a negative control.

(ii) In the second SELISA, the specificity of the MAbs against *P. salmonis* and their cross-reactivity with other non-*P. salmonis* bacterial fish pathogens (Table 2.2) were tested. Whole cell preparations of the different *P. salmonis* isolates shown in Table 2.1 (1 μg ml^{-1}) were applied to four replicate wells and the assay was performed according to Section 2.4.2.3. PBS was used in place of antigen as a negative control.

(d) Comparison of mouse sera against *P. salmonis* type strain LF-89 and a polyclonal against *Orientia tsutsagamushi* by ELISA

An indirect ELISA was used here to examine the cross-reactivity of the *P. salmonis* type strain LF-89 with the mouse serum raised against *O. tsutsagamushi,* (Gilliam, Karp and Kato strains) from Scrub typhus group (STG) of rickettsiaea, kindly gifted by Prof. Akira Tamura, Niigata College of Pharmacy, Japan. Mice PAb sera against the *P. salmonis* type strain were also tested in the same assay as positive control. The mice sera were produced during MAb production as described in Section 5.2.3., using the remaining mice not used in the fusion. Two of three mice (m-2 and m-3) were

immunised IP and boosted IV at three weeks intervals with 100 μl of the purified bacterial suspension in PBS (50 μg protein) heat killed at 58°C for 60 mins. The mice were bled out one week after the final booster injection. One of the mice (m-4) was immunised once IV with 100 μl of the purified bacterial suspension in PBS (50 μg protein) heat killed at 58°C for 60 min and the mouse was killed by exposure to CO_2 and then bled out by cardiac puncture one week later. The serum was collected by centrifugation at 300 x g for 5 min at 4°C after incubating the blood samples at 22°C for 2 h. Serum was stored at -70°C. All sera were used as in two fold dilutions in PBS for this test.

The positive-negative threshold of the reaction was determined as three times the value obtained for the mean negative controls for all ELISA systems described here [a, b, c (i, and ii), d and e]. The reactivities were read with an ELISA reader (Dynatech) at 450 nanometre (nm) Optical density (OD).

5.2.4.2. Analysis of PAb-1 and MAbs by Western blot (WB) analysis

Whole cell purified *P. salmonis* type species LF-89 (100 μg ml^{-1}) was used in the analysis of the MAbs and the PAb-1 prepared in this Chapter. The *P. salmonis* isolates (Table 2.1) (50 μg ml^{-1}) were used to examine the cross reaction of the antibodies. The bacterial samples, which had been subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% precast mini-polyacrylamide gels (Section 2.2.1), were transferred to a nitrocellulose membrane and WB analysis was performed with the different MAbs and PAb-1 as described in Sections 2.3.1.

WB was also used to analyse Proteinase-K digested *P. salmonis* isolates (Table 2.1) incubated with MAb 6A1F4C5 and Proteinase-K digested *P. salmonis* type strain LF-89 incubating with PAb-1. A sample of each *P. salmonis* isolate was digested with 1 μl of stock Proteinase-K solution (1mg ml^{-1} per 10 μl of the SDS-PAGE sample) by incubating for 60 min at 60°C. The digestion was stopped by boiling the samples for 10 min and these were used in SDS-PAGE and then transferred to nitrocellulose paper for WB analysis.

A multiscreen apparatus (BioRad) was used for the analysis of MAbs against *P. salmonis* type species LF-89. Avidin-biotin-peroxidase (ABP) complex was used in the analysis of MAbs against type strain LF-89 and the cross-reaction of MAbs against all *P. salmonis* isolates, while anti-rabbit IgG-HRP (Diagnostics Scotland) was used as the secondary antibody when the cross reaction of PAb was tested against the *P. salmonis* isolates.

WB analysis was also used here to examine the cross-reactivity of the *P. salmonis* isolate R-29 with the mouse serum raised against *O. tsutsagamushi,* (Gilliam, Karp and Kato strains) from STG of rickettsiae, kindly gifted by Prof. Akira Tamura, Niigata College of Pharmacy, Japan. WB analysis was as described Section 2.3.1 using a multiscreen apparatus (BioRad). Mice PAb sera (m-2, m-3 and m-4) (See Section 5.2.4.1 e) were also tested in the same assay.

5.2.4.3. Analysis of MAbs and PAb-1 by a DBI

Analysis of MAbs and PAb-1 produced against *P. salmonis* type strain LF-89 was also performed using a DBA following the protocol described in Section 2.3.2. *P. salmonis*

isolates (Table 2.1) (0.5 μg in 10 μl) was placed in duplicate wells of the dot blot apparatus which already contained 100 μl of Tris buffered saline (TBS). CHSE cell monolayer was used as a negative control for the assay. MAbs and PAb-1 (diluted 1 in 100 in TBS) were applied as the primary antibody solution and anti-mouse IgG-HRP (Diagnostics Scotland) or anti-rabbit IgG-HRP (Diagnostics Scotland) (diluted 1 in 100 in TBS) were used as the secondary antibody, respectively.

5.2.4.4. Analysis of MAbs and PAb-1 by a Line blot immunoassay (LBI)

A LBI was performed to analyse the MAbs and PAb using *P. salmonis* isolates (Table 2.1) according to Section 2.3.3. Antigen (1 μg in 10 μl) was mixed with 10 μl of sample buffer and the mixture was applied onto a nitrocellulose membrane with a quill. *P. salmonis* type strain LF-89, and CHSE cells used as a negative control, were applied to the membrane which was then cut into strips (0.5 cm) or placed in a multiscreen apparatus. The nitrocellulose membrane was incubated first with the MAbs and PAb-1, and then with either anti-rabbit IgG-HRP or anti-mouse IgG-HRP as secondary antibody diluted in 1/100 in TBS, as appropriate.

5.2.4.5. IHC with PAb-1 and MAbs

The IHC procedure was performed according to the method described in the Section 2.5. Paraffin-embedded tissue sections prepared from Atlantic salmon *Salmo salar* affected with Rickettsia-like organisms (RLO) obtained in Ireland (provided from archival material held at the Institute of Aquaculture, University of Stirling), were used for the assay in which MAbs and PAb-1, diluted 1/1000 in PBS, were applied as primary antibodies. One slide was incubated with PBS as a negative control. At the end of the procedure the slides were stained and counter-stained with True blue and Contrast red

(Kirgegaard Perry Laboratories, UK) respectively, and staining was then assessed using light microscopy (LM).

5.2.4.6. IFAT with the PAbs and MAbs

Whole cell preparations of the *P. salmonis* isolates were used to test the cross-reactivity of the MAbs and PAb-1 developed in this Chapter, by MIFAT according to the method outlined in Section 2.6. Semi-purified bacterial smears were placed on the wells of micro-slides (Dynex) and MAbs or PAb-1 (diluted 1/1000 in PBS) were used as primary antibodies. One slide was incubated with PBS as a negative control.

Paraffin-embedded tissue sections (obtained from Atlantic salmon, in Ireland affected by RLO) were prepared from archival material held at the Institute of Aquaculture, University of Stirling, were also used in this Chapter. IFAT was performed according to method described in the Section 2.6. The tissue slides were incubated with MAb supernatant or PAbs (PAb-1 and PAb-2) diluted 1/1000 in PBS, or PBS as negative controls. Slides were then applied by either goat anti-rabbit IgG conjugated to Fluorescein isothiocyanate (FITC) (Sigma) diluted 1/80 in PBS, or 1:50 dilution of FITC labelled donkey anti-mouse IgG (Diagnostic Scotland) in PBS as the secondary antibody as appropriate.

Rabbit PAb developed in this study was sent to Department of Zoology, University of Oxford to test its specificity against Wolbachia, which belongs to family *Rickettsiaceae* and Tribe *Wolbachieae*. This examination was kindly performed by Dr. Stephan Hadfield using IFAT. Briefly, sections of the *Drosophila melanogaster* embryos, infected with Wolbachia, were incubated with the PAb-1 (diluted 1/1000 in PBS) and

then stained with FITC labeled anti-rabbit immunoglobulin as secondary antibody (green) and counterstained with propidium iodide (red nucleic acid stain).

Some acetone fixed microscope slides (12 wells microscope slides) coated with PTFE bearing ovine neutrophils infected with *Ehrlichia phagocytophila,* were kindly gifted by Dr. Zerai Woldehiwet, University of Liverpool. The slides had been fixed with acetone prior to posting. The slides were then examined for the cross-reactivity of the MAbs and PAbs (PAb-1 and PAb-2) with this organism. *E. phagocytophila* belongs to *Ehrlichieae*, which is another member of family *Rickettsiaceae*. *E. phagocytophila* were tested by IFAT as described in Section 2.6. MAbs 6A1F4C5, 6A1F4G3, 8G7 and 6A1G6, and diluted PAbs (1/1000 in PBS) were used as primary antibody in the analysis and anti mouse IgG- FITC (Diagnostics Scotland) or anti-rabbit IgG- FITC (Sigma) diluted 1/40 in PBS were used as secondary antibody. Some wells were incubated with PBS instead of antibody as a negative control.

The results were observed under an Olympus IMT-2 microscope with a reflected fluorescent attachment, and exciter and barrier filters for FITC.

5.2.4.7. Analysis of MAbs by Immuno-gold staining (IGS)

Purified *P. salmonis*, type strain LF-89 and isolate R-29 were used in immuno-gold staining as described in Section 2.7. LR white resin (London Resin Co., Reading, England) was used to prepare the tissue sections in capsules and the samples were then cut into ultra-thin sections and mounted on nickel grids. To block non-specific binding sites, the grids were floated side down on drops of wash buffer (Wb: 0.2 M TBS, 1%Tween, 1% BSA) containing 10% FCS which had been spotted onto a piece of parafilm, placed in a

moist container, and incubated overnight at 4°C. The grids were then transferred to hybridoma supernatants and incubated overnight at 22°C. MAbs 6A1F4C5, 8G7, 7E2 and 6A1G6 were used as primary antibody and anti-mouse IgG gold conjugate, 5 nm (Sigma) was used as the secondary antibody. *P. salmonis* isolate SLGO-95 was also stained with immuno-gold using only MAb 6A1F4C5. The 5 nm immuno-gold label was enhanced using silver enhancing solution (British BioCell Int. Cardiff, UK) for 2 min and the sections were counter-stained using uranyl acetate and lead citrate before examination under a Phillips 301 electron microscope (EM) at 80 kilovolt (kV).

5.3. RESULTS

5.3.1. Characterisation of rabbit anti-*P. salmonis* PAbs

5.3.1.1 Determination of the sensitivity and specificity of rabbit anti-*P. salmonis* PAbs using an indirect ELISA

The specificity and sensitivity of the PAb developed against the *P. salmonis* type strain LF-89 in this study (PAb-1) were established using an indirect ELISA. The sensitivity threshold of PAb-1 against purified *P. salmonis* type species LF-89, the isolate against which the antisera was made, was 1/100 000 (Figure 5.1). The same sensitivity was found with PAb-2 in the ELISA.

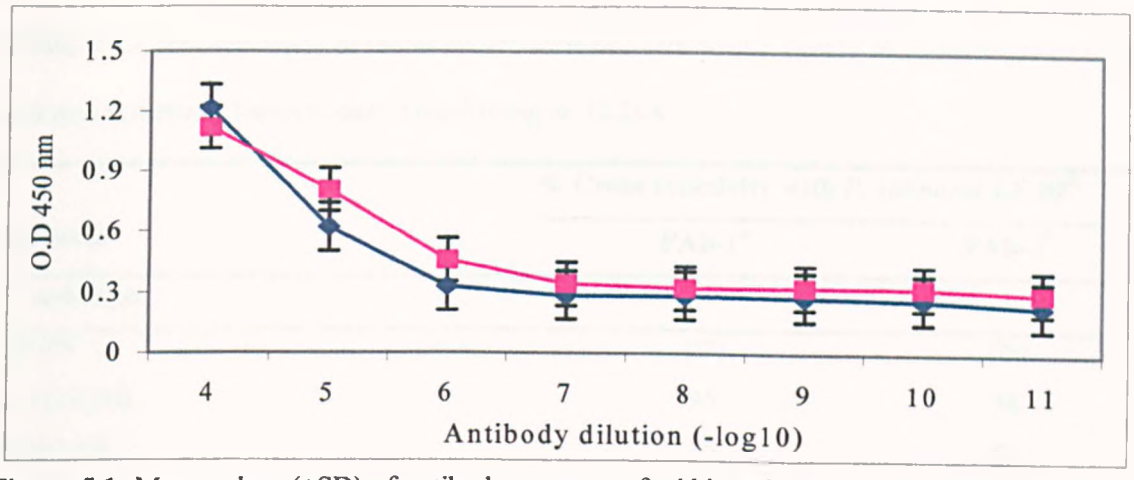


Figure 5.1. Mean values (±SD) of antibody response of rabbit anti-*P. salmonis* serum developed against type strain LF-89: PAb-1 (—♦—) developed in this study and PAb-2 (—■—) kindly gifted by Prof. J. Fryer, Oregon State University, Corvallis, OR, USA.

PAb-1 reacted with all of the *P. salmonis* isolates shown in Table 2.1, which were used in order to establish the level of reactivity of the rabbit serum PAb-1 (Table 5.1). It also appeared to cross-react with non-rickettsial species *Yersinia ruckeri* (31% cross-reactivity), *Aeromonas hydrophila* (34% cross-reactivity), and *Escherichia coli* (48% cross-reactivity). These values are similar to the level of reaction obtained with one of the *P. salmonis* isolates, R-62, which gave an absorbance of 34% of that obtained with the type strain. The other rabbit sera raised against *P. salmonis*, PAb-2 gifted by Dr. John Fryer, reacted with most of the *P. salmonis* isolates examined, however, it gave a low reaction with the Irish isolate AVG5/268 (38%) and the Chilean isolate VQ013 (46%). The reaction obtained with PAb-1 against isolates LF-89, AVG5/268, R980769 and VQ013 was higher than the reaction obtained with PAb-2, however, isolates SLGO-95, R-29, R61 and R-62 gave a higher absorbance with PAb-2 than that obtained with PAb-1. Both PAbs showed similar absorbance levels at 450 nm with isolates SRS-4 and SRS-UACH. Although PAb-2 gave a strong reaction with CHSE cells (126%) unlike PAb-1 (23%), which had a low level of reactivity against these cells, PAb-2 did not cross-reacted with any of the non-*P. salmonis* bacteria examined apart from *Renibacterium salmoninarum*.

Table 5.1. Cross-reactivity of rabbit anti-*P. salmonis* sera with a variety of *P. salmonis* isolates and non-rickettsial bacteria, determined using an ELISA

Bacteria	% Cross-reactivity with *P. salmonis* LF-89[a]	
	PAb-1[b]	PAb-2[b]
P. salmonis		
LF-89	100	100
AVG5/268	95	38
R980769	85	63
VQ013	86	46
SRS-4	73	94
SRS-UACH	98	108
SLGO-95	53	114
R-29	56	87
R-61	55	90
R-62	34	99
Non-*P. salmonis* bacteria		
Aeromonas hydrophila	34	14
Bacillus subtilis	12	24
Edwardsiella tarda	26	13
Escherichia coli	48	17
Nocardia asteroides	9	11
Pseudomonas aeruginosa	30	18
Photobacterium damselae subsp. *piscicida*	18	20
Renibacterium salmoninarum	20	31
Streptococcus faecalis	7	7
Vibrio ordalii	13	16
Yersinia ruckeri	31	13
CHSE-214 cells	23	126
Negative control (PBS)*	7	9

(*) The negative control (PBS) absorbance at 450 nm was 0.105 (the mean of 18 wells) for both analysis.

[a] Each value represents the mean percentage cross-reaction of two wells when compared to the absorbance of the type strain LF-89 at 450 nm. Reactivity was calculated as follows for the mean of duplicate wells:

$$\frac{OD_{450}\text{ of test sample - }OD_{450}\text{ of background (x3)}}{\text{Positive control (}OD_{450}\text{ of type strain LF-89 with - }OD_{450}\text{of background (x3)}} \times 100$$

[b] The values are the absorbance at 450 nm for the type strain LF-89 were 1.448 with PAb-1 and 1.115 with PAb-2

5.3.1.2. Characterisation of the rabbit anti-*P. salmonis* sera using a variety of immunoblotting techniques (Western blot, Dot blot and Line blot)

The various *P. salmonis* isolates screened in this study all reacted with PAb-1 in WB analysis. The number and intensity of the bands differed between the different *P. salmonis* isolates (Figure 5.2), and a number of different bands with molecular weights ranging from 160 to 10 kDa were observed (Table 5.2). All *P. salmonis* isolates had a major band which was intensively stained with the rabbit serum at around 60 kDa (the band spread from between 50 and 75 kDa), while bands were also seen at 83, 51, 40, 36 and 32 kDa, but the staining of these was less intense. The isolates also appeared to have a band at 95 kDa which very slightly stained with isolates SLGO-95 and R-29, and a band was strongly stained at around 72 kDa with isolates R-61 and R-62 which was less intense with the remaining isolates, particularly isolates SLGO-95 and R-29. The type strain LF-89 shared a 115 kDa antigen in common with the Irish isolate AVG5/268, although this band was also faintly stained on the Chilean isolates, SLGO-95 and R-29. Greek isolate R980769 contained a band at around 64 kDa, while this band was present but appeared much paler on the type strain LF-89. The isolates also contained some lower molecular material between 30-10 kDa, which was weakly stained with PAb-1. WB analysis of Proteinase-K digested *P. salmonis* type strain LF-89 with PAb-1 resulted in several bands below 30 kDa.

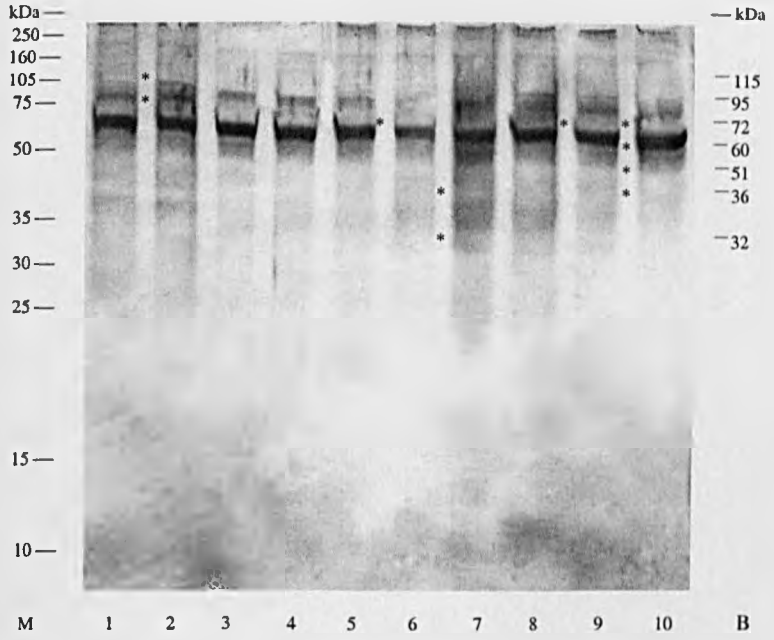


Figure 5.2. WB analysis with anti-*P. salmonis* rabbit sera (PAb-1) against a variety of *P. salmonis* isolates. Lanes: (1) R-62; (2) R-61; (3) R-29; (4) SLGO-95; (5) SRS-UACH; (6) SRS-4; (7) VQ013; (8) R980769; (9) AVG5/268; (10) LF-89; M:Marker (Amersham); (B) Molecular weight of some of the bands reacting with PAbs represented by (*).

A strong positive reaction was obtained with all isolates tested in the Dot blot assay (Figure 5.3), and also strong positive reaction was obtained as a violet line in the Line blot assay (Figure 5.4 a, b). The intensity of the reactions in both analyses is indicated in Table 5.2.

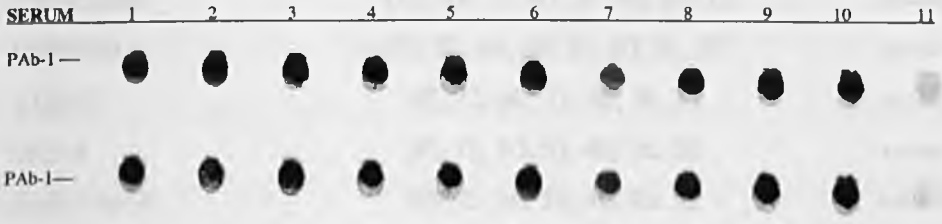


Figure 5.3. DBI with PAb-1 screened against a variety of *P. salmonis* isolates antigen. Lanes: (1) LF-89; (2) AVG5/268; (3) R980769; (4) VQ013; (5) SRS-4; (6) SRS-UACH; (7) SLGO-95; (8)R-29; (9)R-61; (10)R-62; (11) CHSE-214 as control

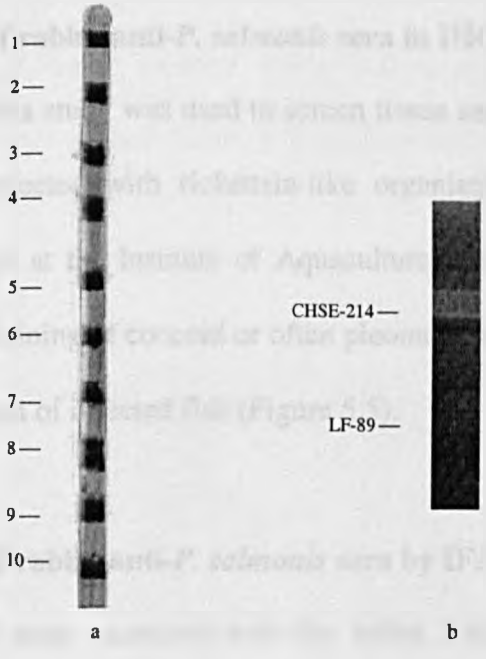


Figure 5.4. (a). LBI with PAb-1 screened against a variety of *P. salmonis* isolates. Lanes: 1) LF-89; (2) AVG5/268; (3) R980769; (4) VQ013; (5) SRS-4; (6) SRS-UACH; (7) SLGO-95; (8) R-29; (9) R-61; (10) R-62; (b) LBI for the analysing of PAb-1 using with *P. salmonis* type strain LF-89 as antigen and CHSE-214 cells as control

Table 5.2. Comparison of the reactivity of rabbit anti-*P. salmonis* sera (PAb-1) with a variety of *P. salmonis* isolates in Western blot, Dot blot, and Line blot analysis

P. salmonis isolates	Major bands recognised in Western blot (kDa)	Reactivity	
		Dot blot	Line blot
LF-89	115, 95, 72, 64, 60, 51, 40, 36, 32	++++	++++
AVG5/268	115, 95, 72, 60, 51, 40, 36, 32	++++	++++
R980769	95, 72, 64, 60, 51, 40, 36, 32	++++	++++
VQ013	95, 72, 60, 51, 40, 36, 32	++++	++++
SRS-4	95, 72, 60, 51, 40, 36, 32	++++	++++
SRS-UACH	95, 72, 60, 51, 40, 36, 32	++++	++++
SLGO-95	115, 95, 72, 60, 51, 40, 36, 32	++++	++++
R-29	115, 95, 72, 60, 51, 40, 36, 32	++++	++++
R-61	95, 72, 60, 51, 40, 36, 32	++++	++++
R-62	95, 72, 60, 51, 40, 36, 32	++++	++++

(++++) Intense Reaction

5.3.1.3. Reactivity of rabbit anti-*P. salmonis* sera in IHC

PAb-1 produced in this study was used to screen tissue sections obtained from Atlantic salmon, naturally infected with rickettsia-like organism in Ireland (provided from archival material held at the Institute of Aquaculture, University of Stirling) by IHC. Light blue positive staining of coccoid or often pleomorphic organisms within host cells was observed in spleen of infected fish (Figure 5.5).

5.3.1.4. Reactivity of rabbit anti-*P. salmonis* sera by IFAT

P. salmonis isolates were examined with the rabbit PAb developed in this Chapter (PAb-1) by MIFAT using micro-slides. Small, pleomorphic and ring-shaped fluorescent organisms were detected in all of the isolates. PAb-1 was able to detect RLO in infected fish tissue using IFAT as shown in Figure 5.6 in which bright

fluorescent pleomorphic RLOs were seen spread throughout the spleen of naturally infected Atlantic salmon.

The cross-reactivity of *Wolbachia pipientis* present in *D. melanogaster* embryos was also examined using the PAb-1. Staining of small material within the embryo was evident (Figure 5.7). However, the green spots observed did not correspond to Wolbachia bacterial genome shown in red, because the green spots were also present in uninfected embryos. It would appear from this analysis and the conditions used here, that, PAb-1 does not cross react with *W. pipientis*.

Micro-slides bearing *E. phagocytophila* infected ovine neutrophils were also screened by MIFAT with PAb-1 and PAb-2 to establish if there is any cross-reactivity between *P. salmonis* and *E. phagocytophila*, another member of the family *Rickettsiacea* (Figure 5.8 a, b). Although, some positive white dots associated with neutrophils were observed on the slides, a similar reaction was obtained with control slides (Figure 5.8 c).

(a)

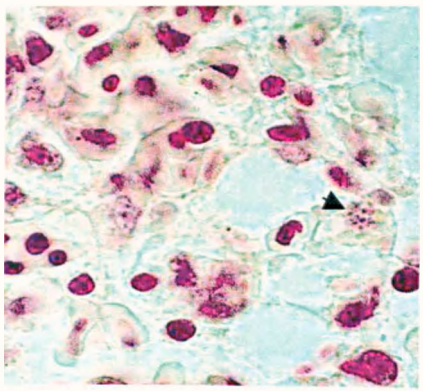


Figure 5.5. IHC analysis of tissue sections from Atlantic salmon from Ireland naturally infected with *P. salmonis,* using with PAb against *P. salmonis* type species LF-89 (see arrow) (magnification x 1400)

(a)

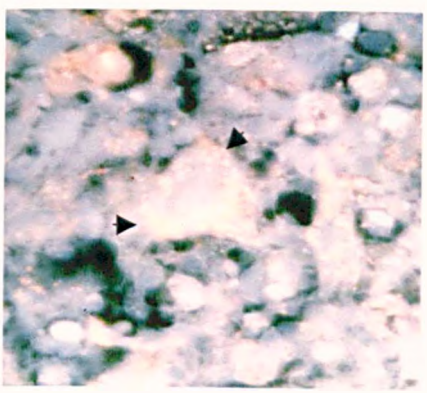


Figure 5.6. Detection of rickettsia-like organisms in tissue sections from naturally infected Atlantic salmon from Ireland by IFAT using with PAb against *P. salmonis* type species LF-89 (see arrows) (magnification x 1000)

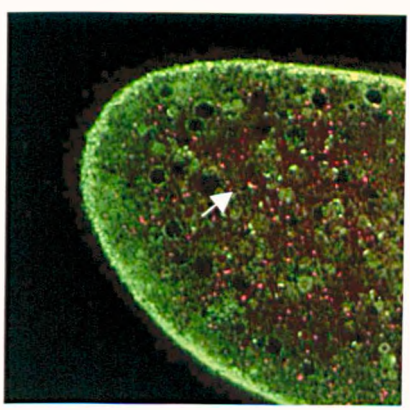


Figure 5.7. *D. melanogaster* embryos infected with Wolbachia analysied with PAb-1 against *P. salmonis* by IFAT (see arrow).

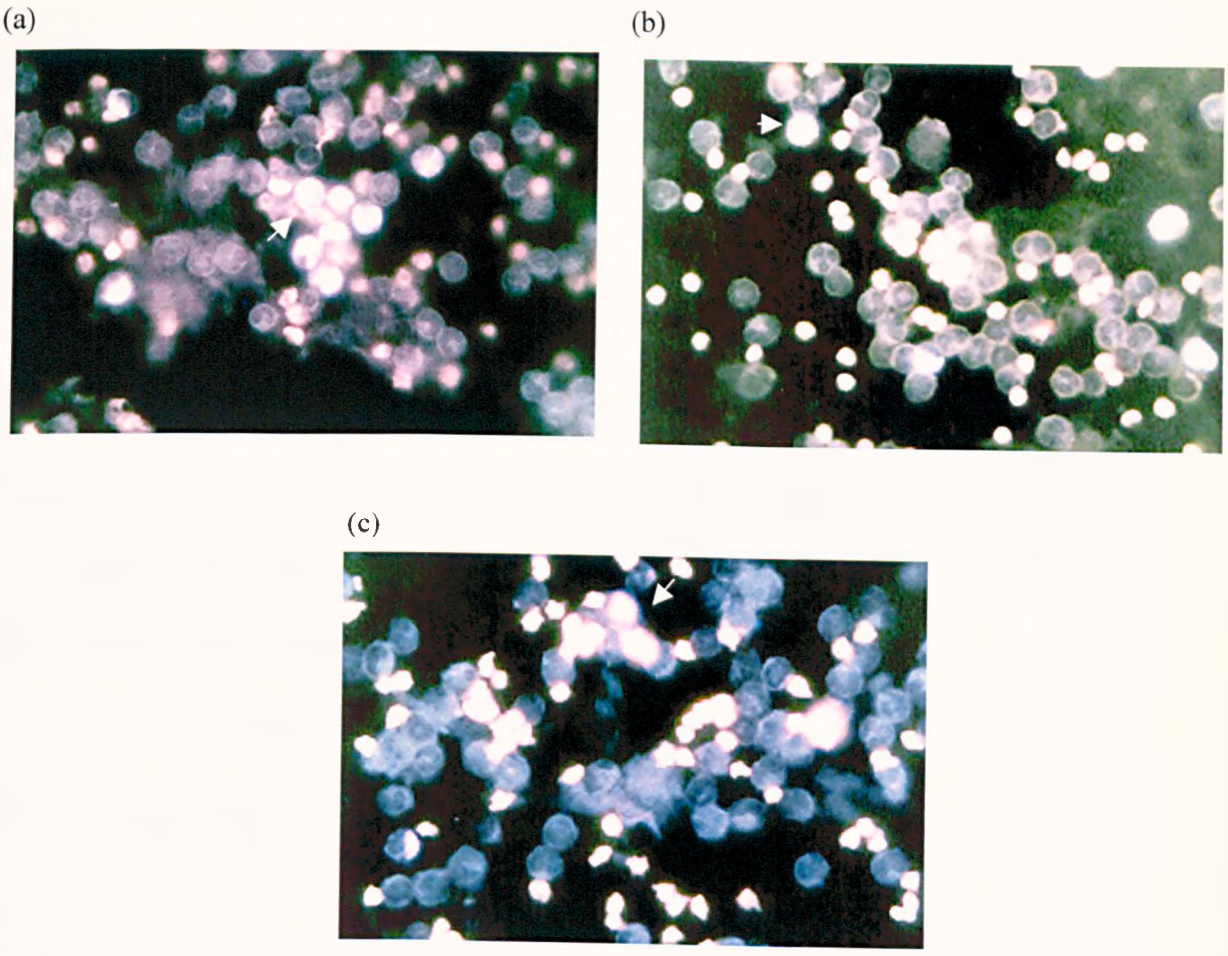


Figure 5.8. Analysis of cross-reactivity of *E. phagocytophila* with PAb against *P. salmonis* type strain LF-89 by MIFAT. (a) PAb-1 and (b) PAb-2 (see arrows indicating some positive white dots) (magnification x 1000); (c) Negative control (see arrow also indicating similar positive reaction) (magnification x 1000).

5.3.2. Characterisation of the MAbs

5.3.2.1 Production of the MAbs

Six MAbs were produced against *P. salmonis* type strain LF-89 in this study and are referred to as 6A1F4C5, 6A1F4G3, 8G7, 7E2, 6A1G6 and 6A1G3. All MAbs were of an IgM isotype.

5.3.2.2. Characterisation of the MAbs by ELISA

(a) Sensitivity of the Sandwich ELISA

A sandwich ELISA was used to detect purified *P. salmonis* type strain LF-89. The bacterium was detected at concentrations as low as 0.1-0.3 $\mu g\ ml^{-1}$ with both whole cell and sonicated bacteria.

(b) Specificity of the MAbs determined by a Sandwich ELISA

The cross-reaction of the MAbs with a variety of different *P. salmonis* isolates and with a number of non-rickettsial bacteria, determined using a sandwich ELISA, are shown in Table 5.3. The MAbs reacted strongly with all of the *P. salmonis* isolates tested apart from isolates SLGO-95, R-29 and R-62. Isolates LF-89, AVG5/268 and SRS-UACH isolates gave a higher response in the assay compare to the others isolates screened with the MAbs. The non-*P. salmonis* bacteria screened with the MAbs gave the same level of reaction as that of the negative control (8-16% cross-reactivity), calculated for each bacteria tested in the same plate.

Table 5.3. The response of the MAbs against a variety of *P. salmonis* isolates and non-rickettsial bacteria determined using a sandwich ELISA

Bacteria	(%) Cross-reactivity of MAbs against *P. salmonis* LF-89[a,b]					
	6A1F4C5	6A1F4G3	8G7	7E2	6A1G6	6A1G3
P. salmonis						
LF-89	100	100	100	100	100	100
AVG5/268	130	143	128	114	111	104
R980769	101	105	88	90	82	82
VQ013	91	93	85	82	67	74
SRS-4	94	100	90	87	83	84
SRS-UACH	121	114	88	76	65	72
SLGO-95	26	26	23	21	20	21
R-29	17	17	13	13	13	14
R-61	100	108	81	87	73	80
R-62	20	17	15	12	12	14
Non-*P. salmonis*						
Aeromonas hydrophila	13	13	11	10	9	9
Bacillus subtilis	16	16	14	13	13	13
Edwardsiella tarda	12	12	10	9	9	9
Escherichia coli	10	11	9	6	8	8
Listonella anguillarun	12	12	11	8	10	9
Nocardia asteroides	12	13	11	9	10	9
Pseudomonas aeruginosa	16	15	14	13	13	12
Photobacterium damselae subsp *piscicida*	11	12	11	11	10	10
Renibacterium salmoninarum	18	18	14	11	12	10
Serratia sp.	11	11	11	10	9	9
Streptococcus faecalis	11	11	10	9	9	8
Vibrio ordalii	11	11	10	5	9	8
Yersinia ruckeri	12	12	11	10	9	9
*Absorbance (450 nm) of *P. salmonis* type strain LF-89	0.952	0.922	1.037	1.153	1.183	1.224

[a]Each value (mean of 4 wells) represents the percentage absorbance compared to type strain LF-89. Reactivity was calculated as follows for the mean of the wells:

$$\frac{OD_{450} \text{ of test sample} - OD_{450} \text{ of background (x3)}}{\text{Positive control } (OD_{450} \text{ of reference isolate}) - OD_{450} \text{ of background (x3)}} \times 100$$

[b]Negative control (mean of 4 wells) had absorbance value of 0.118 at 450 nm.
*Average value (for 4 wells) used as 100% to calculate the cross-reactivity of other bacteria.

(c) Comparison of various sera against *P. salmonis* type strain LF-89 and a polyclonal against *O. tsutsagamushi* using ELISA

The indirect ELISA system was used to test cross-reactivity of sera against *O. tsutsugamushi* and also other rabbit and mice polyclonal sera and MAb supernatant produced against *P. salmonis* type strain LF-89. The reaction of the rabbit polyclonal serum gave a highest response against antigen LF-89 in the ELISA while the mouse polyclonal sera produced against same isolate and against *O. tsutsugamushi* had a titre of 1/256 (Figure 5.9).

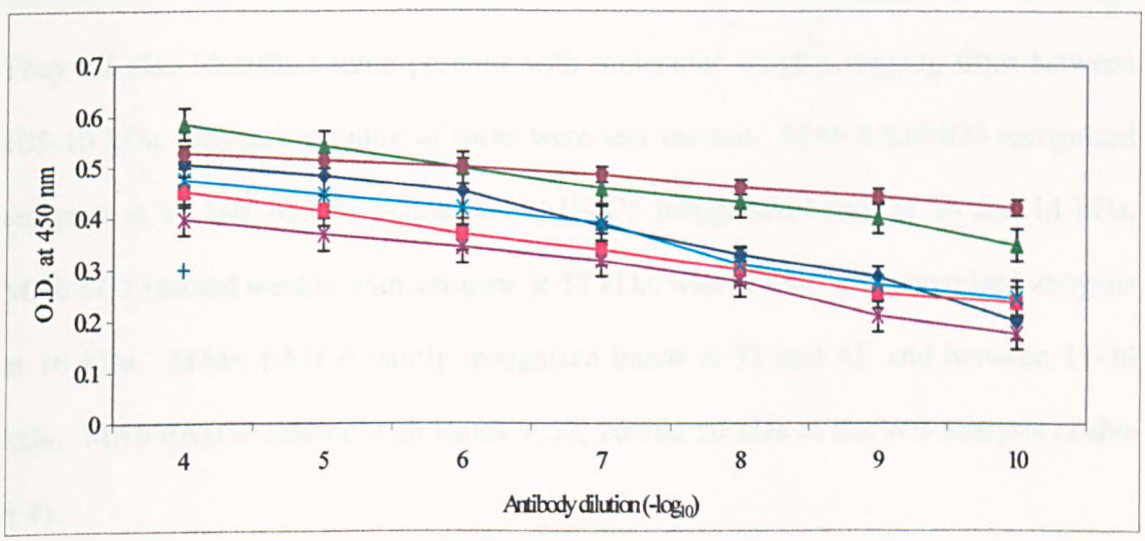


Figure 5.9. Comparison of various sera by ELISA.

Mean values (±SD) of polyclonal sera produced against *O. tsutsugamushi* in mice (—♦—); and mice sera against *P. salmonis* LF-89: m-2 (—■—); m-3 (—▲—); m-4 (—x—); monoclonal antibody supernatant 6A1F4C5 (—*—); PAb-1 produced in rabbit against *P. salmonis* type strain LF-89 (—•—) and Negative control (PBS) (3 x OD_{450} of background) (+)

5.3.2.3. Characterisation of the MAbs using a variety of Immuno blotting techniques

(a) WB

WB analysis of whole cell preparations of *P. salmonis*, type strain LF-89, were performed using the MAbs prepared in the study (6A1F4C5, 6A1F4G3, 8G7, 7E2, 6A1G6 and 6A1G3) and biotin amplification (Figure 5.10). The MAbs gave a slightly different banding profile to each other, although all MAbs gave a strong reaction with antigens in the region between 105 and 75 kDa, mainly around 95 kDa, and they also recognised a fainter band in the region between 160-105 kDa, estimated to be 115 kDa. They all also identified some proteins with molecular weights ranging from between 105-10 kDa, although staining of these were less intense. MAb 6A1F4G3 recognised antigens at 18 and 16 kDa, while the 6A1F4C5 recognised bands at 20 and 14 kDa. MAb 8G7 reacted weakly with antigens at 32 kDa, while MAb 7E2 recognised antigens at 16 kDa. MAbs 6A1G6 faintly recognised bands at 72 and 32, and between 11-30 kDa. MAb 6A1G3 reacted with bands at 32, 26 and 20 kDa in the WB analysis (Table 5.4).

Table 5.4. The molecular weight (kDa) of major antigens recognised by the anti-*P. salmonis* MAbs developed against type strain LF-89 in WB assay

MAbs	Bands (kDa)
6A1F4G3	95, 18, 16
6A1F4C5	95, 20, 14
8G7	95, 32
7E2	95, 16
6A1G6	95, doublet at 72, 32
6A1G3	95, 32, 26, 20

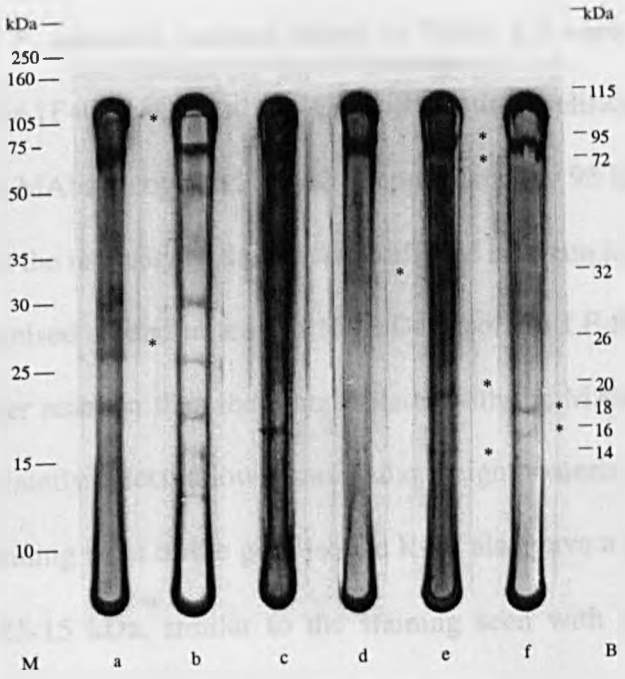


Figure 5.10. WB analysis of MAbs against *P. salmonis* type strain LF-89. Lines: (a) MAb 6A1G3; (b) MAb 6A1G6; (c) MAb 7E2; (d) MAb 8G7; (e) MAb 6A1F4C5; (f) MAb 6A1F4G3 ; M:Marker (Amersham); (B) Molecular weight of bands reacting with MAbs represented by the asterisk.

In order to establish if the MAbs recognise different bands on the different *P. salmonis* isolates, a variety of *P. salmonis* isolates shown in Table 2.1 were screened in WB analysis with MAbs 6A1F4C5, 8G7 and 6A1G6 with biotin amplification (Figure 5.11 a, b and c). All of the MAbs recognised a band at approximately 95 kDa on most of the isolates examined, but the intensity of the reaction differed between isolates (Table 5.5). The MAbs also recognised a band at around 160 kDa. Isolates LF-89, AVG5/268 and VQ013 gave a stronger reaction than the other isolates with the MAbs in WB analysis. MAb 6A1F4C5 also faintly detected lower molecular weight material at between 28-72 kDa located at the running front of the gel. Isolate R-29 also gave a faint reaction with this MAb between 25-15 kDa, similar to the staining seen with type strain LF-89. MAbs 6A1F4C5 and 6A1G6 recognised a pale band at approximately 115 kDa with all of the isolates. This band was also evident with isolates LF-89, AVG5/268 and VQ013 with MAb 8G7.

Table 5.5. Intensity of the reaction of the MAbs with the 95 kDa band on a variety of different *P. salmonis* isolates in WB analysis

	Reactivity of MAbs		
Isolates	**6A1F4C5**	**8G7**	**6A1G6**
LF-89	++	++	+
AVG5/268	+++	++	+
R980769	++	+	+
VQ013	+++	++	+
SRS-4	++	+	+
SRS-UACH	++	+	+
SLGO-95	+++	++	++
R-29	+++	++	++
R-61	++	++	++
R-62	++	++	++

(+) Faint Reaction; (++) Clear Reaction; (+++) Moderate Reaction

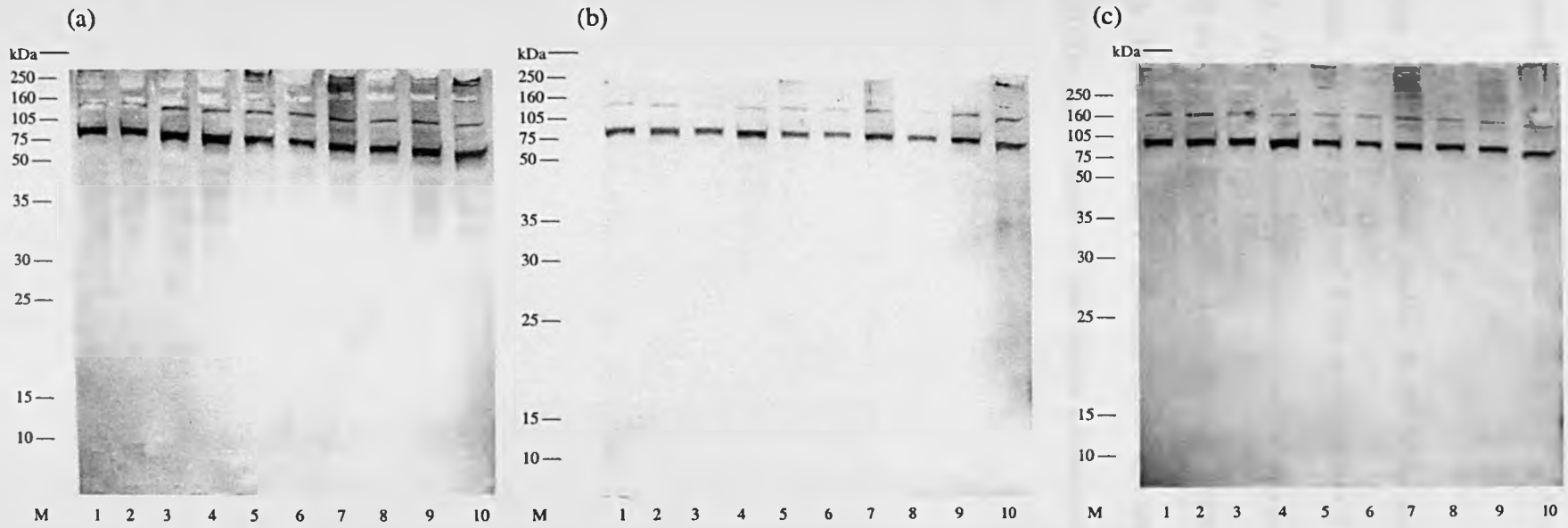


Figure 5.11. WB analysis of *P. salmonis* isolates using MAbs (a) MAb6A1F4C5; (b) MAb 8G7; (c) MAb 6A1G6.

Lanes: *P. salmonis* isolates (1) R-62; (2) R-61; (3) R-29; (4) SLGO-95; (5) SRS-UACH; (6) SRS-4; (7) VQ013; (8) R980769; (9) AVG5/268; (10) LF-89; ; M:Marker (Amersham).

A band between 10-16 kDa was faintly recognised by MAb 6A1F4C5 in WB analysis of Proteinase-K digested P. salmonis isolates (Figure 5.12).

WB analysis of *P. salmonis* isolate R-29 with mouse serum raised against *O. tsutsagamushi,* (Gilliam, Karp and Kato strains) from STG of rickettsiaea, is shown in Figure 5.14. The mouse serum recognised bands with a molecular weight of 95, 60 and 56 kDa as well as a doublet at 72 kDa. Less intensely stained bands were seen at 47, 36, 30 and 20 kDa. Three of the anti *P. salmonis* mouse sera used also recognised protein bands at 95, 72, 60 kDa and a number of paler bands at between 40-14 kDa (Figure 5.13).

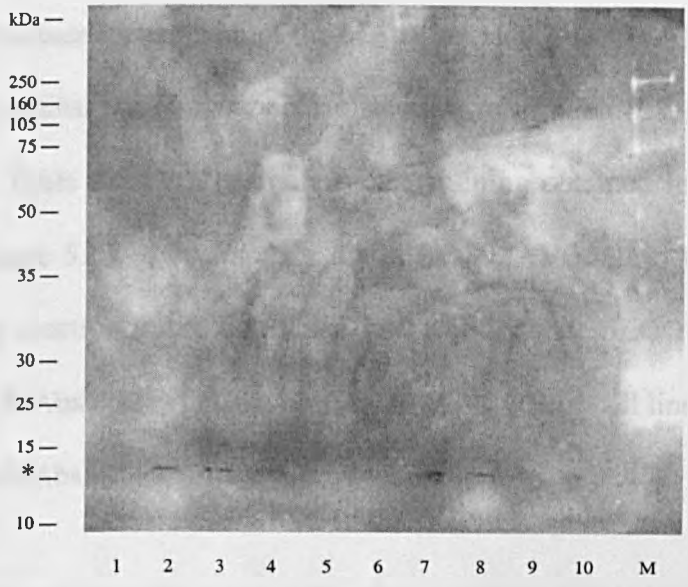


Figure 5.12. WB analysis of Proteinase-K digested *P.salmonis* isolates with the MAb 6A1F4C5; (1) R-62; (2) R-61; (3) R-29; (4) SLGO-95; (5) SRS-UACH; (6) SRS-4; (7) VQ013; (8) R980769; (9) AVG5/268 (4); (10) LF-89; M:Marker (Amersham); (*) shows the band between 10-16 kDa.

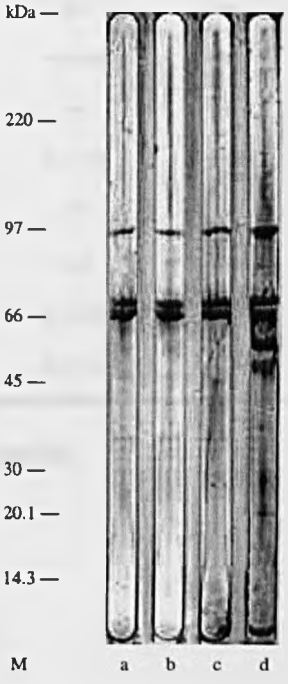


Figure 5.13. Western blotting with *P. salmonis* isolate R-29 using Mouse PAb against *O. tsutsagamushi* and mouse sera raised against *P. salmonis*. Lines: (a) Mouse-4 sera against LF-89; (b) Mouse-3 sera against LF-89; (c) Mouse-2 sera against LF-89; (d) Mouse sera against *O. tsutsugamushi*; M:Marker (Amersham).

(b) DBI

A DBI was used to evaluate the reaction of the MAbs (6A1F4G3, 6A1F4C5, 8G7, 7E2, 6A1G6 and 6A1G3) against the *P. salmonis* isolates shown in Table 2.1, the results of which are shown in Table 5.6. An example of the staining obtained in the dot blot assay is shown in Figure 5.14. *P. salmonis* isolates LF-89, AVG5/268, VQ013, SRS-UACH gave a strong reaction in the dot blot assay, while isolate SLGO-95 was only faintly recognised by MAbs 6A1F4G3, 6A1F4C5 and 8G7. CHSE cell line did not give any reaction with the MAbs.

Table 5.6. Intensity of the staining of a variety of *P. salmonis* isolates with MAbs in DBI

	Reactivity of MAbs*					
Isolates	**6A1F4C5**	**6A1F4G3**	**8G7**	**7E2**	**6A1G6**	**6A1G3**
LF-89	++++	++++	++++	+++	++++	++++
AVG5/268	++++	++++	++++	+++	++++	++++
R980769	+++	+++	++	+	++	++
VQ013	++++	++++	++++	+++	+++	+++
SRS-4	+++	+++	+	+	+	+
SRS-UACH	++++	+++	++++	+++	+++	++++
SLGO-95	+	+	+	-	-	-
R-29	++	++	++	-	+	+
R-61	++++	++++	++++	+++	++++	++++
R-62	+++++	++++	++++	+++	++++	++++

(*) The assay was performed in duplicate wells.
(-) No Reaction
(+) Faint Reaction
(++) Clear Reaction
(+++) Moderate Reaction
(++++) Intense Reaction

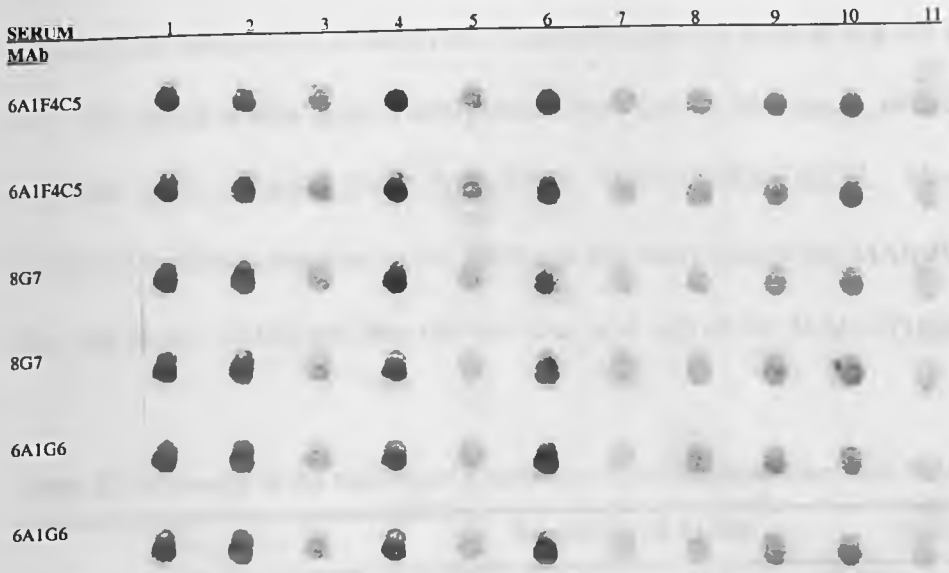


Figure 5.14. DBI with MAbs screened against a variety of *P. salmonis* isolates antigen. Lanes: (1) LF-89; (2) AVG5/268; (3) R980769; (4) VQ013; (5) SRS-4; (6) SRS-UACH; (7) SLGO-95; (8) R-29; (9) R-61; (10) R-62; (11) CHSE-214 as control.

(c) LBI

The line blot analysis of a variety of *P. salmonis* isolates with MAbs are shown in Table 5.7. The result of this assay corresponded with the dot blot assay, with strong line blot reactions with isolates LF-89, AVG5/268, VQ013, SRS-UACH. However, isolates SLGO-95 had faint reaction by the MAbs in the assay except for MAb 7E2 (Figure 5.15 a) in the assay. CHSE cell line did not react with any of the MAbs (Figure 5.15 b).

Table 5.7. Intensity of the staining of a variety of *P. salmonis* isolates with MAbs in the LBI

	Reactivity of MAbs					
Isolates	**6A1F4C5**	**6A1F4G3**	**8G7**	**7E2**	**6A1G6**	**6A1G3**
LF-89	++++	++++	++++	++++	++++	++++
AVG5/268	++++	+++	+++	++++	++++	++++
R980769	+++	+++	+++	++	++	++
VQ013	+++	+++	+++	+++	+++	+++
SRS-4	++	++	+	+	+	+
SRS-UACH	+++	+++	+++	+++	++	++
SLGO-95	+	+	+	-	+	+
R-29	++	++	+	-	+	+
R-61	+++	+++	+++	+	+++	+++
R-62	++++	++++	++++	++	+++	+++

(-) No Reaction
(+) Faint Reaction
(++) Clear Reaction
(+++) Moderate Reaction
(++++) Intense Reaction

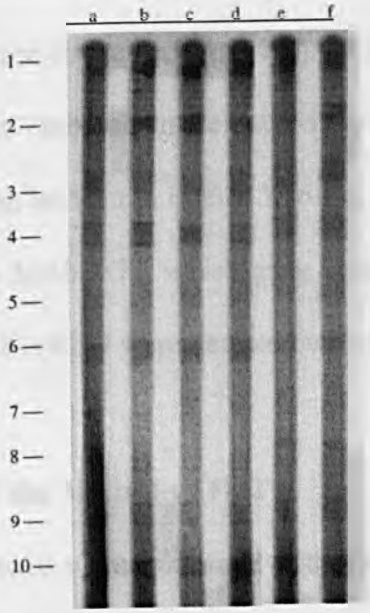


Figure 5.15 a. LBI with MAbs screened against a variety of *P. salmonis* isolates; Lanes, 1) LF-89; (2) AVG5/268; (3) R980769; (4) VQ013; (5) SRS-4; (6) SRS-UACH; (7) SLGO-95; (8) R-29; (9) R-61; (10) R-62. Lanes: (a) 6A1G3 ; (b) 6A1G6; (c) 7E2, (d) 8G7; (e) 6A1F4G3; (f) 6AF4C5

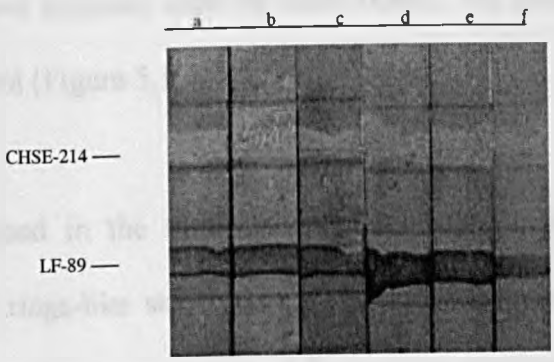


Figure 5.15 b. LBI for the analysing of MAbs using with *P. salmonis* type strain LF-89 as antigen and CHSE-214 cells as control. Lanes: (a) 6A1G3 ; (b) 6A1G6; (c) 7E2, (d) 8G7; (e) 6A1F4G3; (f) 6AF4C5

5.3.2.4. Use of the MAbs in IHC

The MAbs were used to screen tissue sections sampled from Atlantic salmon infected with a RLO during a disease outbreak in Ireland. Examples of the staining obtained with the antibodies in IHC can be seen in Figure 5.16 a-c. Staining was similar between the MAbs in IHC except for MAb 8G7, which gave a much stronger reaction than the other Mabs (Figure 5.16 b). No RLO were detected with the negative control.

5.3.2.5. Characterisation of the MAbs by IFAT

Different isolates of *P. salmonis* were examined with the six MAbs by MIFAT (Table 5.8). Illustrations of the immunofluorescence staining obtained are shown in Figure 5.17 a-f. Differences were evident in the intensity of the staining by the different MAbs with the various *P. salmonis* isolates. *P. salmonis* isolates LF-89, AVG5/268, SRS-4 and SRS-UACH were recognised by MAbs 7E2, 8G7 and 6A1G6, which gave stronger fluorescent than the other *P. salmonis* isolates examined. MAb 8G7 appeared to stain isolate VQ013 more intensely than the other MAbs. No fluorescence was evident with the negative control (Figure 5.17 g).

P. salmonis, stained in the tissue smears and tissue sections, appeared as small, pleomorphic and rings-like structures of fluorescence around the outer edges of the bacterium. MAbs also detected foci of fluorescing microorganisms in tissue sections from Atlantic salmon obtained from a natural outbreak in Ireland (Figure 5.18 a-d).

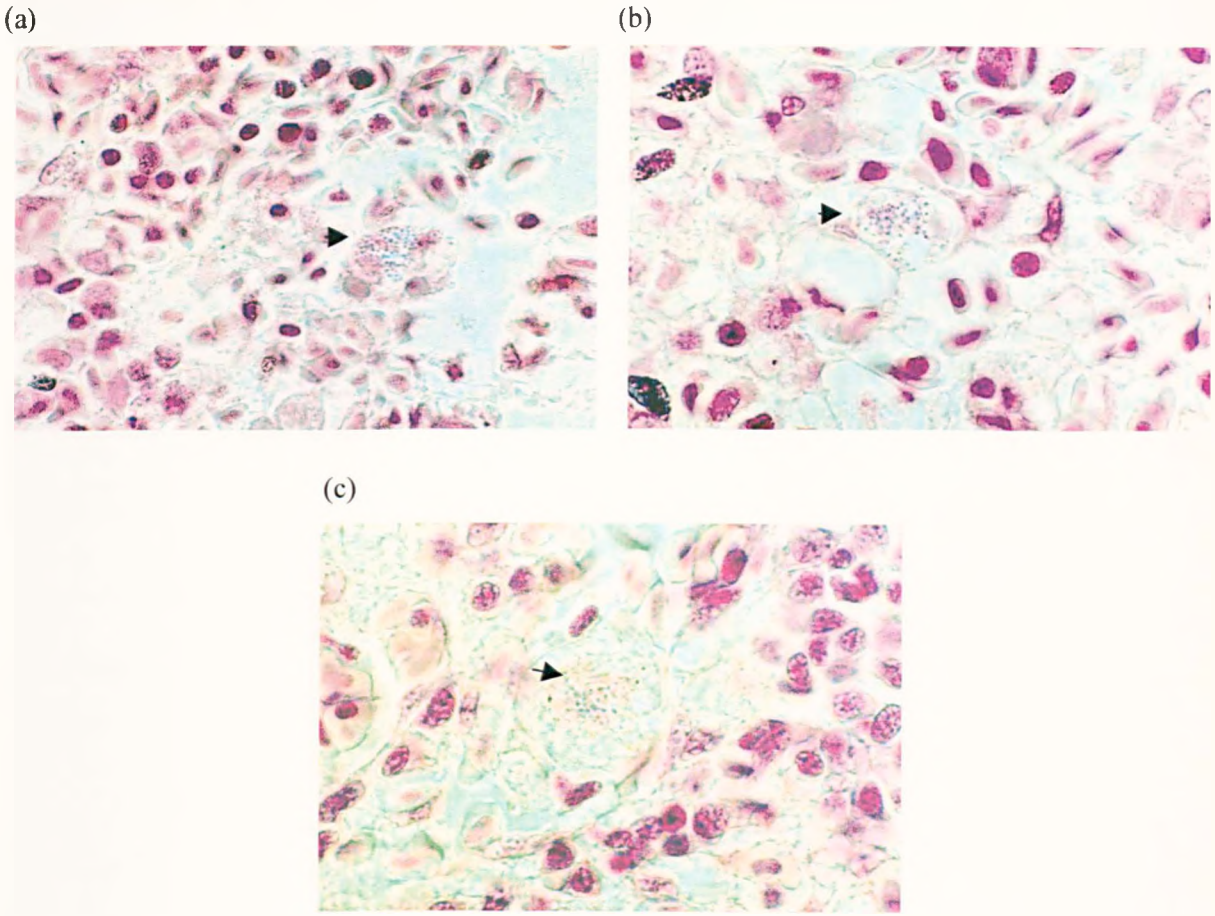


Figure 5.16. IHC analysis of tissue sections from Atlantic salmon naturally infected with *P. salmonis,* using monoclonal antibodies against *P. salmonis* type species LF-89 (see arrows). (a) MAb 7E2 (magnification x 1100); (b) MAb 8G7 (magnification x 1400); (c) 6A1G6 (magnification x 1400); (d) Negative control (magnification x 1100).

MIFAT analysis of *E. phagocytophila* with MAbs developed in this study against *P. salmonis* illustrated in Figure 5.19 a-d. Acridine orange staining of microscope slide wells bearing ovine neutrophils infected with *E. phagocytophila* was shown in Figure 5.19 e and f as control. Fluorescence white dots with MIFAT and some red reaction with Acridine orange staining associated with ovine neutrophils were observed under fluorescence microscopy.

Table 5.8. Reaction of *P. salmonis* isolates with the various MAbs in MIFAT

P. salmonis	**Reactivity of MAbs**					
isolates	**6A1F4C5**	**6A1F4G3**	**8G7**	**7E2**	**6A1G6**	**6A1G3**
LF-89	+	+	++	++	++	+
AVG5/268	+	+	++	++	++	++
R980769	-	+	+	+	+	+
VQ013	+	+	++	+	+	+
SRS-4	-	+	++	++	++	+
SRS-UACH	+	+	++	++	++	+
SLGO-95	+	-	+	-	+	-
R-29	+	-	+	+	+	+
R-61	-	+	+	+	+	+
R-62	-	-	-	-	-	-

(-) No fluorescence; (+) Faint but distinct fluorescence; (++) Clearly fluorescent

5.3.2.6. IGS with MAbs

IGS was carried out using purified *P. salmonis* type species LF-89, which was then examined by EM (Figure 5.20 a-f). MAb 6A1F4C5 appeared to recognise material on the surface and within the cytoplasm associated with the cell surface of the bacterium (Figure 5.20 a-b). A similar response was seen with MAbs 8G7 (Figure 5.20 c-d), 7E2 (Figure 5.20 e), 6A1G6 (Figure 5.20 f). The MAbs also reacted with material on the surface of the Chilean isolate R-29, but to a lesser extend than was seen with the type strain (Figure 5.20 g-k). Slight labelling of surface material was also evident with isolate SLGO-95 with MAb 6A1F4C5 (Figure 5.20 l).

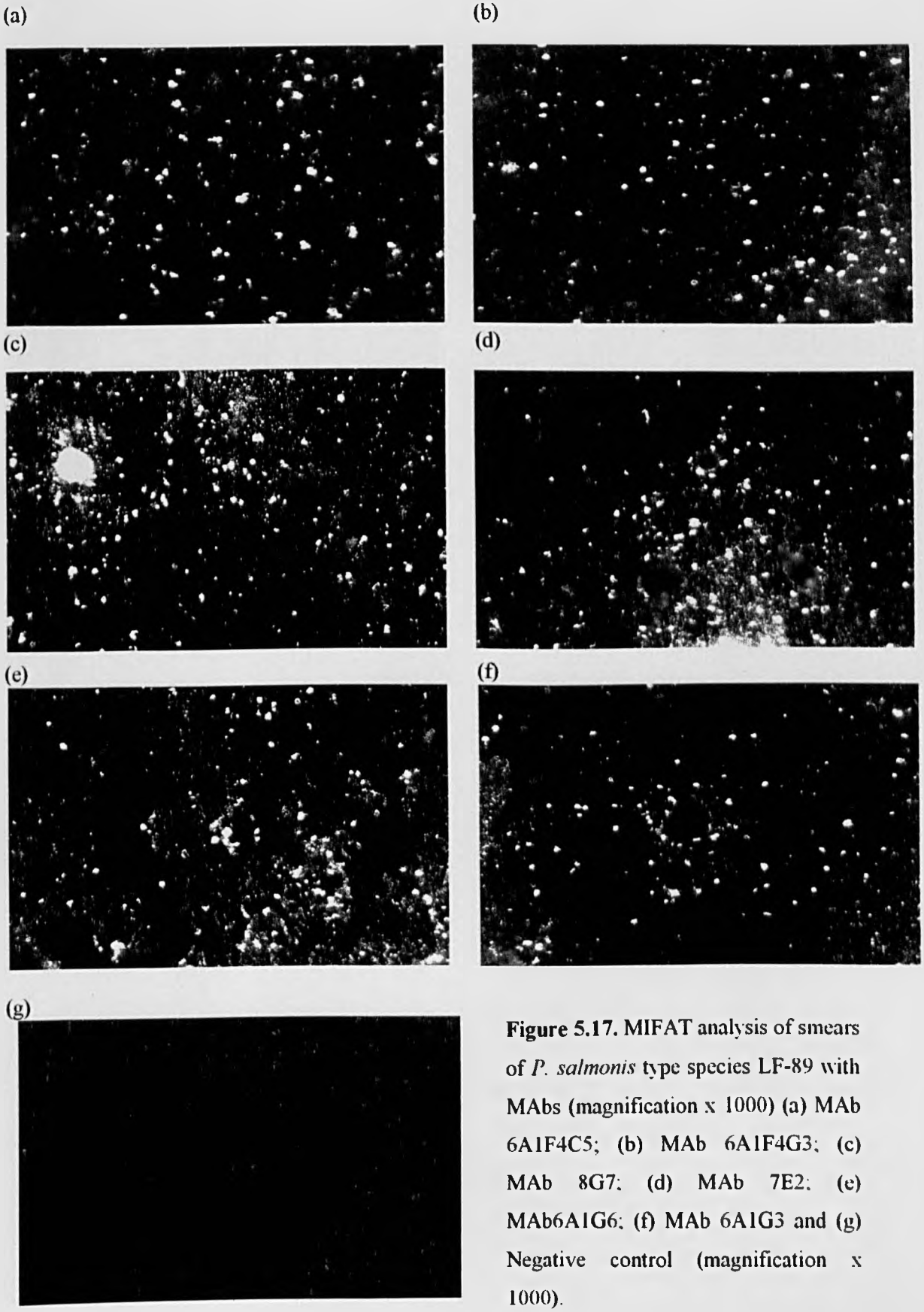


Figure 5.17. MIFAT analysis of smears of *P. salmonis* type species LF-89 with MAbs (magnification x 1000) (a) MAb 6A1F4C5; (b) MAb 6A1F4G3; (c) MAb 8G7; (d) MAb 7E2; (e) MAb6A1G6; (f) MAb 6A1G3 and (g) Negative control (magnification x 1000).

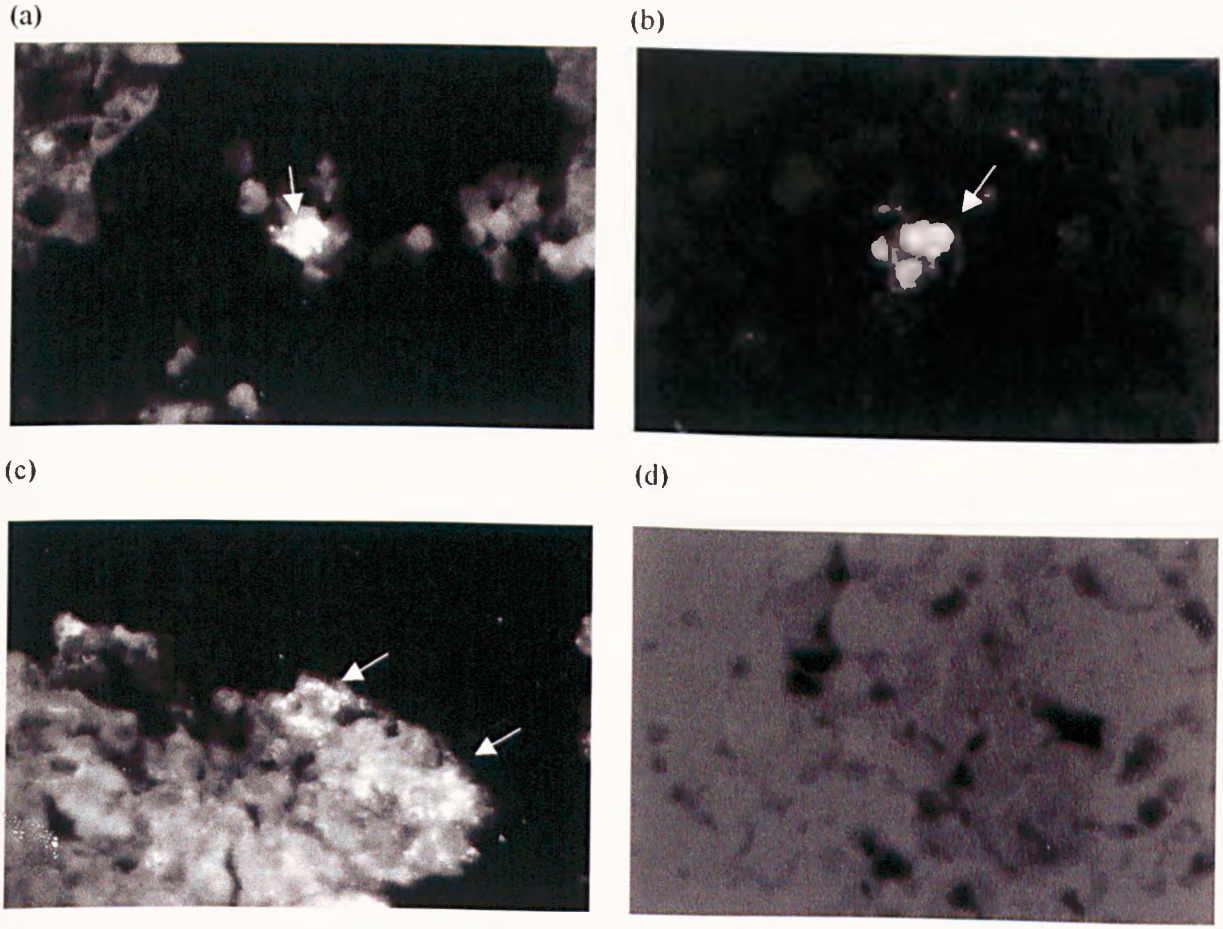


Figure 5.18. Detection of rickettsia-like organism in tissue sections from naturally infected Atlantic salmon from Ireland with MAbs (see arrows) (magnification x 1000) (a) MAb 6A1F4C5; (b) MAb 6A1F4G3; (c) MAb 8G7 and (d) Negative control. (magnification x 1000).

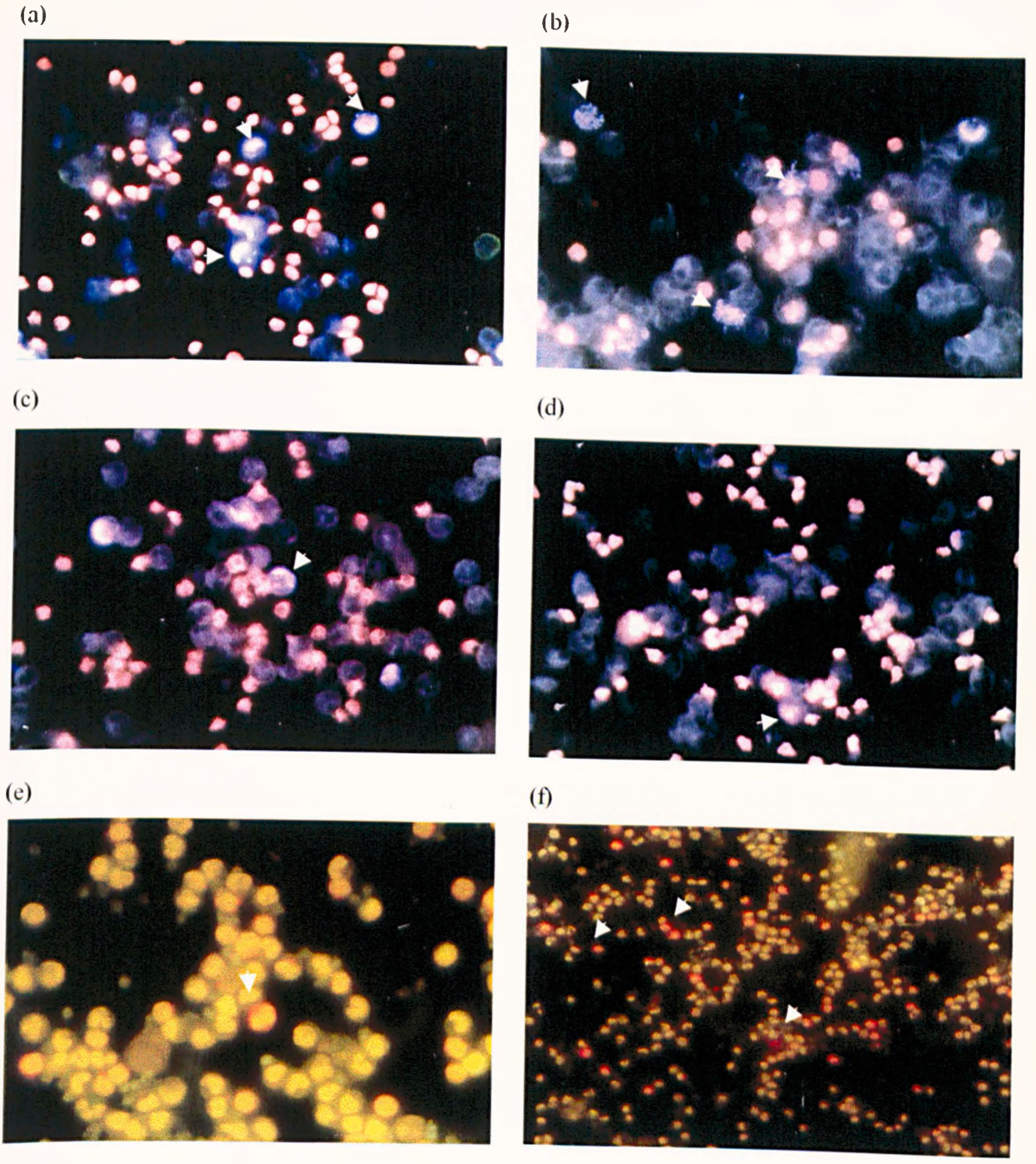


Figure 5.19. Analysis of cross-reactivity of *E. phagocytophila* with MAb against *P. salmonis* type strain LF-89 (see arrows indicating some positive white dots) (magnification x 1000) (a) MAb 6A1F4C5; (b) MAb 6A1F4G3; (c) MAb 6A1G3; (d) Negative control (see arrow also indicating similar positive reaction) (magnification x 1000); and Acridine orange staining of smears bearing ovine neutrophils infected with *E. phagocytophila* (e) (magnification x 1000) and (f) (magnification x 400).

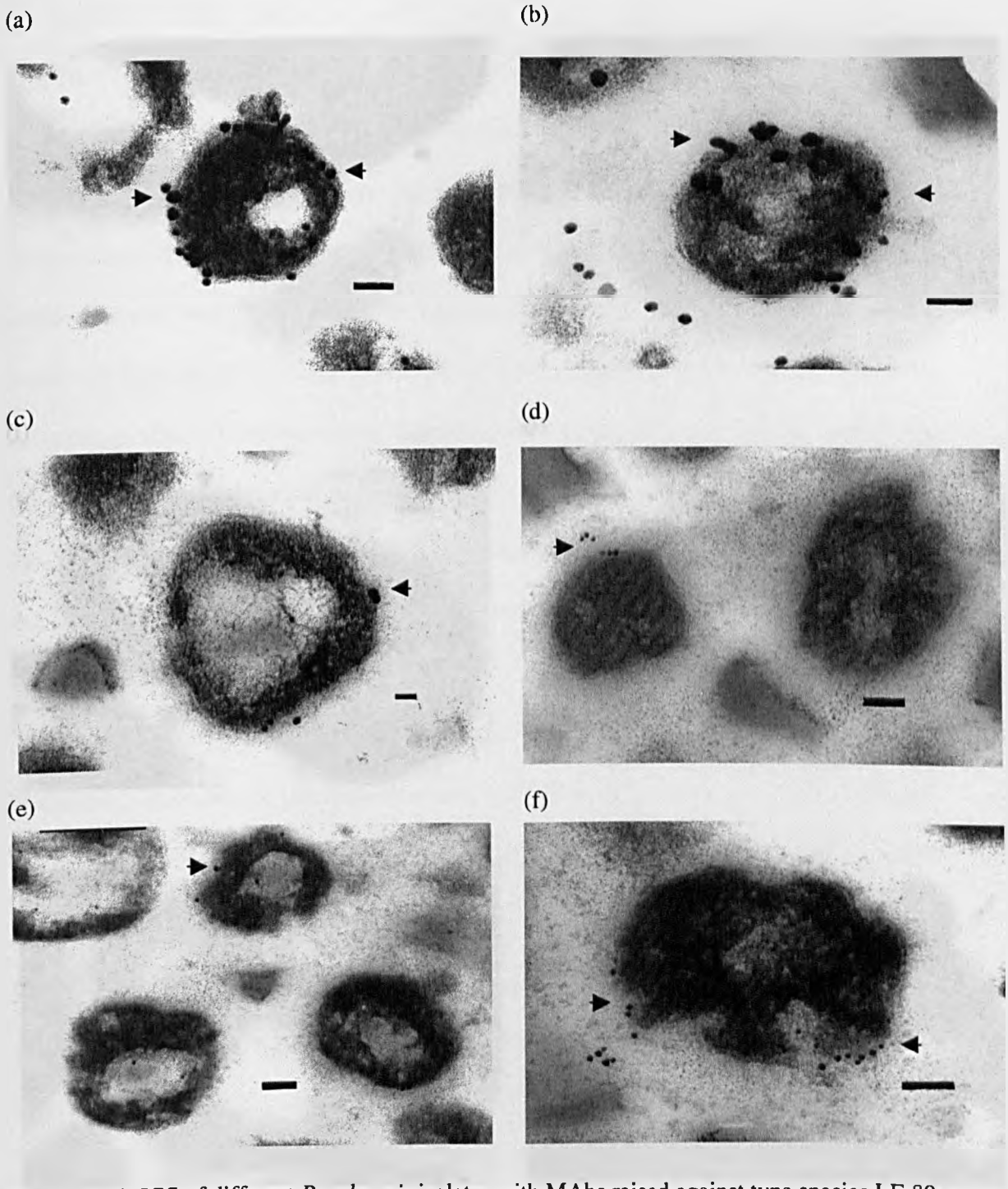


Figure 5.20. IGS of different *P. salmonis* isolates with MAbs raised against type species LF-89. **(a)** and **(b)** *P. salmonis* type strain LF-89 stained with MAb 6A1F4C5 (Bar: 0.1 μm); **(c)** (Bar: 0.05 μm) and **(d)** (Bar: 0.1μm) *P. salmonis* type species LF-89 stained with MAb 8G7; **(e)** *P. salmonis* type species LF-89 stained with MAb 7E2 (Bar: 0.1 μm); **(f)** *P. salmonis* type species LF-89 stained with MAb 6A1G6 (Bar: 0.1 μm) (See arrows indicating MAbs labelled with anti-mouse IgG conjugated to 5 nm gold particles).

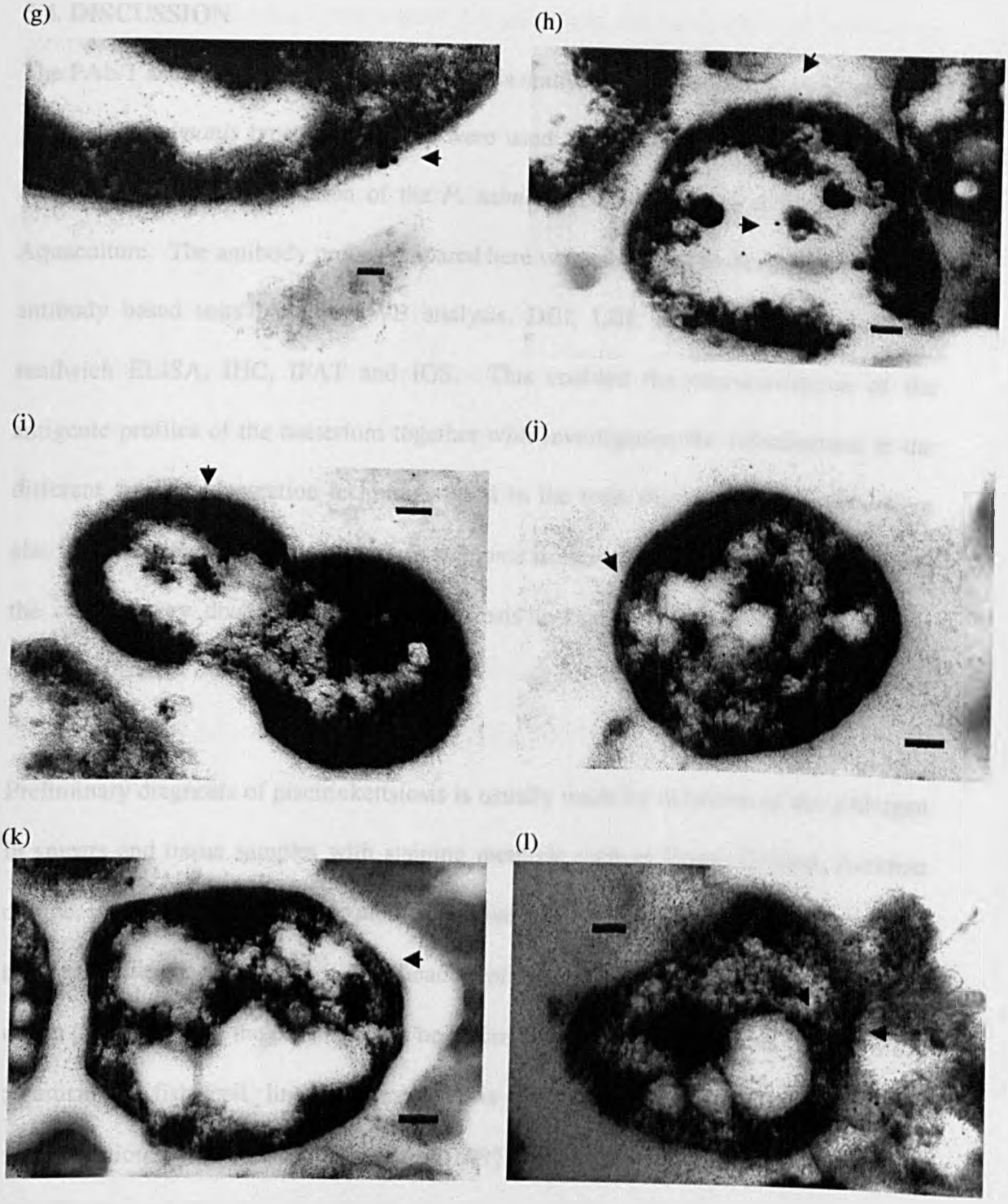


Figure 5.20. (Cont.). **(g)** *P. salmonis* R-29 stained with 6A1F4C5 (Bar: 0.05 μm) ; **(h)** *P. salmonis* R-29 stained with MAb 8G7 (Bar: 0.1 μm); **(i)** and **(j)** *P. salmonis* R-29 stained with MAb 7E2 (Bar: 0.2 μm); **(k)** *P. salmonis* R-29 stained with MAb 6A1G6 (Bar: 0.2 μm); **(l)** *P. salmonis* SLGO-95 stained with 6A1F4C5 (Bar: 0.1 μm) (See arrows indicating MAbs labelled with anti-mouse IgG conjugated to 5 nm gold particles).

5.4. DISCUSSION

The PAb-1 and the six MAbs developed in this study, against whole cell preparations of purified *P. salmonis* type strain LF-89 were used in the identification of *P. salmonis* antigen, and the differentiation of the *P. salmonis* collection held at the Institute of Aquaculture. The antibody probes prepared here were also used to develop a variety of antibody based tests including WB analysis, DBI, LBI, an indirect ELISA and a sandwich ELISA, IHC, IFAT and IGS. This enabled the characterisation of the antigenic profiles of the bacterium together with investigation the effectiveness in the different antigen preparation techniques used in the tests mentioned. The tests were also used to improve the detection of *P. salmonis* isolates more specifically and assist in the confirmatory diagnosis of piscirickettsiosis in complex clinical and sub-clinical cases in different outbreaks worldwide.

Preliminary diagnosis of piscirickettsiosis is usually made by detection of the pathogen in smears and tissue samples with staining methods such as Gram, Giemsa, Acridine orange, Gimenez, Macchiavello, methylene blue and Periodic Acid-Schiff (PAS). The most sensitive method for the identification of *P. salmonis,* and the only method by which the viability of the organism can be determined is still isolation and culture of the organism in fish cell lines, although it is time consuming and susceptible to contamination (Almendras & Fuentealba 1997; Fryer & Lannan 1996). Ideally the identification of *P. salmonis* should be improved to make it more specific, more sensitive and more rapid. Antibody-based methods for the detection of *P. salmonis,* such as IHC and IFAT, may meet these requirements (Alday-Sanz *et al.* 1994; Lannan & Fryer 1991). Detection of bacterial pathogens in fish tissue is an important tool for epidemiological studies. It is also an essential component in antibacterial treatment *in*

vivo (Bernoth 1990). used to detect both fish pathogens and fish antibodies is becoming a more common diagnostic tool in fish pathology.

The ELISA, provides rapid detection of the pathogen, and is practical and economical (Olesen & Jorgensen 1991). The test which can be read with the naked eye or in an ELISA reader (Adams & Thompson 1990; Keysary & Strenger 1997), and also offers a highly sensitive test over other immunoassays (Arkoosh & Kaattari 1990). ELISAs have been used for diagnosing rickettsial disease, particularly the Spotted Fever group (SFG) rickettsia (Jones, Anderson, Olson & Greene 1993) and the method is as sensitive and specific as IFAT for the diagnosis of Rocky Mountain SFG rickettsiae and STG rickettsia (La Scola & Raoult 1999). It is also more sensitive than IFAT for detection of low levels of antibody after vaccination or during late recovery from the disease suggesting its usefulness for sero-epidemiologic studies of rickettsiae (La Scola & Raoult 1999).

An ELISA was developed by Cassigoli (1994) to detect Salmonid rickettsial septicaemia. Both indirect and sandwich ELISA (SELISA) were developed here to examine the reaction of MAbs and PAb-1 with a variety of *P. salmonis* isolates and non-*P. salmonis* bacteria. The specificity and sensitivity of PAb-1 developed against *P. salmonis* type strain LF-89 were analysed using the ELISA. The PAb-1 reacted positively with all *P. salmonis* isolates. Although the rabbit anti-*P. salmonis* serum also reacted with some non-*P. salmonis* bacteria like E. *coli, A. hydophila* and *Y. ruckeri,* the *P. salmonis* isolates examined had a stronger reactivity than non-*P. salmonis* bacteria, except for isolate R-62 which gave a lower reaction than *E. coli.* The reactivity of the rabbit anti-*P. salmonis* serum, kindly gifted by Dr. Fryer (PAb-2), was compared with the rabbit serum prepared

in this Chapter (PAb-1). PAb-2 recognised all *P. salmonis* isolates except for two Chilean isolates VQ013 originating from rainbow trout *Oncorhynchus mykiss* and R-29 isolated from Atlantic salmon which gave lower responses in the ELISA than *R. salmoninarum.* The response of PAb-2 against the other isolate originated from Atlantic salmon, AVG5/268, isolated in Ireland was also low but the responses obtained against CHSE cells was fairly strong, unlike the response seen with PAb-1. Using either PAb-1 or PAb-2 in an ELISA as a diagnostic tool could cause problems because of the cross-reaction obtained with some of the non-*P. salmonis* bacteria. The sensitivity threshold obtained with both rabbit anti-*P. salmonis* sera was similar with purified *P. salmonis* type strain LF-89. When ELISA and SELISA were used for the analysis of the MAbs in this study, the reaction of the MAbs with each of the different *P. salmonis* isolates was very similar, although LF-89, AVG5/268 and SRS-UACH gave a higher reaction in the SELISA than the other *P. salmonis* isolates examined. The isolates SLGO-95, R-29 and R-62 gave the lowest response in the SELISA, while none of the non-*P. salmonis* bacteria gave reacted with any of the MAbs. In the SELISA, *P. salmonis* was detected as low as 0.1-0.3 $\mu g\ ml^{-1}$ concentration, using PAb-1 as captured antibody and anti-mouse IgG-HRP as secondary antibody. Aguayo, Miquel, Aranki, Jamett, Valenzuela and Burzio (2002) used ELISA to detect *P. salmonis* in fish tissue using specific monoclonal antibodies against *P. salmonis* (Jamet *et al.* 2001). Aquayo *et al.* (2002) used a combination of the MAbs in the capture step which were bound to the solid support with an adhesive protein purified from a bivalve mollusc, and one of the MAb conjugated to HRP as a secondary antibody in the sandwich ELISA in which the saturation level was achieved with 300 nanogram (ng) of *P. salmonis* protein $well^{-1}$. When an ELISA was used to test the cross-reactivity of a mouse anti-*O. tsutsagamushi* polyclonal serum with *P. salmonis* type strain LF-89 in this study, the serum had a similar titre to that of the mice sera produced against the type strain.

WB is a useful technique particularly for characterising unknown antigens in sera or tissues and examining antibody specificities. Its main strengthen compared with other immunoassays is its ability to establish the presence of individual antigens. A major disadvantage of this method, however, is that it is qualitative not quantitative (Wiens, Turaga & Kaattari 1990). Also there is a reduction or destruction of antigenic activity due to the denaturing conditions of the assay (Bers & Garfin 1985; Dunbar 1987). Immunoblot analysis is a powerful serodiagnostic method for sero-epidemiology and confirmation of serologic diagnosis obtained by conventional tests of rickettsial diseases (La Scola & Raoult 1999). Immunblot was used here with MAbs and PAb-1 developed against *P. salmonis* type strain LF-89 against a variety of *P. salmonis* isolates to examine antigenic profiles of the bacterium. The use of the ABP complex system greatly increased the sensitivity of the reaction particularly for the antigens in the lower region of SDS-PAGE profile.

PAb-1 raised against the type species LF-89 prepared in this study, recognised bands with molecular weights at 115, 95, 72, 60, 51, 40, 36 and 32 kDa on the isolate in WB analysis. Kuzyk *et al.* (1996) prepared rabbit antiserum against whole cell preparations of *P. salmonis* which had been purified by Percoll density gradient and then formalin killed (5% in PBS). Their serum recognised four different protein bands at 65, 60, 54, 51 kDa and two carbohydrate immunoreactive antigens at 16 and 11 kDa in purified preparations of *P. salmonis.* Barnes *et al.* (1998) also partially characterised the antigens of type strain *P. salmonis* purified using a Diatrizoate meglumine and diatrizoate sodium (DMDS) gradient. They observed antigens at 108, 95, 64, 60, 56, 40, 36, 32 and 20 kDa with rabbit anti *P. salmonis* serum obtained from Lannan *et al.* (1991). The antigen at 60 kDa is common among all studies. The 51 kDa protein

observed in the present study was also observed in the study by Kuzyk *et al.* (1996), as well as the 95 and 32 kDa proteins identified by Barnes *et al.* (1998) with their rabbit anti *P. salmonis* serum. Barnes *et al.* (1998) also observed an antigen with molecular weight of 108 kDa, while antigens at 115 and 95 kDa proteins were identified in this study. A possible explanation for the differences in the number and molecular weights of the bands identified in the different studies could be the fact that different gradients and purification protocols were used to prepare the bacterium and as well as different antiserum, to characterise the bacterium.

When WB analysis was performed on whole cell preparations of *P. salmonis* LF-89, the six MAbs produced here appeared to have a slightly different banding pattern, but all reacted with a large band with a molecular weight 95 kDa and a band also between 105-160 estimated to be 115 kDa. The protein band at 95 kDa was observed by all MAbs on different isolates of *P. salmonis*, but the intensity of the reaction differed between MAbs. The 95 kDa band of isolates R980769, SRS-4, SRS-UACH, R-61 and R-62 gave a weaker reaction with the MAbs, however, the reaction with MAb 6A1F4C5 was more intense. The MAbs appeared to give a slightly different banding profile to each other in the lower region of the gel with LF-89. When biotin amplification was applied, the sensitivity of the reaction greatly increased, particularly in the lower region of the antigen profile. However, when PAb-1 was used as the first antibody, biotin amplification resulted in a strong non-specific reaction. Jones, Mosley, Jeffrey and Stoddart (1987) reported that non-specific reactions sometimes occur as a result of biotin amplification.

WB profiles of the Proteinase-K digested *P. salmonis* isolates were also tested in this study. MAb 6A1F4C5 slightly stained a band at between 10-16 kDa with Proteinase-K digested *P. salmonis*. Kuzyk *et al.* (1996) reported similar results that rabbit antiserum developed against whole-cell *P. salmonis* recognised 16 and 11 kDa immunoreactive carbohydrate antigen. The authors claimed that the 11 kDa antigen is most likely the lipo-ologosaccharide (LOS) component of lipopolysaccharide (LPS). The 16 kDa antigen may represent the core region LOS with one unit of O polysaccharide antigen. High molecular weight carbohydrates generally represent core region LOS with O antigen with a decreasing number of repeat units (Amano, Fujita & Suto 1993). Amona *et al.* (1993) speculated that the fastest-migrating band of LPS from SFG rickettsia did not reacted with the serum, but did react strongly with silver stain suggesting that these are the molecules containing the core oligosaccharide and lipid A portions of LPS without the O polysaccharide. Jamet *et al.* (2001) produced six monoclonal antibodies against *P. salmonis*. The MAbs recognised a range of proteins with molecular weights from 90 to about 10 kDa bands. Four of the MAbs gave bands with sizes of at 42, 60, 85, 90 and 100 kDa while MAb 7G4 reacted with three diffuse bands from 24 to 10 kDa. MAb 8F5 had similar immunoreactivity with MAb 7G4, although it also recognised a protein with apparent molecular weight of 60 kDa. Jamet *et al.* (2001) reported that the immunorectivity of the 10-24 kDa antigens of the bacterium was not destroyed with proteinase K treatment indicating that the bands most likely correspond to LOS components of LPS. Most of the antigens are similar or close to the proteins detected with the MAbs produced in this study. The LF-89 carbohydrate antigen (between 10-16 kDa) reported have may be similar to the LPS at 14 kDa in *Rickettsia typhi* (from Typhus Group Rickettsia), which is reported to be a group-specific antigen (Amona, Williams & Dasch 1998).

Jones, Markham, Groman and Cusack (1998) developed both a MAb (2C3) and rabbit PAb against formalin killed whole cell preparations of *P. salmonis* type species LF-89. The antibodies were analysed by IFAT and WB. Bacteria from cell culture supernatant, infected with *P. salmonis* isolates LF-89, CR288, CR1010 from Chile and ECR0811 from Canadian Atlantic salmon, were easily recognised as small round or pleomorphic organisms by the rabbit antiserum and MAb (2C3) by IFAT. All 4 isolates were examined electrophoretically and were found to be similar to each other. The isolates shared as many as nine immunoreactive bands in common, located at 32, 45, 55, 60, 65, 89, 93, 98 and 125 kDa. After Immunoblotting with the rabbit-anti-*P. salmonis* serum, the isolates, LF-89, CR1010 and ECR0811 were compared and found to have bands at 32, 45, 60, 65, 89, 98 and 125 kDa, and a Proteinase-K resistant band at 12 kDa was also common amongst isolates. MAb (2C3), used by the authors, recognised an epitope on isolates (LF-89, CR288, CR1010 and ECR0811) which was present on a number of different molecular weight bands resulting in a ladder pattern extending from less than 20 kDa to greater than 95 kDa. Treatment of LF-89 with periodic acid removed the immunoreactivity of the MAb. The authors indicated that this MAb recognised a LPS-associated epitope in the isolates, because of co-elution of 2-keto-3-deoxyoctonate and MAb reactivity following the size exclusion chromatography of solubilised *P. salmonis.*

Kuzyk, Thornton and Kay (1999) reported a misdiagnosis of *P. salmonis* in mycoplasma infected cell lines. They claimed that the immunoreactive ladder-like pattern observed by Jones *et al.* (1998) was the pattern of *Mycoplasma pulmanis* V-1 antigen as demonstrated by Watson, Dybvig, Blalock and Casell (1989). Mycoplasmas are very common cell culture contaminants that are not easily detected. Routine culture of used fish cell lines make them open to contamination by mycoplasma-like

organisms. Kuzyk *et al.* (1999) analysed both CHSE-214 and Epithelioma papulosum cyprini *Cyprinus carpio* L (EPC) cell lines contaminated with the immunoreactive ladder-like patterns, for mycoplasma infection by Polymerase chain reaction (PCR), and were able to amplify mycoplasma specific products from the cell lines. They obtained fresh samples of CHSE-214 cells and *P. salmonis* directly from American Tissue Culture Collection (ATCC) and screened them with the rabbit antiserum 79CR prepared against purified *P. salmonis* using density gradient Percoll centrifugation (Kuzyk *et al.* 1996) and no ladder like patterns were observed. Therefore, Kuzyk *et al.* (1999) indicated that the ladder-like pattern was not of *P. salmonis* origin. The authors also pointed out that the anti-*P. salmonis* rabbit serum (82CR) used by Kuzyk *et al.* (1996) was developed against a *P. salmonis* preparation purified from an uncontaminated CHSE-214 cells source of ATCC. Kuzyk *et al.* (1999) as well as Smith (1984) cited by the authors, indicated that mycoplasma V-1 antigens are post-translational carbohydrate modifications and lipo-oglycan polysaccharides on the surface of the organism which are covalently bound to lipids and have a structure and physiologically similar to the LPS of Gram negative bacteria.

Each species of rickettsia has own species-specific antigens which stimulate the production of homologous antibodies. Apart from *Coxiella burnetti*, all species also possess a soluble group of antigens (Buxton & Fraser 1977). Species-specific antigens for rickettsia are based on antigens localised on the surface of the rickettsial cell wall, such as LPS or O antigens which are common to a particular rickettsial group (Teysseire & Raoult 1992). *Rickettsia prowazekii* and *R. typhi* which belong to the typhus group (TG) of rickettsia, have a species-specific antigen with an estimated weight of 120 kDa. This antigen has been found to be heat-stable at 50°C, but heat-labile when subjected to 56°C

for 30 min (Winkler 1990). These surface proteins are highly immunogenic (Ching, Wang, Jan & Dasch 1996), and constitute 10 to 15% of the total rickettsial cellular protein which are believed to interact directly with components of the hosts immune system since they are associated with the rickettsial cell envelop (Dasch 1981). Some of the antigens of SFG rickettsia appear similar to the species-specific antigens of the TG. SFG rickettsia possess two surface proteins with an estimated molecular weight of 128-170 kDa and 110-133 kDa respectively and these consist of both heat labile and heat stable epitopes. A SFG rickettsia, *Rickettsia conarii* has two partial cell surface antigens with a molecular weight of 128 kDa and 112 kDa respectively. Two additional proteins on *R. conarii* at 135 kDa and 115 kDa, both of which are heat stable at 52°C, have also been reported. MAbs against the 115 kDa protein may be involved in adhesion of the rickettsia to the surface of the host cell with reduction of rickettsial attachment to the host cell (Winkler 1990). The high molecular-weight proteins of *R. conarii* have also been reported in the Mediterranean Spotted Fever group (MSFG) of rickettsia with some cross-reactivity occurring with LPS antigens (Teysseire & Raoult 1992). A 60 kDa protein has also been detected in the serum of patients with a malignant form of MSFG. This is a heat-shock protein (Hsp), which has been frequently described for MSFG rickettsia and this Hsps appears to be structurally and immunologically similar to many other heat-shock proteins from a wide range of eukaryotic and prokaryotic organisms. The Hsp antigen promotes translocation, folding and assembly of proteins and in some forms of MSFG, it can cause malignancy (Teysseire & Raoult 1992). Heat label, surface antigens have also been described for *Rickettsia tsutsugamushi*, a scrup typhus group (STG) rickettsia (Winkler 1990).

The MAbs and PAb-1 developed in this study can be used in WB analysis to determine and further investigate the antigens of *P. salmonis* isolates. The major antigens of *P.*

salmonis are proteins of 95, 60, 51, 40, 36, 32 and bands ≤ 20kDa. Some of the antigens recognised on *P. salmonis* have also been identified by other authors working on *P. salmonis* or other rickettsial genera. The 60 kDa antigen is a common protein observed in all the studies relating to *P. salmonis.* The 115 kDa protein of MSFG was also recognised in this study by MAbs and PAb-1 with LF-89.

The 56 kDa protein found on *P. salmonis* isolate R-29 recognised by anti-*O. tsutsugamushi* sera is a major outer membrane protein of *O. tsutsugamushi*, the agent of Scrup tyhpus or tsutsugamushi which is an acute disease. This is a Gram-negative bacterium, and in contrast to other Gram-negative bacteria, it has neither LPS nor a peptidoglycan layer. The ultrastructure of its cell wall differs significantly to that of it closest relatives belonging to the TG and SFG of rickettsia. The former has a thicker outer leaflet compare to its inner leaflet while the latter bacteria are surrounded by a slime layer, presumably composed of carbohydrate in nature. Although *Orientia* isolates are highly variable in their antigenic properties, the major outer membrane protein of *O. tsutsugamushi* is the variable 56 kDa protein which accounts for 10 to 15% of its total protein, suggesting that it is a good candidate for use as a diagnostic antigen as this protein was recognised by sera from most patients infected with Scrub typhus (Weiss & Moulder 1984; Ching, Wang, Eamsila, Kelly & Dasch 1998). PAb-1, MAbs and mouse anti-sera against *P. salmonis* produced in this study did not recognise the 56 kDa antigen on any of the *P. salmonis* isolates examined. However, the bands recognised by the anti-*O. tsutsugamushi* sera on *P. salmonis* isolate R-29 was similar to the antigenic profile of the isolate R-29 when it was analysed with the sera produced against *P. salmonis* type strain LF-89 in this Chapter. This may show an antigenic

similarity between *O. tsutsugamushi* and *P. salmonis*, agreeing with the ELISA results mentioned above.

Large numbers of samples can be screened with line blot and dot blot assays with a minimum effort in a short space of time, and could be performed without the use of microscopes or incubators (Raoult & Dasch 1989; Koay, Tay, Cheong & Yasin 1995; La Scola & Raoult 1999). The line blot assay has a similar sensitivity and specificity to that of the MIFAT, but requires a much lower titer of serum for diagnosis e.g. as with MSFG rickettsiae (Raoult & Dasch 1989). The dot–blot method reported by Hawkers, Niday and Gordon (1982) was first introduced to fish pathology to detect *Vibrio anguillarum* (Cipriano, Pyle, Starliper & Pyle 1985), *Aeromonas salmonicida* (Sakai, Ishii, Atsuta & Kobayashi 1986) and then *R. salmoninarum* (Sakai, Amaaki, Atsuta & Kobayashi 1987). Although the assay is qualitative, it is less specific and sensitive than WB. It has the advantage over ELISA in that it can be more easily applied in the field and is more economic than ELISA (Sakai, *et al.* 1987). This method has been used in field studies to diagnosis STG rickettsia in rural areas (Koay *et al.* 1995).

The line blot and dot blot assays used in this study is the first report of their use to detect *P. salmonis*. They are useful for the rapid and easy detection of *P. salmonis* and PAb-1 developed against *P. salmonis* type strain LF-89, used in the assay gave an intensive reaction with all *P. salmonis* isolates with both methods and did not react with CHSE-214 cells. The DBI and LBI also worked well with the MAbs, where the reaction were similarly for both assays using the *P. salmonis* type species LF-89 (against which the antibodies had been raised) and the other *P. salmonis* isolates

examined. This was with the exception of isolate SLGO-95 only gave a faint reaction with some of the MAbs in both of the assays.

The fluorescent antibody test is widely used for the detection of antigen and antibody in fish. The disadvantages of IFAT or MIFAT is that it needs sophisticated and expensive equipment (De La Fuente, Anda, Rodriguez, Hechemy, Raoult & Casal 1989). IFAT is a preferable serodiagnosis test for rickettsial disease offering more sensitivity and specificity, but only if applied before the initiation of antimicrobial therapy and before the 10^{th} d of the disease. However, MIFAT provides the possibility of detecting antibodies to a number of rickettsial antigens simultaneously (La Scola & Raoult 1996, 1999).

The MAbs and PAb-1 raised in this study against *P. salmonis* type strain LF-89 were also used in IFAT, IHC and IGS to test the sensitivity of these diagnostic methods for piscirickettsiosis and also to examine the interaction between the bacterium and its host. The tests were assessed in this study since they could provide specific determination as well as revealing antigenic properties of the bacterium. IHC has the advantage of allowing histopathological evaluation of lesions to be carried out as well as confirming the presence of the pathogen (Alday-Sanz *et al.* 1994). Alday-Sanz *et al.* (1994) were able to detect RLO within the cells of kidney in Atlantic salmon, experimentally infected in Chile and *naturally infected* in Ireland, using IHC with rabbit anti-sera provided by Dr. Fryer. The MAbs and the PAb-1 raised in this study against *P. salmonis* type strain LF-89 were also successfully used in IHC to confirm the presence of RLO in fish tissue sampled from Atlantic salmon *naturally infected* in Ireland.

IFAT was used in this study in order to determine the capacity of the MAbs and PAb-1 to react with the different isolates of *P. salmonis* and to evaluate the potential of the antibodies to detect the bacterium in fish tissue. The bacterium was recognised by the MAbs and PAb-1 produced against *P. salmonis* type strain LF-89, in IFAT as small, pleomorphic, cocco-bacili or as rings, with staining around the outer edges of the bacterium in tissue smears and tissue sections. Lannan *et al.* (1991) developed an IFAT for the detection of RLO in salmonids using blood films, tissue smears, tissue sections and cell culture smears infected with rickettsia. The rabbit anti-rickettsia serum used was produced against partially purified and formalin killed whole cell preparations of isolate LF-89. Lannan *et al.* (1991) also observed the bacterium in their samples as rings, with fluorescence around the outer edges of the bacterium. Although extracellular rickettsia were present in the tissue smears and cell culture material, and were generally stained more uniformly than intracellular rickettsia, many of the extracellular rickettsia appeared to have irregular outlines and appeared to be damaged in some way. Lannan *et al.* (1991) did not observe any cross-reaction with Gram-negative (*V. anguillarum* and *A. salmonicida*) or Gram-positive bacteria (*Carnobacterium piscicola*) or in kidney smears from fish infected with *R. salmoninarum*.

Almendras, Fuentealba, Jones, Markham and Spangler (1997) used IFAT for the specific detection of *P. salmonis* in experimentally infected fish using polyclonal antiserum produced against *P. salmonis* strain LF-89 donated by Dr. Fryer. They observed foci of fluorescing microorganisms in liver and spleen serosa, occasionally extended into parenchymal tissue. Kuzyk, Thornton and Kay (1997) indicated that *P. salmonis* had antigens localised on the surface of the bacterium since their rabbit sera recognised a large amount of fluorescence around the cell perimeter of the bacterium by IFAT. Jamett *et al.*

(2001) found that the MAbs produced bright ring-shaped with all of the isolates of *P. salmonis* from Chile tested by IFAT. Kidney and brain smears obtained from infected salmon also showed bright fluorescence of the bacterium, with little or no background when tested with MAbs by IFAT in that study (Jamet *et al.* 2001).

The cross-reactivity of the rabbit anti *P. salmonis* serum (PAb-1) developed in this Chapter was examined with other Rickettsial species using IFAT. It did not cross react with *W. pipientis* belonging to tribe *Wolbachieae* family *Rickettsiaceae* presented in *D. melanogaster* embryos. *W. pipientis* belongs to the same genus and tribe as *Wolbachi persica.* When *W. persica* was used for phylogenetic analysis of the *P. salmonis*, using 16 small subunit ribosomal ribonucleic acid (16S rRNA) gene sequences, both were found to be a member of the gamma subgroup of the Proteobacteria and *P. salmonis* type species LF-89 was closely related to *W. persica* (Fryer, Lannan, Giovannoni & Wood 1992). Although, *W. persica* was originally classified as a *Wolbachia* on ultrastructural evidence, later it has been revealed to be distinct from other *Wolbachia* based on its 16S sequence. This could be the reason why PAb-1 did not give any cross-reaction with *W. pipientis.* It was not possible to test the cross-reaction of *W. persica* with PAb-1 by IFAT.

Fryer *et al.* (1992) suggested that *P. salmonis* might be a member of the tribe *Ehrlichieae*, and Lannan *et al.* (1991) also assessed the presence of rickettsia in animals infected with the members of *Ehrlichieae* by IFAT. The serum from a dog infected with canine granilocytic Ehrlichia (*Ehrlichia canis*) had a fluorescent antibody titer of 64 against rickettsia isolated from coho salmon, *Oncorhynchus kisutch,* although it did not react with sera from a horse infected with *Ehrlichia equi* and from a dog infected

with *Neorickettsia helminthoeca,* salmon-poisoning disease. *E. phagocytophila* is in the same genus as *E. canis, E. equi* and *Ehrlichia ristic* all of which were subjected to phlogenetically analysis by Mauel, Giovannoni and Fryer (1999). The rickettsias *E. equi, E. canis* and *E. ristic* have 77.6, 76.7 and 75.6% 16 small subunit ribosomal deoxyribonucleic acid (16S rDNA) similarities to that of *P. salmonis.*

Granulocytic ehrlichiosis is an emerging infectious disease in humans, closely related to *E. phagocytophila* and *E. equi. E. phagocytophila* is also the etiological agent of tick-borne fever in sheep, goat and cattle in Europe (Ravyn, Goodman, Kodner, Westad, Coleman, Engstrom, Nelson & Johnson 1998). Magnarelli, Mather and Yeh (1995) cited that Chen, Dumler, Bakken and Walker (1994) and Bakken, Dumler, Chen, Eckman, Van Etta and Walker (1994) reported that sera from human's with granulocytic ehrlichiosis presumably caused by an infectious organism (closely related organism to *E. phagocytophila* and *E. equi*) did not cross react with *E. canis* or *E. chaffeensis* by indirect fluorescence antibody stainings methods. The primary method used for detecting antibodies to Ehrlichia has been the indirect immunofluorescence assay, in which neutrophils from experimentally infected horses have been examined for the presence of *E. equi* (Ravyn *et al.* 1998). In this study, MIFAT was used to test cross-reactivity of *E. phagocytophila* with the MAb, as well as PAb-1 and PAb-2. Although some microscope slides bearing ovine neutrophils infected with *E. phagocytophila* showed some positive white dots in MIFAT and some red reaction with Acridine orange staining, associated with the neutrophils, slightly similar structures were observed on negative control slides. Antigenic similarity between *E. phagocytophila* and *P. salmonis* type strain LF-89 were therefore not confirmed in this study and requires further analysis.

The result of the IGS with the MAbs showed that they recognised surface material on the bacterium as well as cytoplasmic material associated with the cell surface of the organism. Kuzyk *et al.* (1997) indicated that rabbit anti-*P. salmonis* sera specifically labelled the surface of the bacterium by IGS. Kuzyk *et al.* (1997) also analysed purified *P. salmonis* with an impermeant alkylating reagent, sulfo-NHS-biotin, to determine if any immunoreactive proteins comigrated with major *P. salmonis* surface proteins. Following biotinylated labelling of the proteins, the bacteria were detected by streptavidin-conjugated alkaline phosphatase and one major *P. salmonis* protein observed was evident at 28 kDa with another four located between 45 and 80 kDa. Some other minor proteins were also observed, but the major proteins were different to those seen on the CHSE-214 cells. However, the number of minor biotinylated proteins identified could suggest the some damage of *P. salmonis* occurred during purification (Kuzyk *et al.* 1997).

Although, the PAb-1 showed some cross-reactivity with a few non-*P. salmonis* bacteria, which could just be due to some non-specific binding of the antisera with ELISA, the type species LF-89 and the isolates AVG5/268, VQ013, R980769, SRS-4 and SRS-UACH showed very strong reactivity with the same technique. Absorption of the antisera with the cross-reacting bacteria may have improved the specificity of ELISA, however, no attempt was made here to reduced the cross-reactivity of the antisera. The PAb-1 can be used for the rapid diagnosis of *P. salmonis* with higher sensitivity than in other serodiagnostic tests such as immunblotting, IHC or IFAT using infected fish tissue with appropriate controls. The MAbs produced in this study proved useful to use in serological diagnostic tests since they did not cross-react with any of the non-rickettsial bacteria examined, not even with the non-*P. salmonis* bacteria which had

cross-reaction with PAb-1 and CHSE cells in SELISA in which PAb-1 was used as the captured antibody. The MAbs are also useful for the development of a vaccine against *P. salmonis* with identification of potential immunogenic vaccine antigens. The isolates AVG5/268, VQ013 and SRS-UACH and the type species LF-89 gave a stronger reaction with all MAbs than the other isolates in all techniques used in this study. Isolate R-29 from Atlantic salmon, Chile and SLGO-95 from coho salmon, Chile seemed different to the other isolates since they reacted less intensely in the tests. Isolate SRS-4, R980769 and R-61 also showed some similarities in their reaction in the different tests used. The reason for the slight differences in the reactivity between isolates is possibly due to differences in the virulence of the isolates or recognition of different antigens in the different techniques used. The sensitivity threshold of the ELISA using MAbs developed in this study was slightly higher with sonicated material rather than whole cell preparations of *P. salmonis* type species LF-89 suggesting that more antigens were revealed upon rupturing the bacteria. On the other hand, the reactions of each *P. salmonis* isolate in the different test were comparable, apart from isolate R-62. The reason for the poor reactivity of *P. salmonis* isolate R-62 with the MAbs in SELISA could be due to the polyclonal antiserum (PAb-1) used as the captured antibody did not react very strongly this isolate. This isolate did react strongly with most of the MAbs in the different immunblotting methods (line blot and WB) or without denaturing the antigen (dot blot). However, R-62 did not react with any of the MAbs in IFAT test. While isolate SLGO-95 was not recognised with MAb 6A1G3 in dot blot, it reacted with the MAb in the line blot and WB tests. Differences in the preparation of the antigen used may have resulted with revelations of different antigens in the different serodiagnostic methods. The antibodies used in this study proved useful for the detection of rickettsiae in disease tissue and in the identification of new *P.*

salmonis. MAb 6A1F4C5 or 6A1F4G3 appeared to have a stronger reaction with *P. salmonis* isolates but 8G7 also reacted more strongly in IFAT and IHC besides line blot and dot blot techniques appeared to be the best MAb to use as a diagnostic tool. Further studies could also help to determine whether or not anti-*P. salmonis* MAbs can passively protect fish against piscirickettsiosis.

CHAPTER VI

EXPERIMENTAL CHALLENGE AND IMMUNISATION OF ATLANTIC SALMON WITH *PISCIRICKETTSIA SALMONIS*

6.1. INTRODUCTION

P. salmonis, an obligate intracellular organism, is the causative agent of the epizootic disease, piscirickettsiosis (Fryer & Lannan 1996). Both the intracellular nature of rickettsia and variations in the virulence of different isolates makes it difficult to standardise an effective experimental challenge model for the bacterium. Differences in the experimental conditions used to artificially infect fish make it difficult to compare the outcome of the challenge results reported in the literature, since different *P. salmonis* isolates, different fish species and different doses of the bacterium were used in the challenges reported (Cvitanich, Garate & Smith 1991; Garcés, Larenas, Smith, Sandino, Lannan & Fryer 1991; Smith, Contreras, Garcés, Larenas, Oyanedel, Reno & Fryer 1996; and Almendras, Fuentealba, Jones, Markham & Spangler 1997).

The clinical signs and gross pathology reported in both the naturally and experimentally infected fish are similar (Cvitanich *et al.* 1991), however, the clinical signs observed tend to vary between outbreaks of the disease and also between individual fish (Turnbull 1993). Fish may appear dark in colour, show lethargy, anorexia, respiratory stress, and swim near the surface of the water. Skin lesions range from small areas of raised scales to shallow ulcers and white nodules. Internally, fish may have ascites, peritonitis, general pallor and petechial hemorrhages of the gastrointestinal tract, pyloric caeca, swimbladder, visceral fat and caudal intestine. The most characteristic lesions appear as white to yellow subcapsular nodules found throughout the liver or as diffuse

swelling and multifocal pale areas in the kidney and spleen. The histological changes are usually classified as necrosis and inflammation in the liver, spleen, intestine and kidney. There may be oedema of the hematopoietic tissue with a granulomatous response and renal tubular degeneration. The condition is specifically characterised by disseminated intravascular coagulation with necrosis of thrombi and vascular endothelium, and perivascular cellular inflammation, endocarditis, variable pericarditis and focal hyaline necrosis of the myocardium in the heart; necrosis and inflammation of the lamina properia in the intestine; multifocal epithelial hyperplasia resulting in lamellar fusion in the gills. Varying degrees of necrosis of the epidermis, dermis and underlying musculature and meningoencephalitis can also be observed. Rickettsia-like organisms (RLO) are mostly observed in macrophages and in the cytoplasm of other infected cells, but can also be found extracellularly because of cell lysis (Branson & Nieto Diaz-Munoz 1991; Cvitanich *et al.* 1991; Turnbull 1993; Almendras & Fuentealba 1997; Fryer & Lannan 1996). However, some fish die with very few signs of the disease (Turnbull 1993).

The two first studies to artificially produce piscirickettsiosis by experimentally infecting Atlantic salmon *Salmo salar* and coho salmon *Oncorhynchus kisutch* by intraperitoneal (IP) injection of the bacterium were reported by Cvitanich *et al.* 1991 and Garces *et al.* (1991). Garces *et al.* (1991) injected 0.05 ml aliquots of cell culture medium containing rickettsia at a dose of between $10^{3.3}$-$10^{5.3}$ 50% Tissue culture infective dose ($TCID_{50}$) per fish. The rickettsial isolate used had originated from diseased coho salmon in southern Chile, and had been cultured in CHSE-214 cells. Fish were approximately 10 g in weight. Mortalities which resulted from this challenge, which was carried out in freshwater at a water temperature of 10.5°C, ranged from 88 to 100% over the 42 days

(d) experimental period. Typical signs of the disease were evident in the experimental coho salmon such as lethargy, anorexia, ascites, appearing dark in colour, pale gills and liver, enlarged spleen, congested kidney and spleen, haemorrhages at the base of the fins and petechial haemorrhages on the pyloric caeca, while no signs of the disease were apparent in inoculated Atlantic salmon. In both reports, the authors were able to isolate rickettsia from moribund fish. Cvitanich *et al.* (1991), however, injected coho salmon held at 15°C with a dose of 6 $x10^4$ and 6 $x10^5$ infective RLO units (IRU) using an isolate which originated from moribund coho salmon naturally infected in Puerto Montt, Chile, which had been cultured in *Oncorhyncus keta* heart (CHH-1) cells. Infected fish died between 7 and 9 days and 10 and 11 days post-infection (dpif) when maintained in seawater or in freshwater, respectively. Experimentally infected fish showed similar signs of the disease to those seen in naturally infected fish. Smith *et al.* (1996) also experimentally infected coho salmon and rainbow trout *Oncorhynchus mykiss* in freshwater to examine the virulence of *P. salmonis* LF-89. They used a ten fold serial dilution of a rickettsial supernatant (from 10^3 to 10^{-3} $TCID_{50}$ ml^{-1}), administered by IP injection (0.05 ml $fish^{-1}$) and fish were maintained at a water temperature of 12°C. Deaths began to occur in infected fish in the third week of the trial, with typical signs of the disease evident in both species. Sixty percent of coho salmon and 28.3% of rainbow trout, injected with the highest concentration of pathogen (10^3 $TCID_{50}$ ml^{-1}), died within 75 dpif.

Since the first isolation of *P. salmonis* in 1989, similar organisms have been described world wide, but the associated outbreaks were not as serious as those that occurred in Chile. To determine if this was due to differences in virulence among isolates of *P. salmonis*, a study was conducted by House, Bartholomew, Winton & Fryer (1999).

They compared three *P. salmonis* isolates; the type strain LF-89, isolate ATL-4-91 obtained from netpen-raised Atlantic salmon in Canada (serial dilutions of 10^5, 10^4 and 10^3 $TCID_{50}$ ml^{-1}) and isolate NOR-92 obtained from Atlantic salmon in Norway (serial dilutions of $10^{4.6}$, $10^{3.6}$ and $10^{2.6}$ $TCID_{50}$ ml^{-1}). Three replicates of 30 coho salmon were injected IP (0.1 ml) with each of the three concentrations of each bacterium and fish were maintained in freshwater at 11°C. The fish injected with the highest concentration of isolates LF-89 and ATL-4-91 had the highest level of mortality with 97 and 92% of fish dying respectively, 41 dpif, while 41% of fish injected with isolate NOR-92 at a concentration of $10^{4.6}$ $TCID_{50}$ ml^{-1} died over same period. However, by Day 87 post-inoculation, 70% of fish in this group had died. House *et al.* (1999) reported that the differences in virulence among the isolates were statistically significant ($p < 0.0001$), and have been important for the management of affected stocks of fish. *P. salmonis* were re-isolated from mortalities and all three isolates caused typical signs of piscirickettsiosis with no obvious difference in gross pathology between the isolates.

After the first report of rickettsial disease in salmon cultured in seawater netpen farms in Chile in 1989 (Fryer, Lannan, Garcés, Larenas & Smith 1990), the disease has since been reported in both salmonids and non-salmonid fish in different locations and different aquatic environments world-wide (Fryer & Lannan 1996). Despite the increasing number of reports of the disease in recent years, knowledge about the aetiological agent and the immune response of fish against the microorganism are still limited, although two different vaccination trials have been reported by Smith, Lannan, Garcés, Jarpa, Larenas, Caswell-Reno, Whipple and Fryer 1995 and Smith, Contreras, Larenas, Aguillon, Garcés, Perez and Fryer 1997. In the first study, a field trial was performed in which formalised *P. salmonis* ($10^{6.7}$ $TCID_{50}$ ml^{-1}) were administered by IP

injection, either once or twice into coho salmon weighing 16-19 g, held at 7-8°C. The vaccinated group experienced a lower level of cumulative mortalities than the non-vaccinated group when fish were naturally challenged by transferring them from freshwater to a seawater site with a history of endemic piscirickettsiosis. The fish were kept at the site for the 20 week trial period, to be naturally exposed to the pathogen.

In the second trial formalin killed bacteria at different concentrations ($10^{6.4}$ and $10^{7.1}$ $TCID_{50}$ ml^{-1}) were injected IP into rainbow trout either with or without a booster injection four weeks after the first immunisation. The fish were then artificially challenged using an IP injection of *P. salmonis* four weeks after the booster vaccination. The cumulative mortality level seen in the control fish was low (20%), but was higher than the level obtained in the vaccinated fish which ranged from 2 to 4%. The antibody response of the vaccinated fish was measured using a radioimmunoassay in which sera against *P. salmonis* obtained from vaccinated coho salmon and rainbow trout as well as naturally infected salmonids was tested. The sera from immunised and experimentally challenged fish had counts of approximately 700 to 2500 count per minute (cpm). The pooled sera from naturally infected fish which had survived a disease outbreak also had an antibody response approximately 2000 and 4000 cpm, for rainbow trout and coho salmon respectively, whereas the negative control sera from both species had counts less than 900 cpm (Smith *et al.* 1997).

Vaccination has been shown to be a useful tool for controlling infectious diseases in fish (Ellis 1988). Although *P. salmonis* appears sensitive to some antibiotics, their use has not been successful in controlling the disease. To develop a successful vaccine against the organism further knowledge is required about the aetiological agent

involved and the antibody response elicited against the organism by the fish as the disease progresses. The current study was used to establish a reproducible challenge model for the bacterium, so as to allow the pathogenesis of the disease and the virulence of to be examined. The antibody response of immunised fish against various *P. salmonis* isolates was also examined using Enzyme-linked immunosorbent assay (ELISA), Western blot (WB), Indirect fluorescent antibody technique (IFAT), Immunohistochemistry (IHC), and Immuno-gold staining (IGS) to determine if there were any differences between the reactivity of antisera against various *P. salmonis* isolates. Plaque neutralisation assay (PNA) was also performed in this study to determine if the presence of *P. salmonis*-specific antibodies in fish sera prevents the infection of the bacterium. This study is the first report to describe the antibody response of Atlantic salmon and the potential of using these sera to identify and to characterise the bacterium.

6.2 MATERIAL AND METHODS

6.2.1. Experimental challenge and re-isolation of rickettsia

6.2.1.1. Bacterial preparation

The *P. salmonis* isolates outlined in Table 2.1 were used in the current study. The bacteria were grown in chinook salmon, *Oncorhynchus tshawytscha* embryo (CHSE-214) cells as described in Section 2.1.1.2. Briefly, the tissue culture flasks (500 cm^2), were seeded with CHSE-214 cells at a concentration of 5 $x10^4$ cells ml^{-1}, and incubated for 48 hours (h) at 22°C until a monolayer of cells with 50-70% confluence was established. The monolayer was then inoculated with supernatant recovered from *P. salmonis*-infected cell cultures (see Section 2.1.1.2). After an extensive cytopathic effect (CPE) was obtained (approximately 90%), between 10 and 15 d, the supernatants

were collected from each flask using centrifugation at 20 000 x g for 30 min at 4°C. The suspended pellet in PBS (5.2 millimolar (mM) KH_2PO_4, 8.1 mM Na_2HPO_4, 116 mM NaCl, 10 mM KCl, pH 7.0) was then homogenised, vortexed and centrifuged at low speed (210 x g) for 10 minutes (min) at 4°C (Mistral 3000i, MES) to remove the host cell debris (Section 3.2.2). The rickettsia in the supernatant were then collected and washed twice with PBS, centrifuging at 20 000 x g for 30 min at 4°C to eliminate Foetal calf serum (FCS) residues. Protein concentrations of the rickettsial preparations were determined using a protein determination kit (BioRad Richmond, C, USA) (Section 2.1.3.1). The sterility of the bacterial preparation was confirmed by looking for a CPE on CHSE-214 cells, and also by culturing on tryptone soya agar (TSA) and TSA with 1% (w/v) NaCl.

6.2.1.2. Experimental infection

Atlantic salmon (20-25 g) used for the infection experiment, were maintained in a flow-through filtrated seawater system had been and was maintained at 11°C. Groups of ten fish were placed in separate tanks (50 l) and each group were inoculated with one of the rickettsial isolates shown in Table 2.1 (except the isolate SLGO-95), by IP injection at a dose of $10^{4.6}$ $TCID_{50}$ ml^{-1} (0.1 ml $fish^{-1}$). Non-injected fish were used as the control group and were held in a separate tank under the same conditions as the infected fish. Pre-infected sera were collected before injection, then one fish was sampled from each group 15 dpif. The presence of bacteria in the kidney of sampled fish was confirmed using an IFAT (see Section 6.2.1.3). Attempts were made to re-isolate bacteria from the kidney of infected fish at this time as described below. However, no mortalities occurred in any of the groups apart from 2 dead fish infected with the isolate SRS-4 at 27 dpif. Since the levels of mortalities which occurred were not significant, fish were

given a second injection of *P. salmonis* 4 weeks after the first injection. The dose and experimental conditions are as described for the first infection. One fish from every group was sampled for IFAT and an attempt was made to re-isolate the bacterium from the kidney at this time. One more fish infected with isolate SRS-4 died 12 d after the second infection while 1 fish infected with isolate R-62 died 20 d after the second injection. Two months after the initial injection, blood and fish tissue samples were collected from all remaining fish and the experiment was terminated. Serum was collected from the blood samples by centrifugation at 13 000 x g for 10 min in a microcentrifuge after storing overnight at 4°C. Tissues (kidney, liver, spleen) were also sampled at 2 weeks, 1 month and 2 months over the course of the experiment, fixed in 10% buffered formalin and embedded in wax. These were used to examine pathological changes histologically under a light microscopy (LM).

6.2.1.3. Indirect fluorescent antibody screening of infected samples

Air-dried smears, prepared from infected kidneys, were fixed in absolute methanol for 5 min. Samples were incubated with rabbit anti-*P. salmonis* (kindly provided by Prof. J. Fryer, Oregon State University, Corvallis, OR, USA) and diluted 1/1000 in PBS (0.02 M Phosphate, 0.15 M NaCl, pH 7.2), for 60 min in a humidified chamber at 22°C. Negative controls of PBS were also included. Slides were rinsed thoroughly with PBS, left to stand for 5 min in PBS, then finally rinsed once again before applying goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Inc., St Louis, Missouri USA) diluted 1/80 in PBS, to the smears for 30 min. Slides were incubated in the dark in a humidified chamber at 22°C during this time, after which they were again washed with PBS as above. Smears were observed for the presence of

bacteria under an Olympus IMT-2 microscope with a reflected fluorescent attachment, and exciter and barrier filters for FITC.

6.2.1.4. Re-isolation of *P. salmonis* from infected tissue

Bacteria were re-isolated from infected kidney tissue by aseptically removing kidney samples and homogenising them in 1:10 with Minimal Essential Medium (MEM) (w/v). The homogenates were left to stand for 5 min at 22°C to allow large particles to settle out, before placing 0.5 ml of the suspension onto a CHSE-214 cell monolayer, prepared previously in 25 cm^2 flasks as described in Section 2.1.1.2. The flasks were incubated at 15°C for 2 h, before removing the supernatant and adding 5 ml of fresh MEM supplemented with 2% (v/v) FCS and 2 mM L-glutamine. The cultures were incubated at 15°C for 20 to 30 d and observed daily for the development of a CPE.

6.2.1.5. Determination of anti-*P. salmonis* antibody levels in the serum of infected fish and levels of *P. salmonis* present in their tissue using ELISA

Blood or kidney tissue was collected from each of the sixty seven remaining fish. Samples were analysed by ELISA to determine serum antibody titres of infected fish and level of *P. salmonis* present in infected tissue respectively. Each fish was given an identification number to allow serum antibodies and the level of *P.* salmonis in their tissue to be compared for individual fish.

Experimental fish infected with isolate LF-89 are referred to as L-1 to L-8; fish infected with isolate AVG5/268 are referred to as A-1 to A-8; fish infected with isolate R980769 are referred to as R-1 to R-8; fish infected with isolate VQ013 are referred to as V-1 to V-8; fish infected with isolate SRS-4 are referred to as Sr-1 to Sr-5; fish infected with

isolate SRS-UACH are referred to as Su-1 to Su-8; fish infected with isolate R29 are referred to as R9-1 to R9-8; fish infected with isolate R61 are referred to as R1-1 to R1-8 and fish infected with isolate R62 are referred to as R2-1 to R2-7.

(a) The first ELISA described in Section 2.4.1 was used to measure the serum antibody response of fish experimentally infected with *P. salmonis*. Briefly ninety-six-well ELISA plates were coated with antigen using each of the isolates used to experimental challenge the fish (1.5 microgram (μg) ml^{-1} protein). Two-fold dilutions of each of the fish sera sampled for the different groups of fish, including the control group, and the pre-injected fish serum were added to duplicate wells of the ELISA plates. Rabbit polyclonal antibody (PAb) developed against *P. salmonis* in Chapter V was used as a positive control for the assay, while PBS was used as a negative control. The plates were incubated with an anti-salmon IgM monoclonal antibody (MAb) (clone 4C10) (Courtesy of Dr. Ann Thuvander, Department of Pathology, Swedish University of Agricultural Science, S-75007 Uppsala, Sweden) [100 microliter (μl) $well^{-1}$], followed by an Horseradish peroxidase (HRP)-conjugated anti-mouse-IgG (Diagnostics Scotland) diluted 1/1000 in PBS [0.02 Molar (M) Phosphate, 0.15 M NaCl, pH 7.2]. Anti-rabbit IgG-HRP (Diagnostics Scotland) diluted 1/1000 in PBS was added to the wells containing the rabbit serum.

(b) Cross-reactivity of antisera obtained from infected fish against *P. salmonis* type strain LF-89 was also measured using a sandwich ELISA. The assay was performed using the protocol described in Section 2.4.2. Acid-treated rabbit polyclonal antibody (10 μg ml^{-1}) produced in Chapter V was used as the capture antibody. Percoll density gradient purified *P. salmonis*, isolate LF-89, (see Section 3.2.2), was used as antigen

(10 μg ml^{-1}), and each of the anti *P. salmonis* fish sera, diluted 1/20 in PBS, were applied to duplicate wells of the ELISA plate. The plates were then incubated with MAb 4C10 supernatant followed by HRP-conjugated anti-mouse-IgG (Diagnostics Scotland). Control fish serum and MAb 6A1F4C5 produced in Chapter V against type strain LF-89 used as negative and positive control, respectively.

(c) The third ELISA system used was a sandwich assay (see Section 2.4.2) developed to measure the level of bacteria present in the kidney of experimentally infected fish. Acid-treated rabbit polyclonal antibody (10 μg ml^{-1}) produced in Chapter V was used as the capture antibody. The ELISA plates were then incubated with kidney tissue, sampled from experimental fish, which had been homogenised and diluted with PBS (100 μl well^{-1}). A cocktail of monoclonal antibodies (MAbs) developed in Chapter V (6A1F4C5, 6A1F4G3, 8G7, 6A1F4G6) was used as the secondary antibody to detect rickettsia present in the tissue, and HRP-conjugated anti-mouse-IgG (Diagnostics Scotland) was used to detect binding of the MAbs to rickettsia. The assay was also performed using preparations of *P. salmonis* LF-89 and kidney from uninfected fish spiked with purified *P. salmonis* LF-89 as positive controls, or kidney tissue sampled from uninfected fish as a negative control.

6.2.1.6. Western blot (WB) analysis using sera sampled from experimentally infected fish

WB analysis, as described in Section 2.3.1, was used here to examine the antigen profiles of *P. salmonis* isolate R-29 recognised by the anti-*P. salmonis* sera sampled from fish remaining at the end of the experimental period (See Section 6.2.1.2). The fish sera were diluted 1/20 in Tris buffer saline (TBS), pH 7.5 for the analysis using a

multiscreen apparatus (BioRad) and Avidin-Biotin-Peroxidase complex (ABP) was used to amplify the reaction.

6.2.1.7. Haematoxylin and Eosin (H&E) staining of tissue sections

Wax embedded blocks of tissue sampled from experimentally infected fish at 15 d, 1 month and 2 months post-primary injection, were cut to a thickness of 0.5 micrometer (μm). The sections were air-dried in the oven and then dewaxed in xylene (5 min), dehydrated in absolute alcohol (2 min) and methylated spirits (1.5 min), and rinsed in tap water (2 min) before staining with Haematoxylin (5 min). The sections were then rinsed with tap water. After 3 quick dips in acid alcohol (1%), the sections were washed with tap water (1 min), Scott's tap water (1 min) and again in tap water (1 min), respectively. The sections were stained with Eosin (5 min), washed briefly with tap water (0.5 min) and dipped in methylated spirits (0.5 min). The sections were dehydrated with 70% (2 min) and 100% alcohol (1.5 min) and placed in xylene for 5 min, after which the slides were mounted. The air-dried sections were also examined by IHC according to the protocol described in the Section 2.9.

6.2.2. Immunisation of fish with *P. salmonis*

6.2.2.1. Bacterial preparation

P. salmonis isolates, LF-89 (type-strain), SLGO-95 and R-29, detailed in Table 2.1, were used for this study. The bacterial samples were prepared as described in Section 6.2.1.1. Rickettsia were pelleted by centrifugation at 20 000 x g for 30 min at 4°C, resuspended in PBS (5.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 116 mM NaCl, 10 mM KCl, pH 7.0). The bacteria were then heat killed at 58°C for 60 min. Protein concentrations of the rickettsial preparations were determined using a protein determination kit

(BioRad Richmond, C, USA) (Section 2.1.3.1). The viability and sterility of the bacterial preparation was confirmed by looking for a CPE on CHSE-214 cells, and also by culturing it on TSA and TSA with 1 % (w/v) NaCl, respectively.

6.2.2.2. Immunisation of fish

Atlantic salmon *(Salmo salar)* parr (9-12 g) were maintained in a flow-through filtered freshwater system at 15-17°C. The tank volume used was 25 l. Thirty six fish (three tanks with 12 fish per tank) were used for each isolate. The fish were immunised IP (0.1 ml $fish^{-1}$) with the preparations of the heat–killed rickettsia at a concentration of 10^6 $TCID_{50}$ ml^{-1} *P. salmonis* (LF-89, SLGO-95 and R-29) mixed 1:1 (v/v) with Adjuvant Montanide ISA 711 (Aquaculture Vaccines Ltd).

Four different groups of fish were used as controls. One group containing 12 fish was injected with PBS mixed 1:1 with adjuvant, one group of 12 fish was injected with a mixture of CHSE-214 cells mixed 1:1 with adjuvant, one group of fish (6 fish) was injected with only PBS and the final group (12 fish) were maintained without injection. All injected fish received 0.1 ml of sample.

Blood was sampled from experimental fish 4 times at 2 weeks intervals throughout the 8-week experimental period. Fish were randomly selected, with 80% of the population sampled at each sampling period. At the end of the eighth week, blood was sampled from all fish and the experiment was terminated.

6.2.2.3. ELISA with immunised fish serum

The ELISA described in Section 2.4.1 was used to examine the antibody response of fish immunised with the different *P. salmonis* isolates. Briefly ninety-six wells ELISA plates coated with *P. salmonis* isolates LF-89, SLGO-95 and R-29 (5 μg ml^{-1} protein). Two-fold dilutions of individual fish sera sampled from all groups were prepared, using PBS as the diluent, and these were added to the wells of the ELISA plates in duplicates. Unimmunised fish sera diluted 1/20 in PBS was added to the plates as a negative control and PBS was also used as a negative control. They were then incubated with anti-trout IgM MAb 4C10 (Courtesy of Dr. Ann Thuvander, Department of Pathology, Swedish University of Agricultural Science, S-75007 Uppsala, Sweden) and with HRP-conjugated anti-mouse-IgG (Diagnostics Scotland). The cross reactivity of these sera with all of the *P. salmonis* isolates shown in Table 2.1 was also examined using this ELISA. The reactions were read with an ELISA reader (Dynatech) at 450 nanometer (nm).

6.2.2.4. IHC with immunised fish sera

The IHC procedure was performed according to the method described in the Section 2.5. Briefly, paraffin-embedded tissue sections prepared from Atlantic salmon, affected by RLOs in Ireland, were used in the application. Anti *P. salmonis* fish sera diluted 1/10 in PBS (0.02 M Phosphate, 0.15 M NaCl, pH 7.2), were applied to the sections and incubated for 2 h in humidified chamber at 22°C. One slide was incubated with PBS instead of antiserum as a negative control. The slides were washed three times with TBS, pH 7.5 and incubated with MAb supernatant of cell line, 4C10 (100 μl well^{-1}) for 2 h at 22°C. The slides were washed three times again with TBS. Anti-mouse-HRP (1/50 in TBS) (Diagnostics Scotland) was applied to the slides. At the end of the

procedure the reaction was developed and counter stained using True blue and Contrast red (Kirgegaard Perry Laboratories, UK) respectively, after which slides were examined under a LM.

6.2.2.5. IFAT analysis of tissue sections using immunised fish sera

The IFAT analysis was performed according to the method described in the Section 2.6. Paraffin-embedded tissue sections prepared from Atlantic salmon, affected by RLOs in Ireland were used. FS-1, FS-2 and FS-3 diluted 1/10 in PBS (0.02 M Phosphate, 0.15 M NaCl, pH 7.2), were used as primary antibody. The control slide was incubated with only PBS. The results were observed under an Olympus IMT-2 microscope with a reflected fluorescent attachment, and exciter and barrier filters for FITC.

6.2.2.6. WB analysis of *P. salmonis* using immunised fish sera

WB analysis was performed as described in Sections 2.3.1 using the fish sera raised against the different *P. salmonis* isolates as described in Section 6.2.2.2. The fish sera were diluted 1/20 in TBS for the analysis. All analyses were amplified with ABP.

6.2.2.7. Immuno-gold staining (IGS) of *P. salmonis* using immunised fish sera

IGS of *P. salmonis* was performed as described in Section 2.7 using purified *P. salmonis* (type strain LF-89 and isolates R-29 and SLGO-95) and immunised fish sera. LR white resin (London Resin Co., Reading, England) was used to prepare the tissue sections in capsules and the samples were then cut into ultra-thin sections and mounted on nickel grids. The grids were floated up-side down on drops of wash buffer [Wb: 0.2 M TBS, 1%Tween, 1% Bovine serum albumine (BSA)] containing 10% FCS which had been spotted onto a piece of a parafilm placed in a moist container, to block non-

specific binding, overnight at 4°C. The grids were placed in the immunised fish sera, including control fish serum, diluted 1/10 in the Wb and incubated overnight at 22°C. The sections were washed twice with Wb by floating them in a 24-well plate with occasional agitation for 90 min each wash. The grids were then incubated in cell supernatant from MAb cell line 4C10 against salmon IgM (Courtesy of Dr. Ann Thuvander, Department of Pathology, Swedish University of Agricultural Science, S-75007 Uppsala, Sweden), overnight at 22°C, and washed as above. The samples were placed side-down on drops of anti-mouse IgG gold conjugate, 5 nm (Sigma), diluted 1/40 in the wash buffer and again incubated overnight at 22°C. The samples were washed as described above, and then held under a constant flow of distilled water for a *few seconds.*

The 5 nm immunogold labelling was enhanced by placing on drops of silver enhancing solution (British BioCell Int. Cardiff, UK) for 2 min, and the reaction was stopped by immersing the grids through a series of three vials containing distilled water. The sections were counter-stained using uranyl acetate and lead citrate, and viewed under a Phillips 301 electron microscope (EM) at 80 kilovolt (kV).

6.2.2.8. Plaque neutralisation assay (PNA) with immunised fish sera

Six 96-well tissue culture plates were seeded with CHSE-214 cells at a concentration of 5 $x10^4$ cells ml^{-1}, and incubated for between 24 and 48 h at 22°C until a healthy monolayer with 50-70% confluence was established. Two fold serial dilutions of either anti-*P. salmonis* fish sera, normal fish serum as control, MAb 6A1F4C5 supernatant without dilution or rabbit anti *P. salmonis* PAb, 1/1000 in PBS (Gibco) (Chapter V), (100 μl of each) were mixed with supernatant (100 μl $well^{-1}$) recovered from *P.*

salmonis R-29 cultures already added to the wells of plates which were not containing any cell monolayers. Each sample was set up in triplicate. The plates were incubated for 60 min at 15°C with the fish serum, but for 60 min at 37°C with the MAb and PAb. The culture medium was removed from wells of the plates carrying CHSE-214 cell monolayer and the cell monolayers were washed with PBS to eliminate any dead cells. The antigen-antibody mixtures were loaded on the CHSE-214 cell monolayers in the wells. After centrifuging the plate at 200 x g for 10 min, duplicate plates were then incubated for 2 h at 15 °C. The supernatant was removed and a 2.5% (v/v) methylcellulose overlay (Appendix 5.1) was added onto the wells at 250 μl well^{-1}. *P. salmonis* infected cell culture supernatant and uninfected cells were used as positive and negative control respectively.

At the end of the 17 d incubation period, incubating at 15°C, the well contents was fixed with 10 % buffered formalin (v/v) for 15 min and the liquid then removed. The wells were stained with 1% (w/v) Crystal Violet solution (Appendix 5.2) (Sigma) for 15 min and the reaction stopped by washing the wells with tap water. The number of plaques present in the cell monolayer were counted under a LM.

6.3. RESULTS

6.3.1. Experimental infection

6.3.1.1. Re-isolation of *P. salmonis* from infected fish

Only four of the fish experimentally infected with *P. salmonis,* died over the 8 week period of the experimental challenge. These consisted of three fish experimentally infected with isolate SRS-4 and one fish infected with isolate R-62. It was possible to recover the bacterium from fish sampled 2 weeks after injection, and smears prepared

from kidney of injected animals were positive for the bacteria by IFAT at this time. It was also possible to obtain a CPE in CHSE cells inoculated with infected fish tissue one month post-infection.

6.3.1.2. ELISA to determinate level of anti *P. salmonis* antibody in serum and *P. salmonis* levels in infected tissue of experimentally challenged fish

The experiment was terminated two months after initially challenging fish with the bacterium and remaining surviving fish (67 fish) were all bled at this time. Sera collected from the fish were analysed by ELISA using the *P. salmonis* isolate with which the fish had been injected. The mean absorbance at 450 nm of wells incubated with PBS instead of sera as negative control was 0.150. Any value three times or greater than this value was considered positive, and therefore, sera with an Optical density (OD) lower than 0.450 OD_{450} were considered negative. Sera from 31 of the 67 remaining fish had an OD value greater than 0.450 (Figure 6.1) when diluted 1/16 in PBS. The mean absorbance at 450 nm of pre-injected fish sera and control sera were 0.160 and 0.180 respectively. The mean absorbance of the rabbit anti-*P. salmonis* serum used as a positive control was 1.433 at 450 nm with all *P. salmonis* isolates.

Results from the ELISA revealed that 46% of the sera from fish infected with different *P. salmonis* isolates had positive levels of antibody when tested against the *P. salmonis* isolate with which they had been challenged. The frequency distribution of the antibody titers in the sera of survivors tested against the *P. salmonis* isolate with which they had been infected shown in Table 6.1.

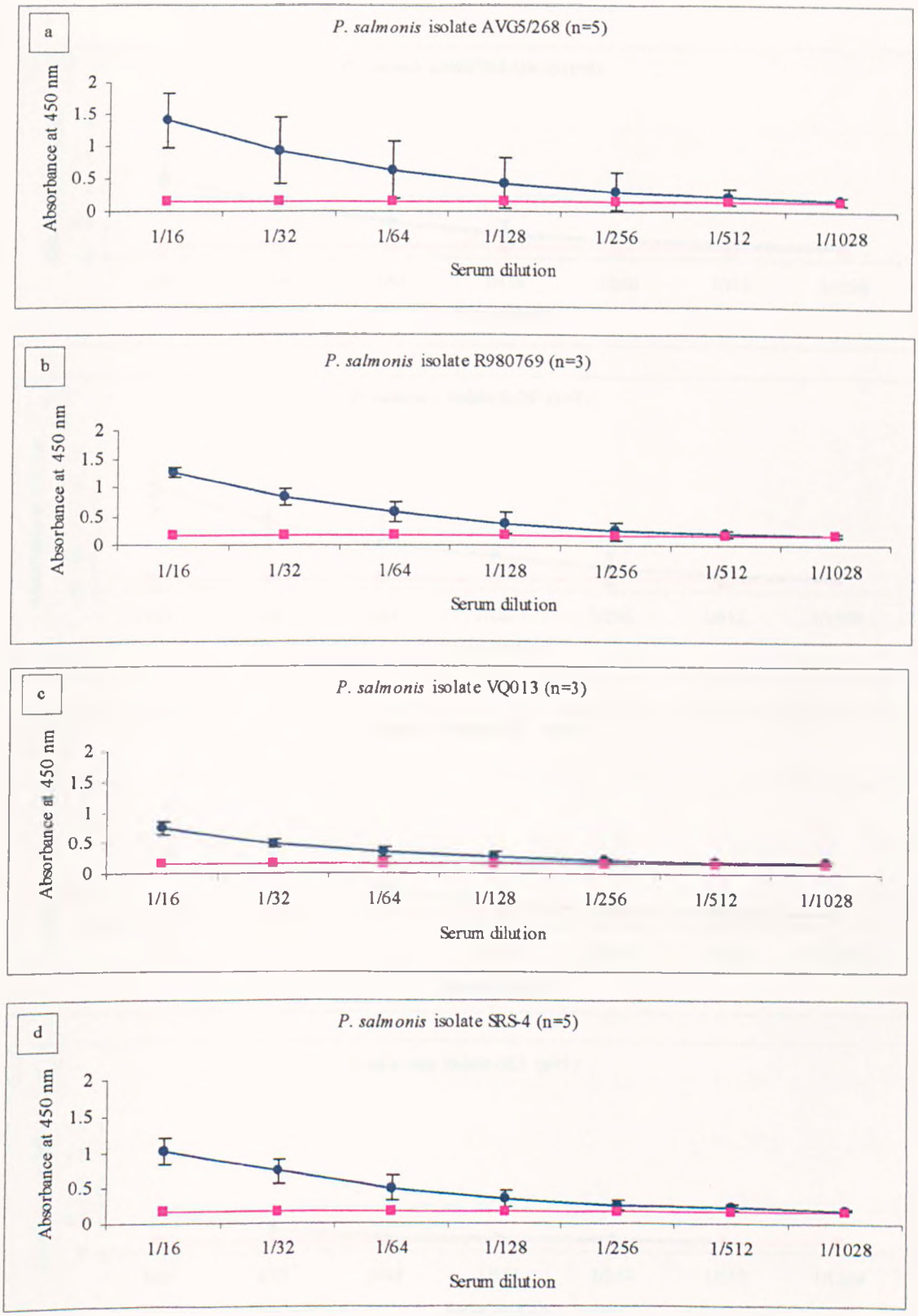


Figure 6.1. Mean values (± SD) of two fold series of anti-*P. salmonis* antibodies in positive fish sera of fish surviving at the end of the experimental challenge with *P. salmonis* isolates (a) AVG5/268; (b) R980769; (c) VQ013 and (d) SRS-4 determined by ELISA. (•) Sera from challenged fish; (■) Sera from fish prior to challenging with *P. salmonis*.

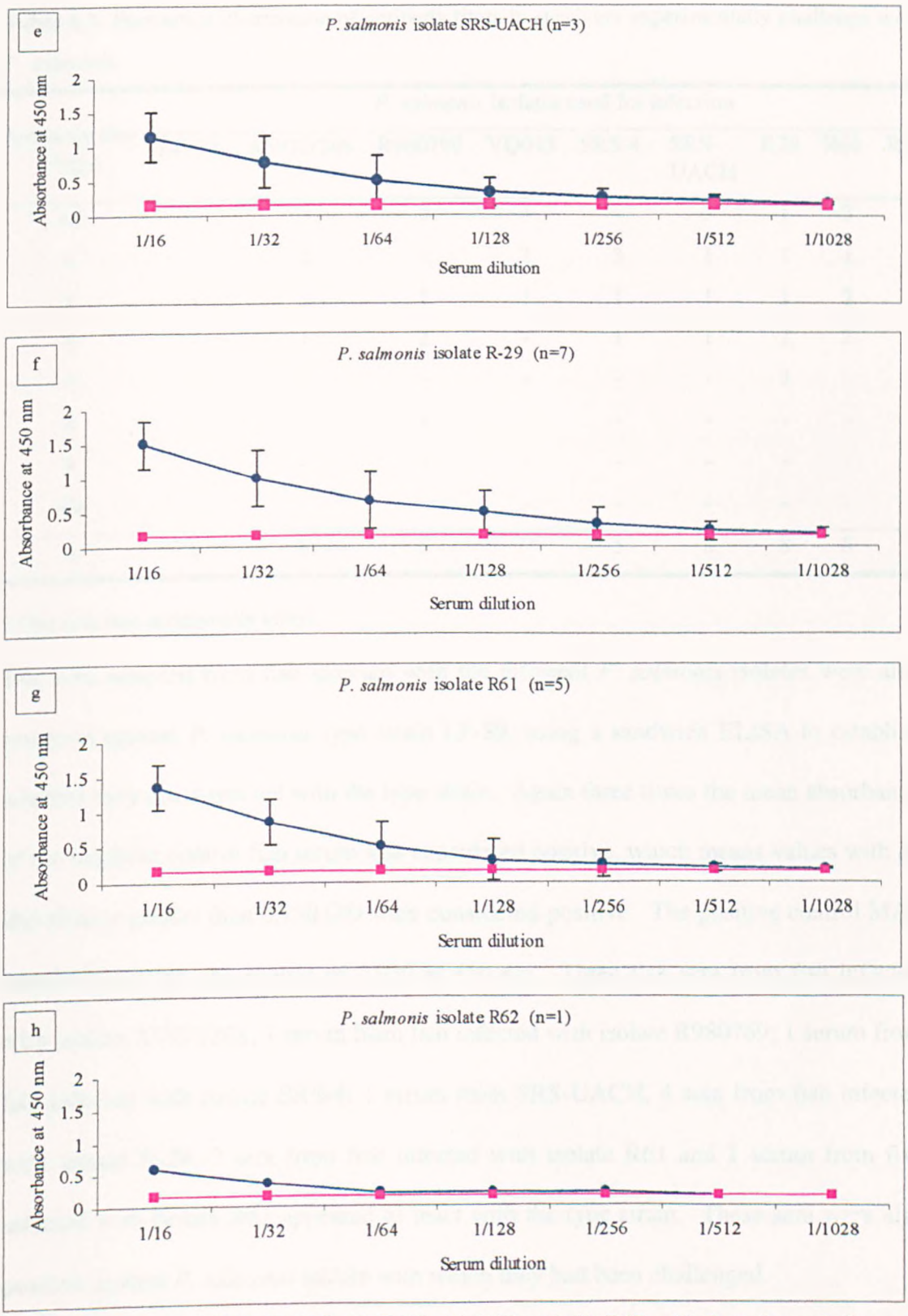


Figure 6.1 Cont. (e) SRS-UACH; (f) R-29; (g) R61 and (h) R62. (•) Sera from challenged fish; (■) Sera from fish prior to challenging with *P. salmonis*.

Table 6.1. Frequency distribution of antibody titers in survivors experimentally challenge with *P. salmonis*

Antibody titer ($-\log_2$)	*P. salmonis* isolates used for infection								
	LF-89	AVG5/268	R980769	VQ013	SRS-4	SRS-UACH	R29	R61	R62
<4	8	3	5	4	-	5	1	3	7
4	-	2	-	2	3	1	1	1	1
5	-	1	1	1	1	1	1	2	-
6	-	1	2	-	1	1	2	2	-
7	-	1	-	-	-	-	3		-
8	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
n	8	8	8	7*	5	8	8	8	7

* One fish was accidentally killed

The sera sampled from fish injected with the different *P. salmonis* isolates were also screened against *P. salmonis* type strain LF-89, using a sandwich ELISA to establish whether they cross-reacted with the type strain. Again three times the mean absorbance of the negative control fish serum was considered positive, which means values with an absorbance greater than 0.750 OD were considered positive. The positive control MAb 6A1F4C5 had an absorbance of 0.950 at 450 nm. Three fish sera from fish infected with isolate AVG5/268; 1 serum from fish infected with isolate R980769; 1 serum from fish infected with isolate SRS-4; 1 serum from SRS-UACH, 4 sera from fish infected with isolate R-29; 3 sera from fish infected with isolate R61 and 1 serum from fish infected with isolate R62 appeared to react with the type strain. These sera were also positive against *P. salmonis* isolate with which they had been challenged.

The differences in the antibody response between sera from different groups and the presence of bacteria in the kidney of fish experimentally infected with different *P.*

salmonis isolates were analysed using non parametric methods as data were shown not to be normally distributed (Kolmogorov-Smirnov, $p<0.05$) and the variance to be heterogenious (Levene Statistic, $p<0.05$). All statistical analysis was carried out using fish sera collected at the end of the challenge experiment and diluted 1/16 in PBS. Data obtained from replicate wells in ELISA plates showed there to be no significant differences between replicate groups when compared using Wilcoxon's Matched Pairs Test ($p>0.05$). As a result of that, only one replicate was used for all further analysis. The differences between the groups were analysed using Kruskal-Wallis Test and Post Hoc tests were carried out using Mean Ranks Multiple Comparisons Test. All statistical analysis were carried out in STATISTICA. Kruskal-Wallis Test showed that there was a statistically significant difference between sera from different groups either experimentally infected with *P. salmonis* isolates or non-infected fish (H: 36.72, d.f: 9, $P<0.001$). The differences were explored using Mean Ranks Multiple Comparisons Test. Statistically significant differences was observed between sera from fish infected with LF-89 and with isolate R-29, and sera from fish infected with isolate R-62 and with isolate R-29 ($3.357<Z<3.996$, $P<0.005$). However, statistically significant differences were observed only between sera from fish infected with *P. salmonis* isolates SRS-4, R29 and R61 and non-infected fish ($3.406<Z<4.886$, $P<0.005$).

The presence of bacteria in the kidney of infected fish was established at the end of the trial using a sandwich ELISA. The level of bacteria presence in kidney samples, expressed as an absorbance at 450 nm, is shown in Figure 6.2. The differences in the level of *P. salmonis* in the kidneys of fish from the different groups was analysed using Kruskal-Wallis Test as mentioned above. It was shown that there was a statistically significant difference between the level of bacteria present in fish groups either infected

with *P. salmonis* isolates or non-infected (H: 42.80, d.f: 9, P<0.001). Mean Ranks Multiple Comparisons Test was used to explore the differences between the groups. The test showed there to be significant differences between the presence of bacteria in kidney of fish infected with *P. salmonis* isolate LF-89 and with isolates R908769, SRS-UACH, R29, R61, R,62 and the level of bacteria in kidney of non-infected fish (3.277<Z<4.466, P<0.005). The test also showed the significant differences between the presence of bacteria in kidney of fish infected with *P. salmonis* isolate AVG5/268 and with isolates SRS-UACH and R-29 (3.752<Z<3.832, P<0.005) (Table 6.2).

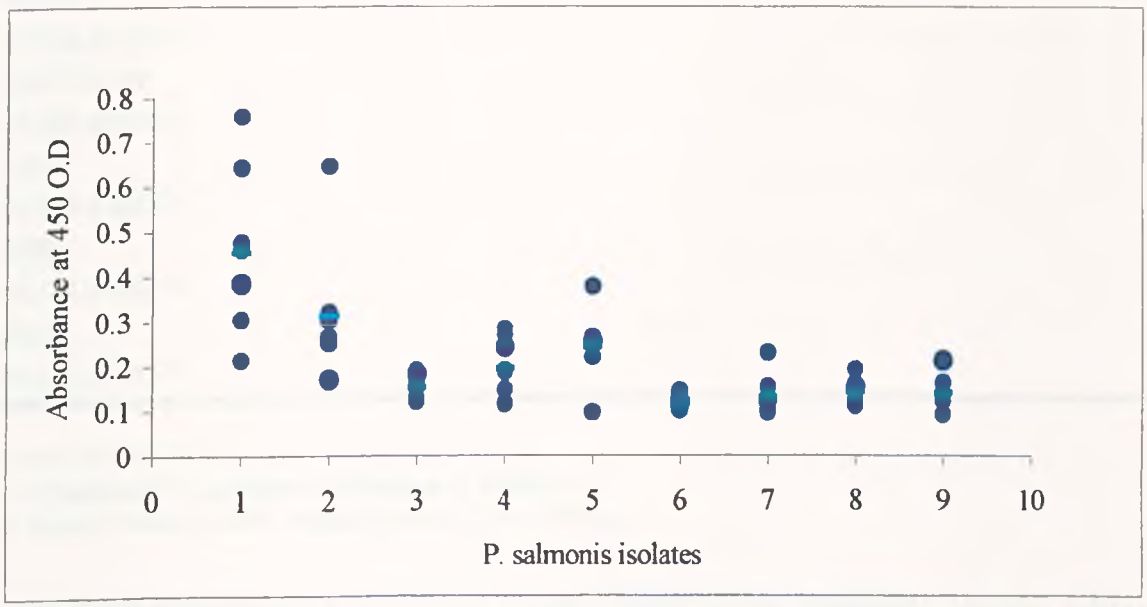


Figure 6.2. Level of *P. salmonis* in kidney sampled from experimentally infected survivors at the end of the challenge period measured using a sandwich ELISA. *P. salmonis* isolates (1) LF-89; (2) AVG5/268; (3) R980769; (4) VQ013; (5) SRS-4; (6) SRS-UACH; (7) R29; (8) R61 and (9) R62. (•) indicates individual fish; (—) the mean value for fish within each group

Table 6.2. Significant differences in the level of *P. salmonis* present in the kidneys of experimental fish at the end of the challenge period

	P. salmonis isolates used for infection								
P. salmonis Isolates*	LF-89	AVG5/268	R980769	VQ013	SRS-4	SRS-UACH	R29	R61	R62
LF-89 (0.453 ± 0.176)		-	+	-	-	+	+	+	+
AVG5/268 (0.314 ± 0.14)	-		-	-	-	+	+	-	-
R980769 (0.151 ± 0.024)	+	-		-	-	-	-	-	-
VQ013 (0.204 ± 0.062)	-	-	-		-	-	-	-	-
SRS-4 (0.241 ± 0.101)	-	-	-	-		-	-	-	-
SRS-UACH (0.122 ± 0.013)	+	+	-	-	-		-	-	-
R29 (0.131 ± 0.043)	+	+	-	-	-	-		-	-
R61 (0.141 ± 0.027)	+	-	-	-	-	-	-		-
R62 (0.141 ± 0.037)	+	-	-	-	-	-	-	-	

(-) No differences
(+) Statistically significant differences at $P<0.05$
* Mean Values (± SD) obtained from ELISA at OD_{450}

The mean absorbance at 450 nm of kidney sampled from uninfected fish was 0.141. Three times this value, 0.423, is close to the value which corresponds to uninfected kidney tissue spiked with 10^4-10^5 $TCID_{50}$ ml^{-1} bacteria (LF-89). Kidney spiked with 10^7 $TCID_{50}$ ml^{-1} of bacteria gave an absorbance of 1.358. The relationship between the presence of bacteria in the kidney of experimental fish with that of its serum antibody level, using a 1/16 dilution of serum in PBS, screened against the *P. salmonis* with which it had been injected, was examined. The OD_{450} value obtained for antibody levels of the twenty-nine positive fish sera was 0.996 ±0.33, while the OD_{450} value for bacteria present in the kidney of these same fish was 0.193±0.11. The correlation

coefficient (r) of this relationship was 0.09731, which was not statistically significant when analysed by a t-test at a significance level of 0.05%.

6.3.1.3. WB analysis of fish sera from experimentally challenged fish

WB analysis was used to examine the antigen profiles recognised by the anti-*P. salmonis* fish sera, which were selected based on their response in the ELISA and/or their cross-reaction with the type strain LF-89 (Figure 6.3). *P. salmonis* isolate R-29 was used as antigen in this experiment since this was the only isolate which produced a positive response with sera from seven of the eight fish tested in the ELISA. All of the sera screened in the WB gave a strong reaction with a doublet band at around 72 kilodalton (kDa). Another strong band was recognized at 95 kDa, while minor bands were recognised at 60, 56, 51, 40 and 36 kDa with the fish sera to differing degrees. However, the 60 kDa band was strongly recognised by sera from fish immunised with *P. salmonis* isolates AVG5/268-2, R29-4, R29-7, R61-7 and R61-7. Another band at 20 kDa was faintly recognised with the fish sera, although this was more strongly stained with sera from fish challenged with *P. salmonis* isolates R62-7, R980769-5 and LF-89-5.

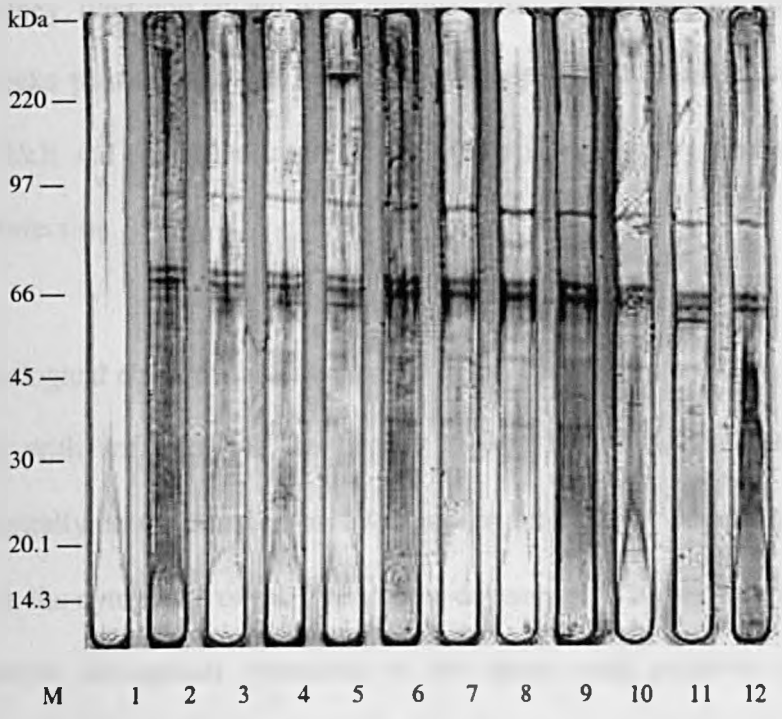


Figure 6.3. WB analysis of fish sera sampled from fish experimentally infected with *P. salmonis.* Lanes: (M) Marker (Amersham); (1) control fish serum; (2) fish serum R62-7; (3) fish serum R61-7; (4) fish serum R-29-7; (5) fish serum R-29-4; (6) fish serum R-29-2; (7) fish serum SRS-UACH-2; (8) fish serum SRS-4-5; (9) fish serum VQ013-6; (10) fish serum R980769-5; (11) fish serum AVG5/268-2; (12) fish serum LF-89-5

6.3.1.4. Histopathology of the experimentally infected fish

Samples of kidney, liver and spleen were obtained from experimentally infected fish at 2, 4 and 8 weeks post-inoculation (wpi). Sections prepared from the samples were stained with H&E and examined under LM for histopathologically changes due to the experimental infection.

No gross pathological change was detected, with the exception that livers of some fish appeared pale with softening of the hepatic tissue, and some fish had dorsal fin damaged. Internally, low-grade necrosis were observed 2 wpi as disseminated vacuolar degeneration in the cytoplasm of the liver, some degeneration of haemopoietic tissue of kidney, dilatation subcapsular sinusoids in the spleen and extensive melanin and hemosiderin pigmentation of haemopoietic tissue. When tissues sampled from fish 4 weeks after the first injection were examined under LM, mild necrosis was observed together with some vacuolar degeneration in the liver, degenerative haemopoietic tissue of kidney and an increased number of dilated subcapsular sinusoids in spleen (Figure 6.4). Widespread necrosis in haemopoietic tissue was evident in fish injected with isolate SRS-4 which had died in the third week of the experiment. Greater pathological changes were present in liver (Figure 6.5), spleen (Figure 6.6) and kidney (Figure 6.7) tissues sampled at the end of the experiment at 8 weeks. Sections of the same tissue blocks were used in IHC to confirm the presence of the bacteria.

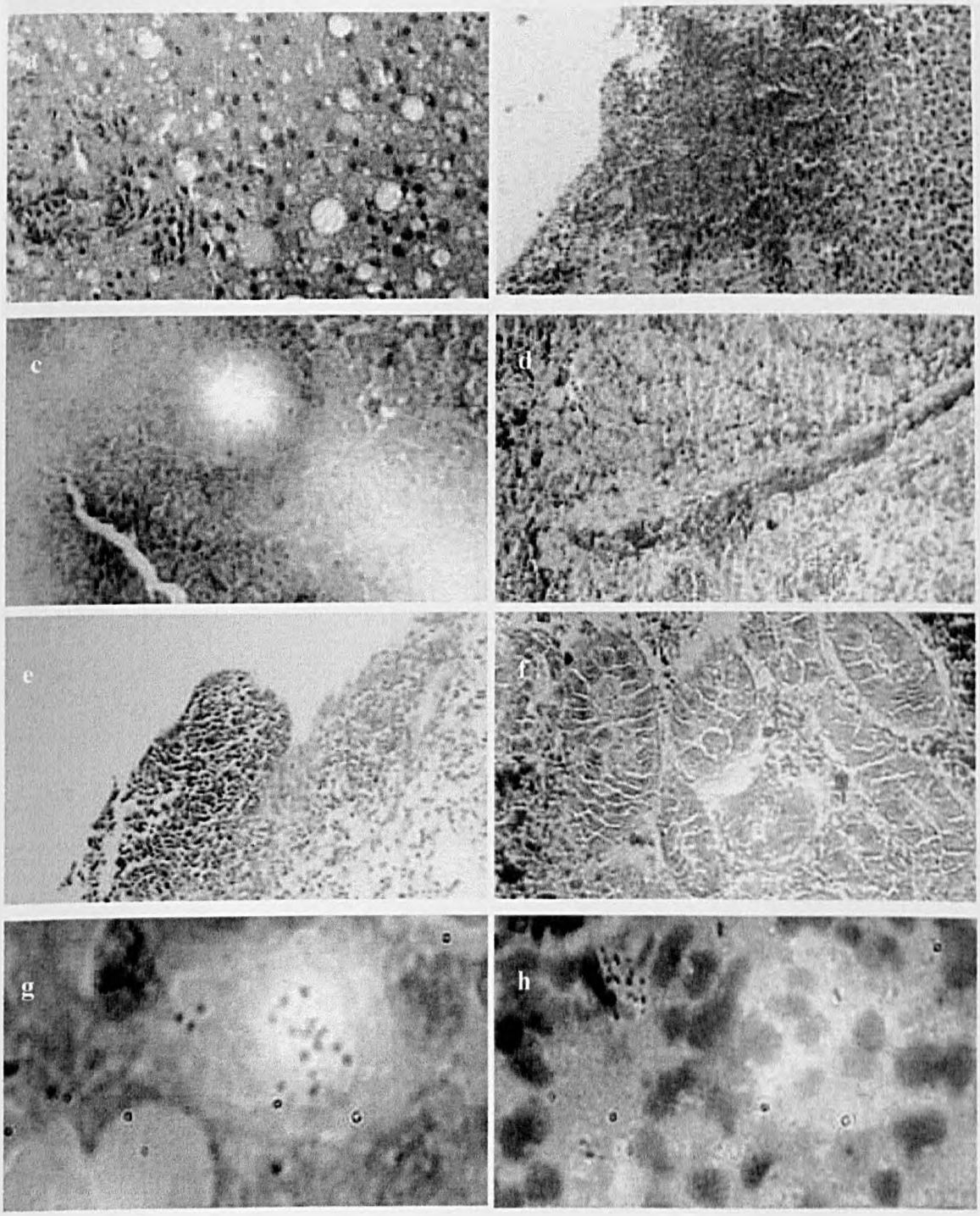


Figure 6.4. Histopathology of Atlantic salmon tissue, stained with H&E;(a) liver from fish infected with isolate R-62 showed vacuolar degeneration and fibrosis (sampled 2 wpi) (magnification x 100); (b) liver tissue with massively increased cellularity that could be due to invasion of inflammatory cells or an increase in the number of haematopoietic cells as a response to anemia in fish infected with isolate VQ013 (sampled 4wpi) (magnification x 40); (c) necrotic area of coagulative necrosis in the center of a granuloma starting to form in liver tissue in a fish infected with *P. salmonis* isolate AVG5/268 (sampled 4 wpi) (magnification x 40); (d) infiltration of inflammatory cells with strands of fibrin in spleen from fish infected with isolate SRS-4 (sampled 2 wpi) (magnification x 40); (e) spleen from fish infected with isolate R-29 showing swelling and dilatation of subcapsular sinusoids and markedly depleted white pulp (sampled 2wpi) (magnification x 40); (f) tubular degeneration of kidney from fish infected with isolate SRS-4 (sampled 4wpi) (magnification x 100); (g) RLOs in infected splenic tissue from AVG5/268 experimentally inoculated fish (magnification x 1000); (h RLOs in spleen sampled from fish which died 27 dpi with *P. salmonis* isolate SRS-4 (magnification x 400, analysed with IHC using PAb).

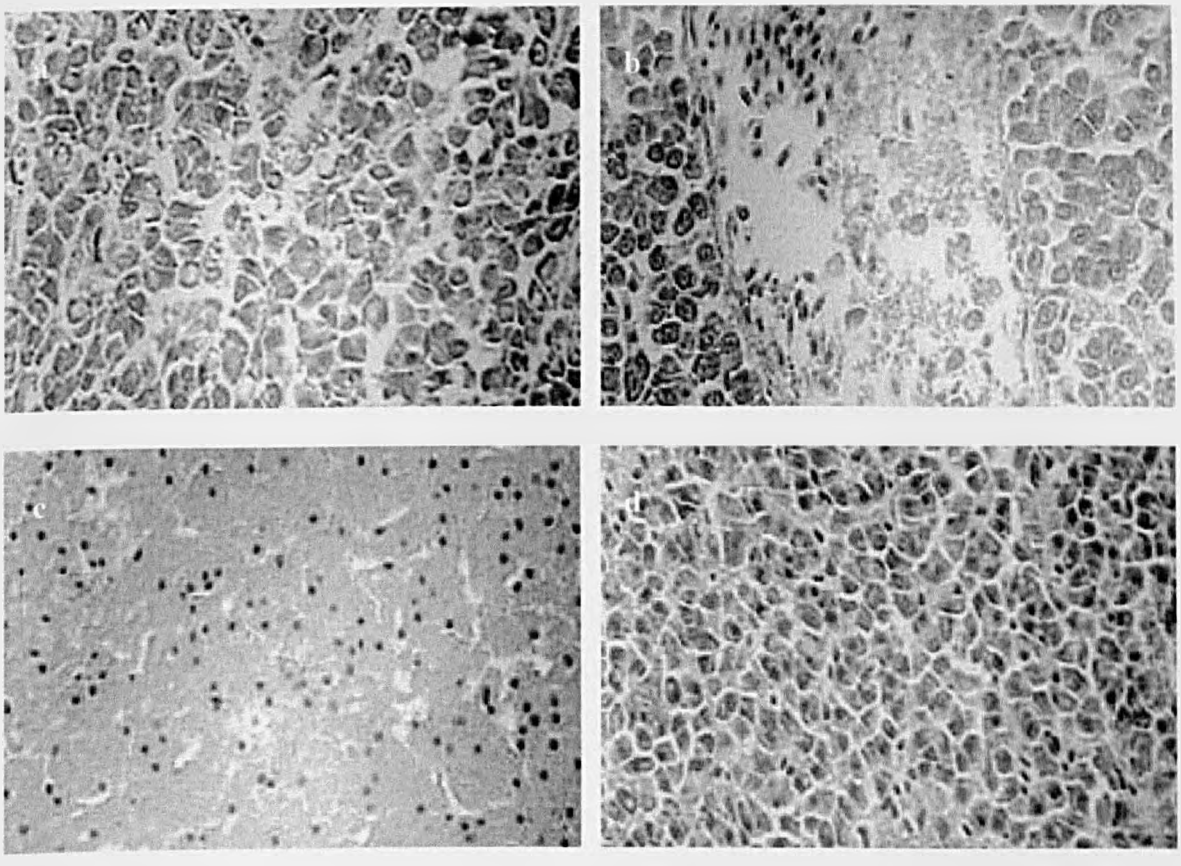


Figure 6.5. Histopathology of liver tissue from experimentally infected fish at the end of the challenge experiment (8wpi) stained with H&E (a) intravacuolar degeneration of hepatic cells in haematopoietic tissue sampled from fish infected with *P. salmonis* isolate SRS-UACH (magnification x 100); (b) note that destruction of vessel wall and perivascular necrosis with low grade necrotic thrombus in haematopoietic tissue sampled from fish infected with isolate LF-89 (magnification x 100); (c) an atrophic liver with some eosinophilic cells sampled from fish infected with isolate R62 (magnification x 100) and (d) liver tissue from fish sampled from control tank at the end of the experiment (magnification x 100)

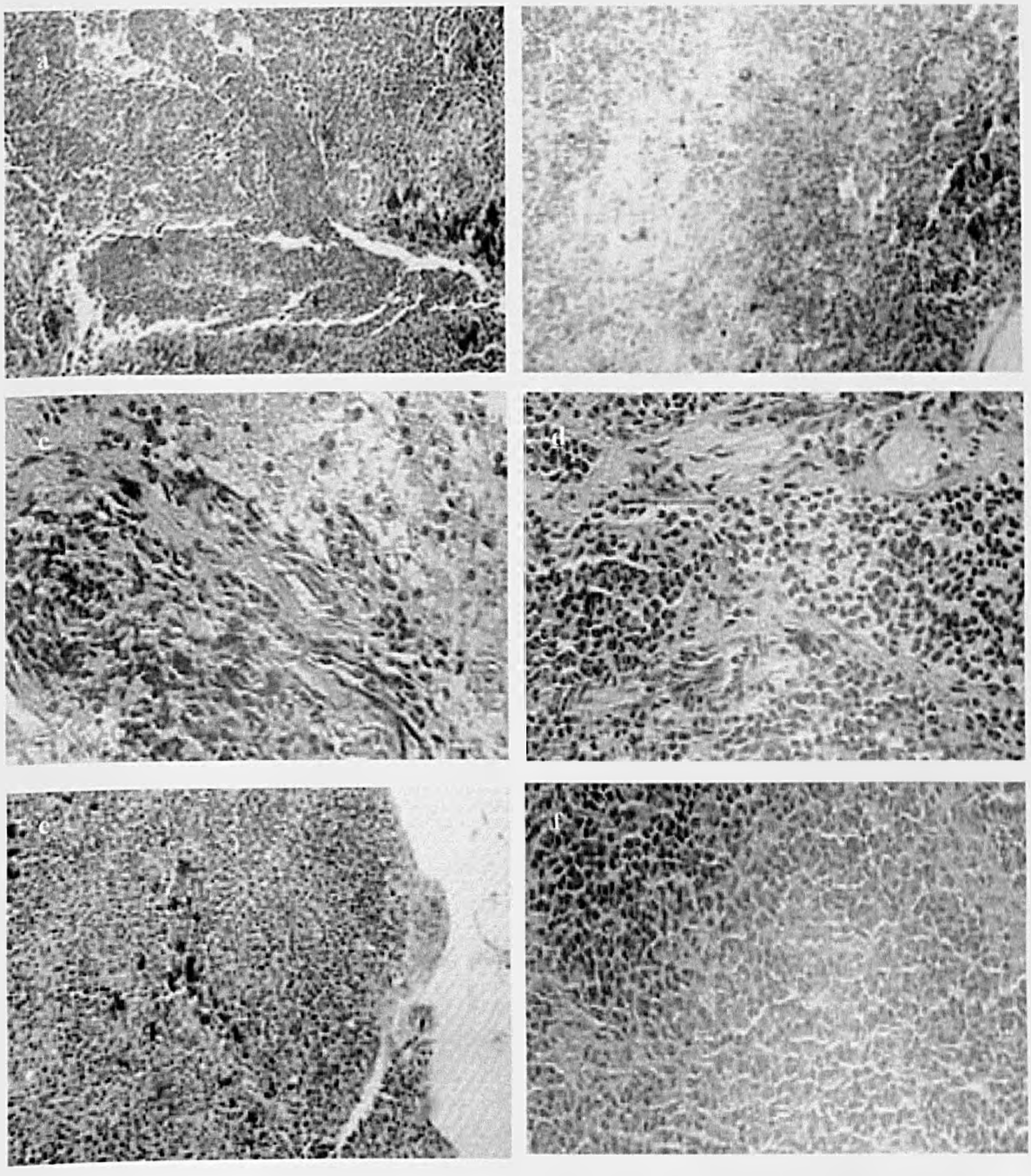


Figure 6.6. Histopathology of spleen tissue from experimentally infected fish at the end of the challenge experiment (8wpi) stained with H&E (a) congestion of red pulp sinuses in spleen from fish infected with *P. salmonis* isolate LF-89 (8 wpi) (magnification x 40); (b) necrotic area in splenic parenchyma from fish infected with isolate R-29 (magnification x 40); (c) chronic inflammatory and exudative response in spleen from fish infected with isolate R-29 (magnification x 100); (d) chronic inflammation in spleen from fish infected with isolate R61 (magnification x 100); (e) some RLO showed in dilated splenic parenchyma from fish infected with isolate R61 (magnificationx 400) and (f) spleen tissue from fish sampled from control tank at the end of the experiment (magnification x 100)

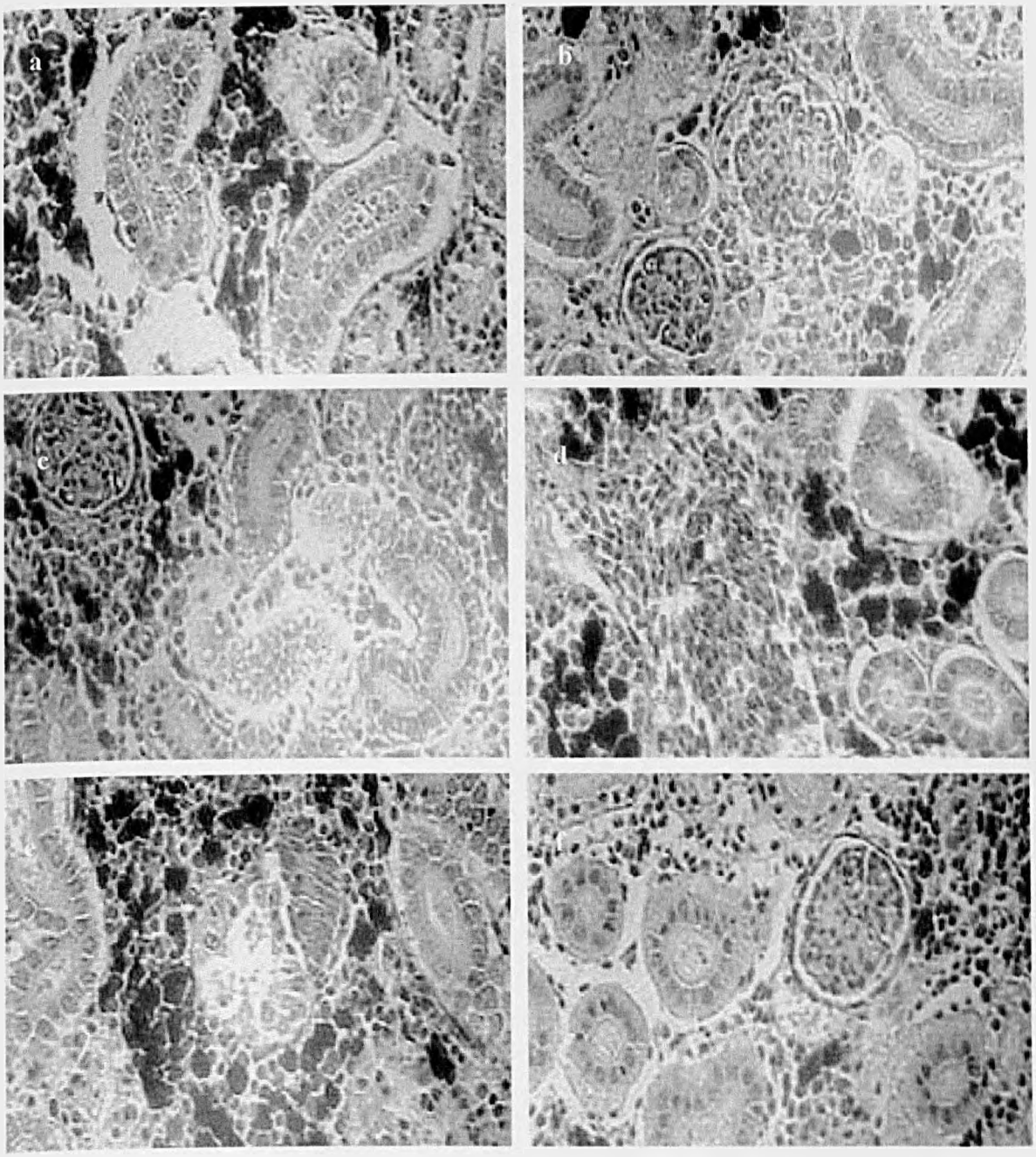


Figure 6.7. Histopathology of kidney tissue from experimentally infected fish at the end of the challenge experiment (8wpi) stained with H&E (a) degenerative renal tubules with cellular inclusion in kidney sampled from fish infected with *P. salmonis* isolate R-62 (magnification x 100); (b) one normal one thickening of basement membrane and occlusion of the glomerular space in kidney sampled from fish infected with isolate AVG5/268; (c) hyaline droplet degeneration of epithelial cells in renal tubules in kidney sampled from fish infected with isolate AVG5/268 (magnification x 100); (d) fibrin thrombis within renal haemopoietic tissue with perivascular necrosis and increased red blood cells in kidney sampled from fish infected with isolate R980769 (magnification x 100); (e) shrunken glomeruli with necrotic glomerular lining cells in kidney sampled from fish infected with isolate R61 (magnification x 100) and (f) kidney tissue from fish sampled from control tank at the end of the experiment (magnification x 100)

6.3.2. Immunisation of fish with different *P. salmonis* isolates

At 8 weeks post-immunisation (pi), blood was collected from all fish, and sera from fish which belonged to the same group were pooled. Sera FS1 refers to sera pooled from fish immunised with *P. salmonis* isolate LF-89 and adjuvant; FS2 refers to sera pooled from fish immunised with *P. salmonis* isolate SLGO-95 and adjuvant; FS3 refers to sera from fish immunised with *P. salmonis* isolate R29 and adjuvant; CLA refers to sera pooled from fish injected with CHSE-214 and adjuvant; PBSA refers to sera pooled from fish injected with PBS and adjuvant; PBS refers to sera pooled from fish injected with only PBS and C refers to pooled sera collected from uninjected fish (See Section 6.2.2.2).

6.3.2.1. Antibody response of immunised fish

The antibody response of immunised fish sera was measured by ELISA using *P. salmonis* isolates LF-89, SLGO-95 and R-29 to coat the ELISA plate. The titre obtained with each serum over the course of immunisation experiment (i.e. 2, 4, 6 and 8 weeks pi) is shown in Table 6.3.

The highest response was obtained with sera sampled from fish immunised with isolate R-29 (FS-3). Sera FS3 had an antibody titre of 4 ($-\log_2$), 4 weeks pi, increasing to 5 ($-\log_2$) at Week 5 pi and 6 ($-\log_2$) at Week 8 pi. This also gave a response against type strain LF-89 and isolate SLGO-95, when tested against these bacteria in the ELISA. Sera from fish immunised with the type strain LF-89 (FS1) gave the lowest response against both itself and against isolates SLGO-95 and R-29. The antibody titre of fish immunised with isolate SLGO-95 (FS2) was 4 ($-\log_2$) at Week 4 pi and this did not change over the course of the experimental period. Sera from fish injected with either

negative control. Anything below three times the negative value was considered negative. Sera from fish immunised with CHSE mixed with adjuvant gave the highest value among the different control groups.

Table 6.3. The antibody titers of sera ($-\log_2$) from fish immunised with *P. salmonis* isolates LF-89, SLGO-95 and R-29 measured by ELISA.

2. WEEK							
Antigen[1]/Sera[2]	FS1	FS2	FS3	CLA	PBSA	PBS	C
LF-89	-[3]	-	-	-	-	-	-
SLGO-95	-	-	-	-	-	-	-
R-29	-	-	-	-	-	-	-
4.WEEK							
Antigen/Sera	FS1	FS2	FS3	CLA	PBSA	PBS	C
LF-89	-	-	4	-	-	-	-
SLGO-95	-	4	4	-	-	-	-
R-29	-	-	4	-	-	-	-
6.WEEK							
Antigen/Sera	FS1	FS2	FS3	CLA	PBSA	PBS	C
LF-89	-	-	5	-	-	-	-
SLGO-95	-	4	5	-	-	-	-
R-29	-	-	5	-	-	-	-
8. WEEK							
Antigen/Sera	FS1	FS2	FS3	CLA	PBSA	PBS	C
LF-89	-	4	4	-	-	-	-
SLGO-95	-	4	4	-	-	-	-
R-29	-	4	6	-	-	-	-

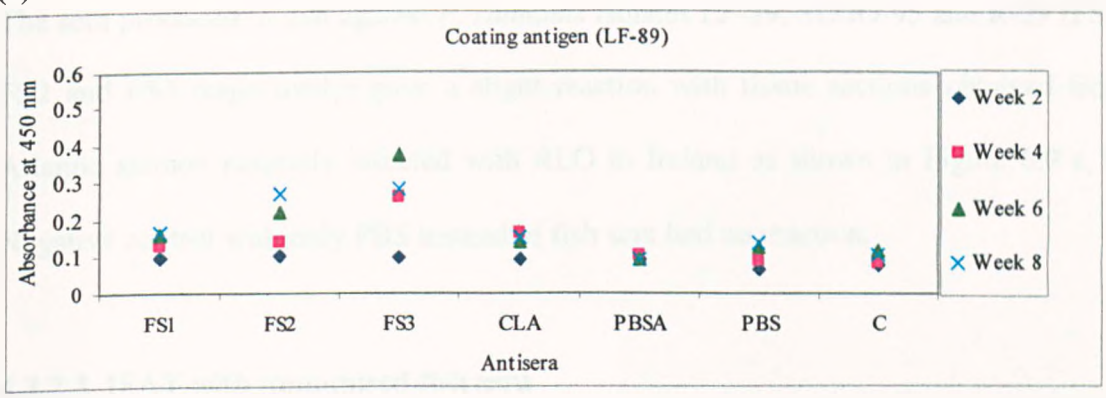
[1] Antigen used to coat ELISA plate

[2] FS1: pooled sera from fish immunised with *P. salmonis* isolate LF-89 and adjuvant; FS2: pooled sera from fish immunised with *P. salmonis* isolate SLGO-95 and adjuvant; FS3: pooled sera from fish immunised with *P. salmonis* isolate R29 and adjuvant; CLA: pooled sera from fish injected with CHSE-214 and adjuvant; PBSA: pooled sera from fish injected with PBS and adjuvant; PBS: pooled sera from fish injected with only PBS; C: uninjected fish sera pooled.

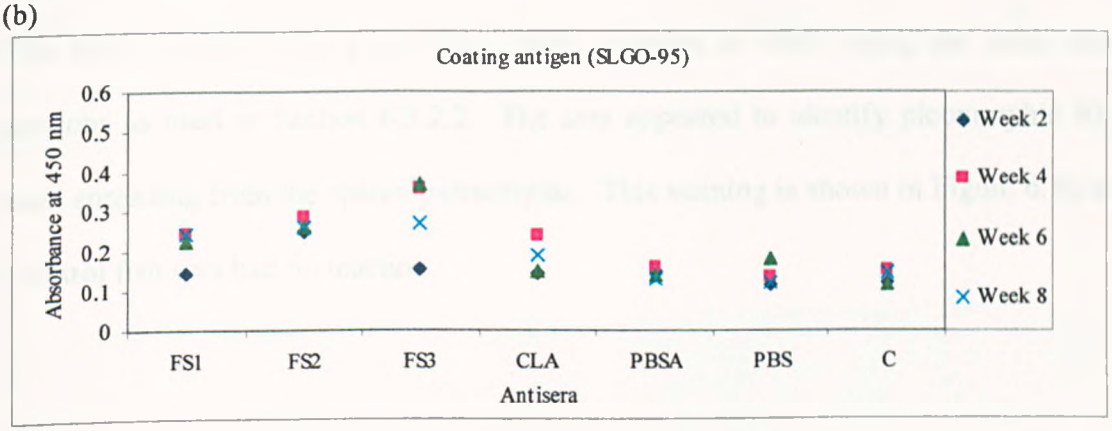
[3] Antibody response lower than 1/16

The fish sera (FS1, FS2, FS3) and C collected at week 8 was also diluted 1/20 in PBS, and screened against all of the *P. salmonis* isolates shown in Table 2.1 by ELISA, sera FS-3 produced against isolate R-29, gave the highest response against all isolates tested. The mean absorbance of sera C was 0.115. Any value greater than this was considered positive. All of the *P. salmonis* isolates tested gave a positive reaction with sera FS3, while sera FS2 only gave a positive reaction with isolate SLGO-95 against which it had been produced. Anti sera FS1 had a negative response with all of the *P. salmonis* isolates tested.

(a)



(b)



(c)

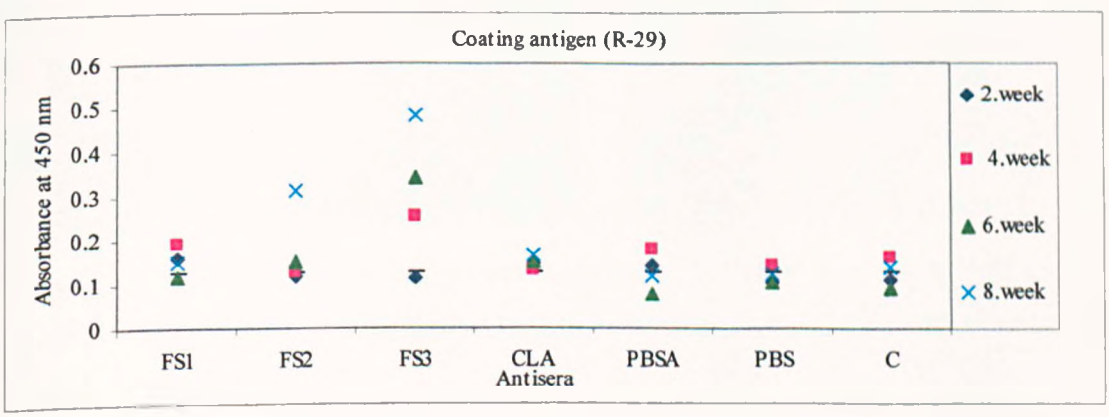


Figure 6.8. Antibody response of fish serum (1/16 dilution in PBS) against different *P. salmonis* isolates measured by ELISA. (a) *P. salmonis* isolate LF-89 used as coating antigen; (b) *P. salmonis* isolate SLGO-95 used as coating antigen; (c) *P. salmonis* isolate R-29 used as coating antigen. FS1: pooled sera from fish immunised with *P. salmonis* isolate LF-89 and adjuvant; FS2: pooled sera from fish immunised with *P. salmonis* isolate SLGO-95 and adjuvant; FS3: pooled sera from fish immunised with *P. salmonis* isolate R29 and adjuvant; CLA: pooled sera from fish injected with CHSE-214 and adjuvant; PBSA: pooled sera from fish injected with PBS and adjuvant; PBS: pooled sera from fish injected with only PBS; C: uninjected fish sera.

6.3.2.2. IHC with immunised fish sera

The sera produced in fish against *P. salmonis* isolates LF-89, SLGO-95 and R-29 (FS1, FS2 and FS3 respectively) gave a slight reaction with tissue sections obtained from Atlantic salmon naturally infected with RLO in Ireland as shown in Figure 6.9 a, b. Negative control with only PBS instead of fish sera had no reaction.

6.3.2.3. IFAT with immunised fish sera

The fish sera, FS-1, FS-2 and FS-3, were screened in IFAT using the same tissue sections as used in Section 6.3.2.2. The sera appeared to identify pleomorphic RLO seen spreading from the spleen parenchyma. This staining is shown in Figure 6.10 a-b. Control fish sera had no reaction.

(a) (b)

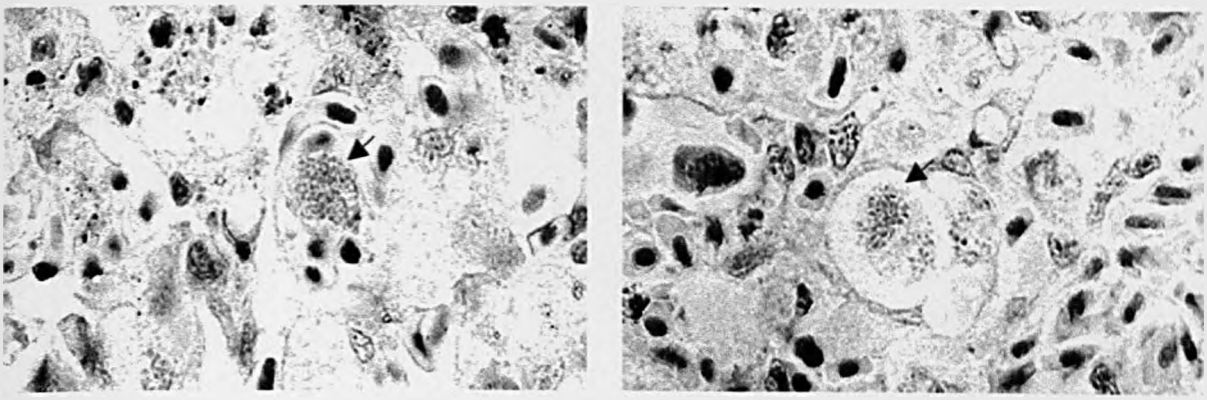


Figure 6.9. IHC using fish sera produced against *P. salmonis* with tissue sections sampled from RLO infected Atlantic salmon in Ireland (see arrows). (a) FS-3 (Magnification x 1100). ; (b) FS-1 (Magnification x 1100)

(a) (b)

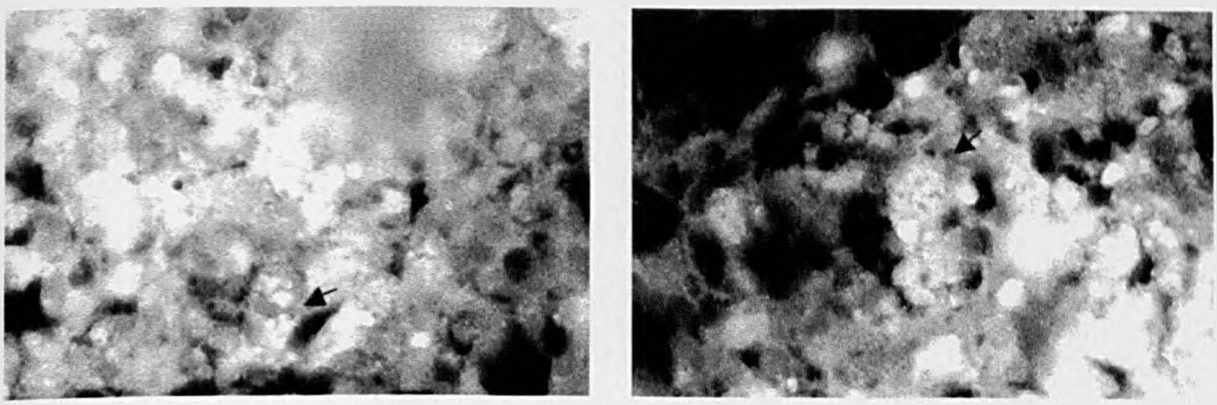


Figure 6.10. IFAT using fish sera produced against *P. salmonis* with tissue sections from Atlantic salmon infected with RLO in Ireland (see arrows). (a) FS-3 (Magnification x 1000); (b) FS-2 (Magnification x 1000)

6.3.2.4. WB analysis of *P. salmonis* using immunised fish sera

The antigen profiles of *P. salmonis* obtained with the immunised fish sera (See Section 6.2.2.2) were examined in WB analysis. When *P. salmonis* isolates LF-89, SLGO-95 and R-29 were used as antigen with anti sera FS1, FS2 and FS3 raised against isolates LF-89, SLGO-95 and R-29 respectively, a band could be seen at 95 kDa and a double band at around 72 kDa (Figure 6.11). Although the bands appeared paler with anti sera FS3, this may have been due to the slightly higher background obtained with this blot. Bands between 30 and 66 kDa were also slightly recognised by the anti sera against the three isolates. However a band at around 60 kDa gave a much stronger reaction with FS3 than obtained with other fish sera. Another band was recognised at around 20 kDa on *P. salmonis* isolates screened with the 3 groups of sera to differing degrees.

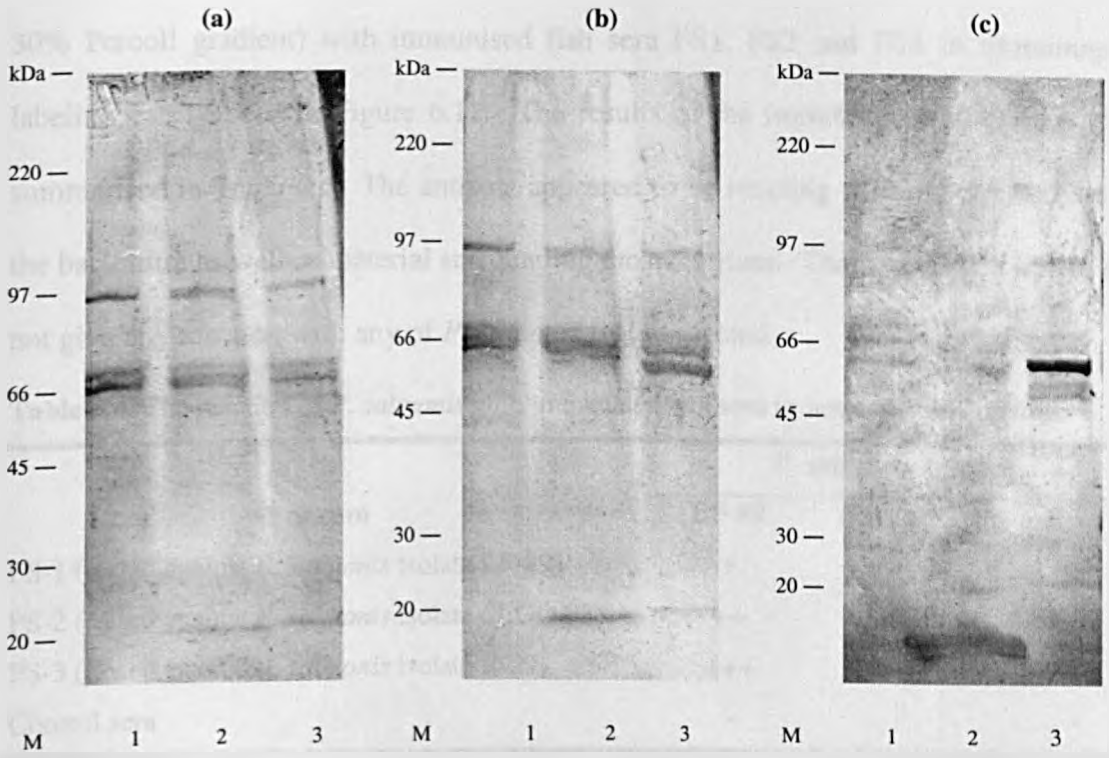


Figure 6.11. WB analysis using immunised fish sera raised against *P. salmonis* isolates (a) LF-89 (FS1); (b) SLGO-95 (FS2) and (c) R-29 (FS3) and screened against *P. salmonis* isolates (1) R-29; (2) SLGO-95 and (3) LF-89; M:Marker (Amersham).

6.3.2.5. Immunogold labeling of *P. salmonis* isolates with immunised fish sera

The reaction of purified *P. salmonis* isolates LF-89, SLGO-95 and R-29 (purified on a 30% Percoll gradient) with immunised fish sera FS1, FS2 and FS3 in immunogold labeling can be seen in Figure 6.12. The results of the immunogold labeling is also summarised in Table 6.4. The antisera appeared to be reacting with the cell surface of the bacterium as well as material surrounding the bacterium. The control fish serum did not give any reaction with any of *P.salmonis* isolates tested.

Table 6.4. The reaction of *P. salmonis* with immunised fish sera in immunogold labeling

Serum	*P. salmonis* isolates		
	LF-89	SLGO-95	R-29
FS-1 (raised against *P. salmonis* isolate LF-89)	+	+	++
FS-2 (raised against *P. salmonis* isolate SLGO-95)	++	++	+++
FS-3 (raised against *P. salmonis* isolate R-29)	+++	++	+++
Control sera	-	-	-

(-) No reaction
(+) Slightly reaction
(++) Moderate reaction
(+++) Strong reaction

6.3.2.6. PNA with immunised fish sera

At the end of the PNA, 6 plaques (approximately 2 mm in size with an expanded comet shape) were seen in the wells inoculated with isolate R-29, diluted 1/64 in PBS, used as a positive control. Plaque numbers were calculated as mean value of three wells for each dilution of each sample. No plaques were observed in the wells containing R-29 mixed with MAb, R-29 mixed with PAb, or when only CHSE-214 cells were used. While, 3 plaques were observed in the wells containing R-29 mixed with control fish sera, only 1 plaque was detected in wells inoculated with R-29 mixed with FS3 at a 1/64 dilution. On the other hand, wells containing isolate R-29 mixed with FS2 diluted 1/64 contained 2 plaques, while R-29 mixed with fish sera FS1 contained 3 plaques (Figure 6.13).

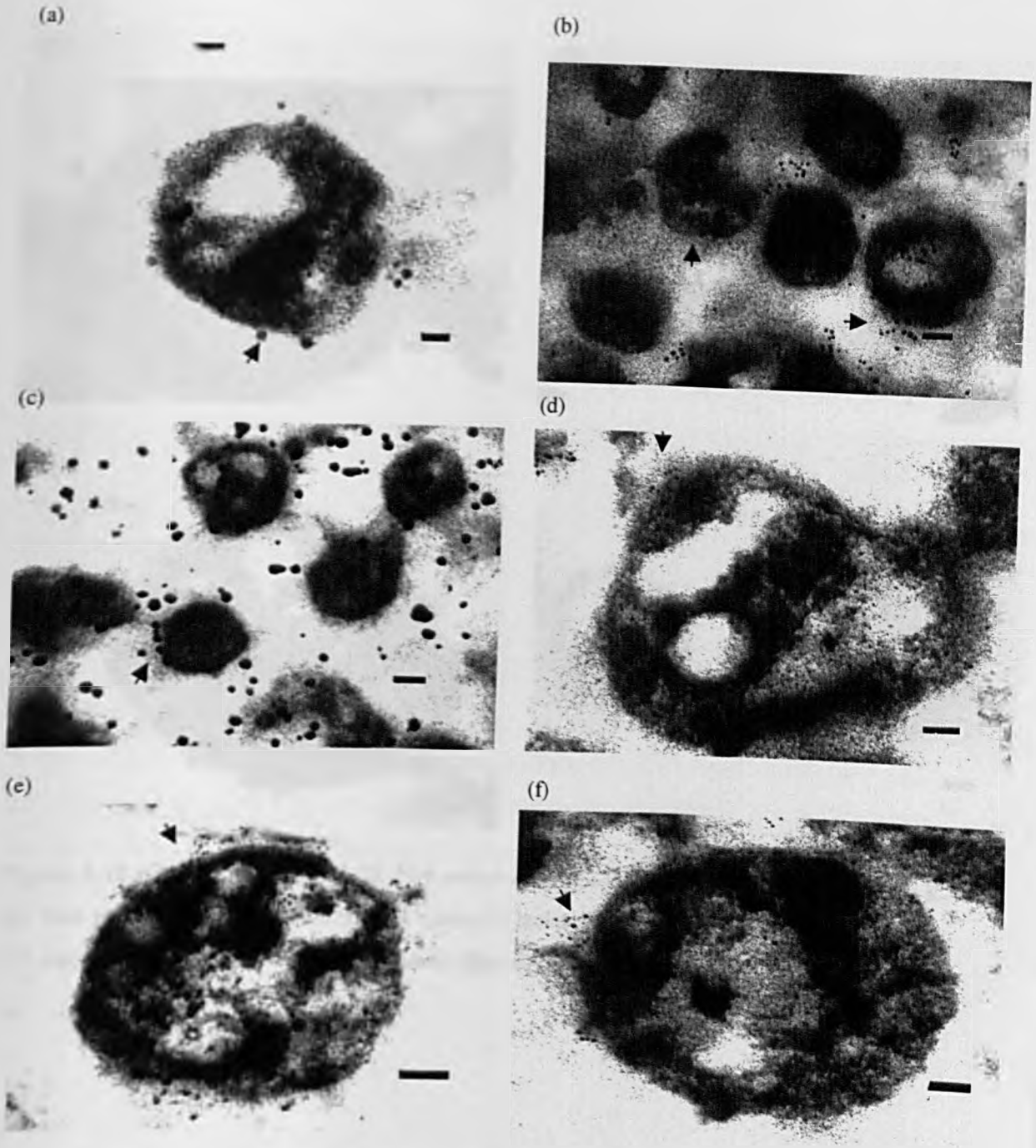


Figure 6.12. Immunogold analysis of immunised fish sera, FS-1, FS-2 and FS-3, with *P. salmonis* isolates LF-89, SLGO-95 and R-29 (see arrows indicating fish serum labelled with anti-mouse IgG conjugated to 5 nm gold particles); (a) Isolate LF-89; Fish serum against LF-89 (Bar: 0.05 μm); (b) Isolate LF-89; Fish serum against SLGO-95 (Bar: 0.1 μm); (c) Isolate LF-89; Fish serum against R-29 (Bar: 0.1 μm); (d) Isolate SLGO-95; Fish serum against LF-89 (Bar: 0.1 μm); (e) Isolate SLGO-95; Fish serum against SLGO-95 (Bar: 0.2 μm); (f) Isolate SLGO-95; Fish serum against R-29 (Bar: 0.1 μm).

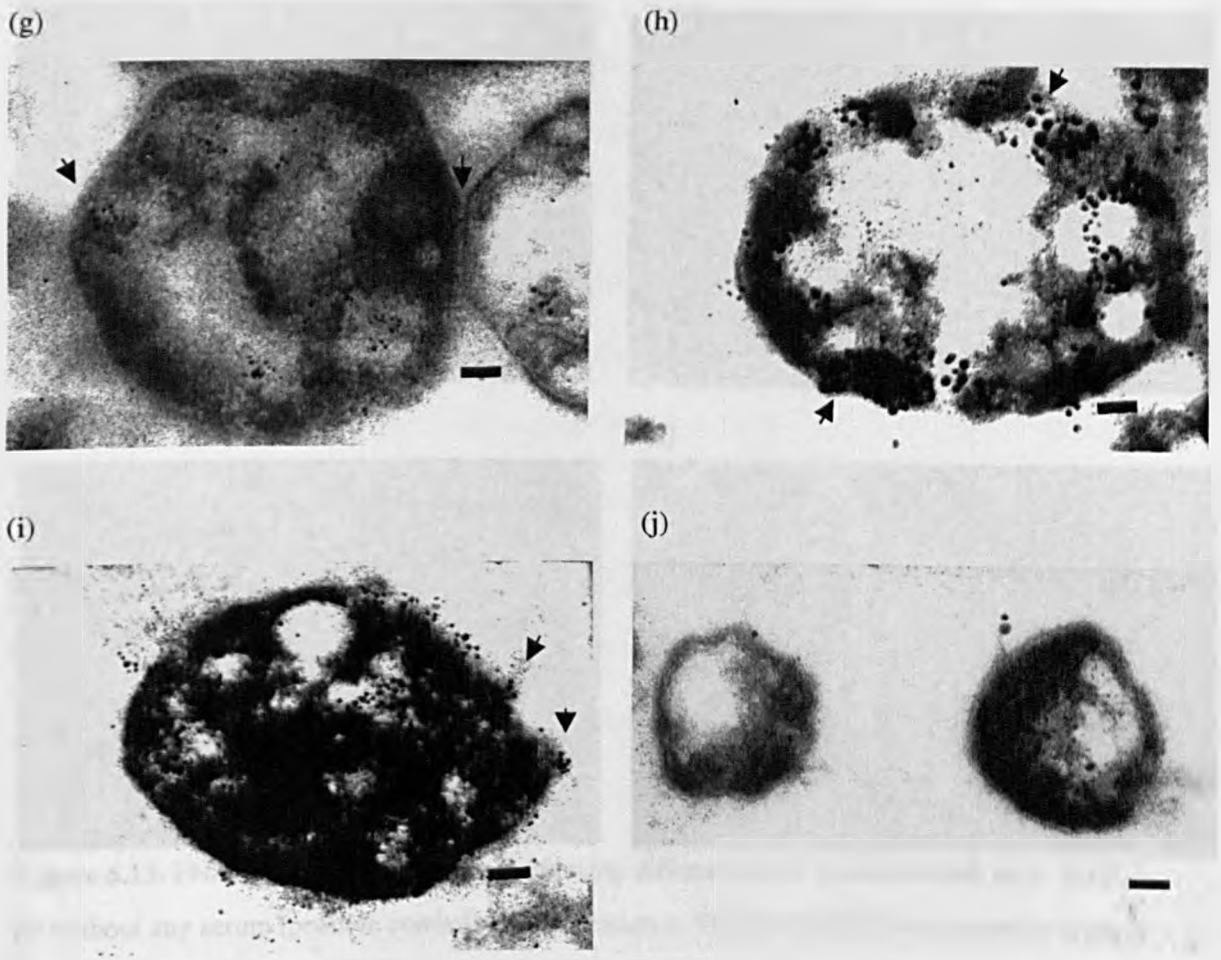


Figure 6.12 (Cont.). (g) Isolate R-29; Fish serum against LF-89 (Bar: 0.1 μm); (h) Isolate R-29; Fish serum against SLGO-95 (Bar: 0.1 μm); (i) Isolate R-29; Fish serum against R-29 (Bar: 0.1 μm); (j) Isolate LF-89; Control fish serum (Bar: 0.1 μm).

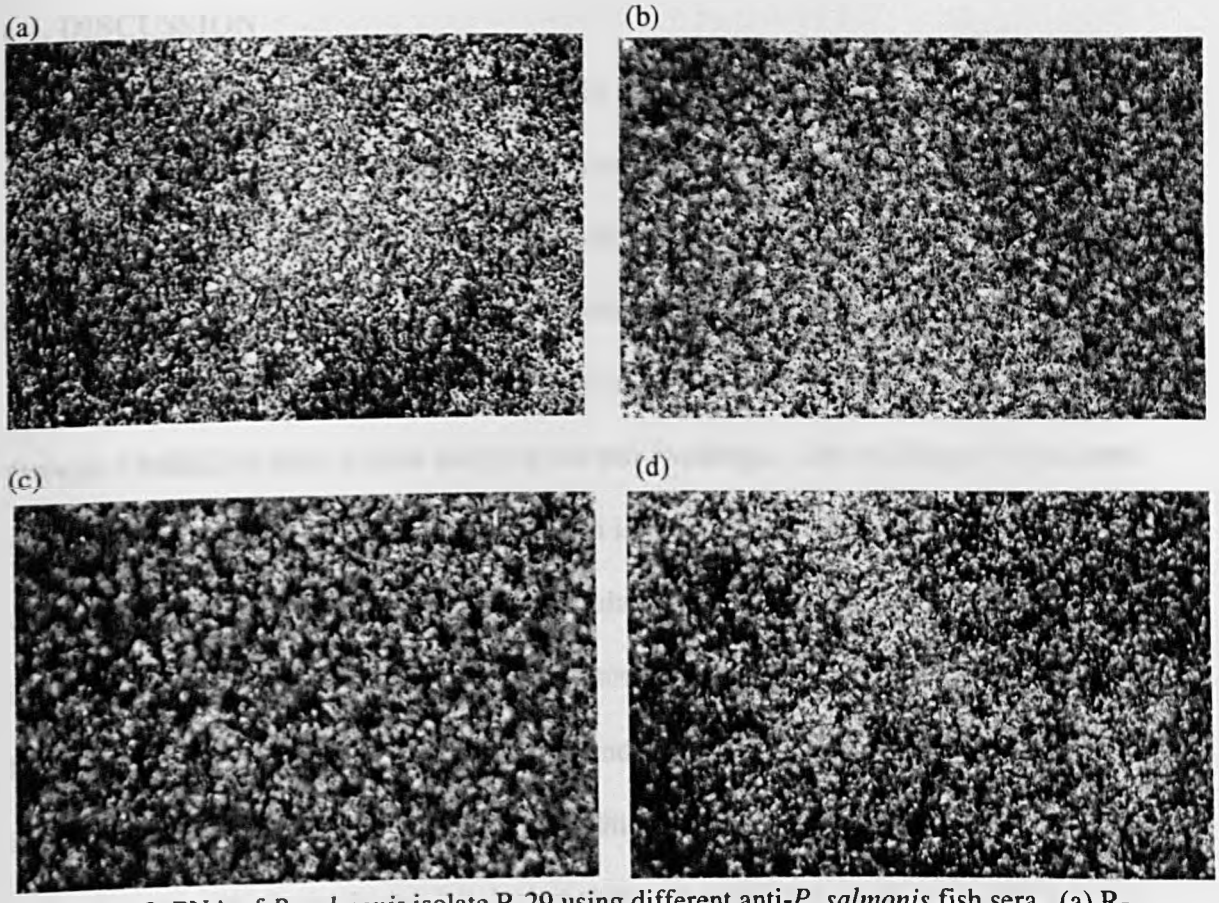


Figure 6.13. PNA of *P. salmonis* isolate R-29 using different anti-*P. salmonis* fish sera. (a) R-29 without any serum (positive control) (magnification x 50); (b) CHSE-214 as negative control (magnification x 50); (c) R-29 mixed with fish sera FS3 raised against R-29 (magnification x 50); (d) R-29 mixed control fish sera as additional negative control (magnification x 50).

6.4. DISCUSSION

Two different experiments were carried out in this Chapter, an experimental challenge and immunisation of Atlantic salmon with a number of different *P. salmonis* isolates. Nine isolates of *P. salmonis* isolated from coho salmon, rainbow trout, Atlantic salmon or sea bass, sampled during outbreaks of rickettsiosis in different geographical locations were used in the experimental challenge. All isolates were first passaged several times through CHSE-214 cells, before carrying out the challenge. The challenge experiment resulted in 30% mortalities in Atlantic salmon injected with *P. salmonis* isolate SRS-4, and 10% in Atlantic salmon injected with isolate R-62. Although, no fish died when inoculated with the other *P. salmonis* isolates, the bacterium could be detected by ELISA, in the kidney of fish sampled at the end of the experiment 8 wpi. No fish died in the control group. Since the level of mortalities was low in the challenge experiment, it was not possible to reach any conclusion about the virulence of the different isolates. However, it was possible to re-isolate the bacterium from infected kidney tissue during the first 4 wpi. The bacterium also observed in kidney smears by IFAT at this period. The pathology caused by the bacterium was observed in tissue sections sampled from challenge fish and stained with H&E or IHC.

It was also shown that fish were able to develop an antibody response against the bacterium in the continuous presence of *P. salmonis*. Results of the ELISA used to measure the antibody response in fish serum collected from experimentally challenged fish, indicated that 46% of experimental animals had developed a humoral response against the strain with which they had been injected. Sera raised against some of the different *P. salmonis* isolates also cross-reacted with the type strain LF-89 in ELISA. Seven of the eight fish infected with isolate R-29 produced a high antibody response

against this isolate (R-29), and some of these sera cross-reacted with the type strain LF-89. Isolate R-29 seemed to be the most immunogenic isolate between the *P. salmonis* isolates used in the experimental challenge. Levels of bacteria in the kidney of experimentally infected fish were found to be highest in fish injected with the type strain LF-89 by ELISA using a cocktail of MAbs developed in Chapter V. However, these fish did not appear to have a very high antibody response against the type strain when measured by ELISA. The differences in the antibody titre between sera collected from all fish infected with *P. salmonis* isolates and the variance of the presence of bacteria in kidney of the fish was statistically significant at a 0.1% significance level (Kruskal-Wallis Test). Post Hoc tests showed a statistically significant difference at the 5% significance level between fish sera from fish infected with LF-89 and R-29, and R-62 and R-29. Differences were observed in the level of the bacteria present in the kidney between fish infected with the type strain LF-89 and isolates R980769, SRS-UACH, R29, R61, R62, and *P. salmonis* isolate AVG5/268 and isolates SRS-UACH and R29 at a 5% significance level. There appeared to be differences between the antigenicity of different *P. salmonis* isolates and the ability of the fish to clear the different *P. salmonis* isolates after experimentally infection. However, the relationship between the presence of bacteria in the kidney of each experimental fish with that of its serum antibody, measured at a 1/16 dilution in PBS against *P. salmonis* with which it had been injected, was not statistically significant.

In WB analysis, a doublet at 72 kDa and bands at approximately 95, 60, 56, 51, 40, 36 and 20 kDa in differing degrees were recognised by fish sera which were positive in the ELISA.

The pathogenesis of piscirickettsiosis has not been fully described in the literature. One of the aims of this study was to examine the pathogenicity of the *P. salmonis* in Atlantic salmon. This study is the first time that histological changes in Atlantic salmon experimentally infected with *P. salmonis* in seawater have been described. No marked gross pathology was observed, except for the pale appearance of livers, kind of softening in the hepatic tissue and dorsal fin damage in some fish during the experimental challenge. Bleie (1995) reported that although internally findings are quite similar in different species of salmon, Atlantic salmon interestingly seemed to lack the signs of external gross pathology seen in coho and other Pacific salmon species. This was similar to what had been reported from RLO-outbreaks in Atlantic salmon in Chile, Canada, Ireland and Norway (Bleie, 1995). Although, gross lesions, characterised with cream-coloured to yellowish subcapsular nodules scattered diffusely through the liver in heavily infected fish (Cvitanich *et al.* 1991; Olsen, Melby, Speilberg, Evensen & Hastein 1997), were described as one of the major diagnostic symptoms of salmonid rickettsial septicaemia, this pathology is not always present (Lannan & Fryer 1993; Turnbull 1993; Almendras, Fuentealba, Markham & Speare 2000). Pale discoloration of the liver, one of recognised rickettsial gross pathologies (Garcés *et al.* 1991; Turnbull 1993) was one of the only gross pathology together with enlarged spleen with mild haemorrhagia observed in salmon post-smolts infected in a scottish sea loch in 1995 (Aldridge 1999). Garcés *et al.* (1991) did not observe any clinical signs of disease in Atlantic salmon (10 g) experimentally infected with rickettsia ($10^{5.3}$ $TCID_{50}$ $fish^{-1}$ with IP route), although the typical disease signs (such as anemic moribund fish with pale gills and liver, enlarged spleen, congested spleen and kidney, hemorraghes in the base of fins and pyloric caeca) were present in the coho salmon which were infected with rickettsiae by the same route and with the same

dilution. However, the rickettsiae were recovered in pure culture from moribund fish from both species while 100% mortality occurred by Day 39 in Atlantic salmon and Day 33 in coho salmon in that experiment (Garces *et al.* 1991). Almendras *et al.* (1997) on the other hand observed 57% mortalities in Atlantic salmon (20 ± 2.3 g) infected IP with the bacterium (100 µl with 1.48 x 10^2 $TCID_{50}$ ml^{-1}). They reported a capsular (serosal) pattern of *P. salmonis* infection in liver and spleen in fish 7 days post-inoculation (dpi), and also infection of leucocytes and other parenchymal cells of the spleen, liver and kidney with *P. salmonis.* In the present study, samples of kidney, liver and spleen sampled from experimentally infected fish 2, 4 and 8 wpi were examined for histopathological changes due to the bacterium. The histopathology observed was similar to that seen during natural outbreaks of piscirickettsiosis, with the degeneration of haemopoietic tissue. Vacuolar degeneration in the cytoplasm, increased cellularity in the liver and degenerative haemopoietic tissue and spreading melanin pigmentation in kidney were observed. Swelling and dilatation of subcapsular sinusoids in the spleen with inflammation and mild-grade necrosis in haemopoietic tissue developed. Perivascular necrosis with fibrin thrombi and chronic inflammation was observed by the end of the 8 weeks experimental period. In mammals, damage to endothelial cells, localised thrombus formation and the release of erythrocytes into surrounding tissues have been described in rickettsial infections (Buxton & Fraser 1977).

Coagulative necrosis with hepatocellular vacuolation were prominent features of pathology in the liver in this experimental challenge. Massively increased cellularity in the liver could be due to invasion of inflammatory cells or an increase in the number of haematopoietic cells, as a response to anemia (Bleie 1995). Tubular degeneration and degenerate glomeruli may be due to some degree of glomerulonephritis in renal tissue.

Unlike any other piscirickettsiosis infections, dilatation of subcapsular sinusoids in spleen was observed in this experimental infection. These changes often appear in such organs of old fish due to fibrosis of the ellipsoids sheaths, congestion of the red pulp sinuses with red blood cells and depletion of white pulp (Roberts 1989). The remaining fibrotic network seen in the spleens of fish 8 wpi may be due to a chronic state of *P. salmonis*. Almendras *et al.* (2000) also reported a capsular type of pathology in the liver of inoculated fish. IP inoculation of many *Rickettsiaea* species has been reported to produce an infection and result in an inflammatory reaction in the peritoneal lining (Almendras *et al.* 2000; Walker, Popov, Wen & Feng 1994).

Almendras *et al.* (2000) reported the presence of liver lesions in juvenile Atlantic salmon experimentally infected with *P. salmonis* in freshwater. They observed no significant morphological changes in the liver with the exception of slight thickening of the capsule by inflammatory cells 1 wpi, while diffuse thickening of the capsule was evident 2 wpi with the presence of multifocal to localised areas of coagulative necrosis. On the other hand, histological changes consisted of vasculitis, the presence of fibrin thrombi, vacuolated hepatocytes and focal areas of necrosis in the third wpi. The authors suggested that *P. salmonis* infects its hosts locally and is then carried through the circulatory system within leucocytes and reaches the main organs by infecting the endothelial cells of blood vessels, causing endothelial damage. This damage induces vasculitis, leakage through the endothelia, and the formation of fibrin thrombi.

No obvious difference was seen in the pathology of the different isolates used in the experimental infection. House *et al.* (1999) observed no obvious difference in gross

pathology between isolates when they compared the virulence of a number of different *P. salmonis* isolates.

Although two vaccination trials have been reported with coho salmon and rainbow trout by Smith *et al.* (1995, 1997), knowledge about the immune response of fish against the microorganism is still limited. This study is the first report to describe the antibody response of Atlantic salmon against the bacterium and to determine the reactivity of antisera raised against *P. salmonis* in these fish.

In the immunization experiment, Atlantic salmon were held in freshwater and immunised with heat-killed *P. salmonis* isolates, type strain LF-89, SLGO-95 and R-29. The antibody titers in sera from these fish were then determined by ELISA. The highest antibody response was obtained in sera raised against *P. salmonis* isolate R-29 (FS3), as was also observed in the challenge experiment in which fish were infected with live bacterium. Sera FS3 also reacted strongly with isolates LF-89 and SLGO-95. Fish immunised with isolate SLGO-95 (FS2) had an antibody titre of 4 ($-\log_2$), while fish immunised with isolate R29 (FS3) had a titre of 6 ($-\log_2$). Sera from fish immunised with the type strain LF-89 (FS1) gave the lowest response. Sera raised against isolate R-29, used at a 1 in 20 dilution, also gave a higher antibody response against all *P. salmonis* isolates (LF-89, AVG5/268, R980769, VQ013, SRS-UACH, SLGO-95, R61, R62, R29), compared to sera against type strain LF-89 and SLGO-95.

Sera from fish immunised with *P. salmonis* isolates LF-89, SLGO-95 and R-29 appeared to recognise a double band with a molecular weight at 72 kDa and bands at approximately 95 and 20 kDa in WB analysis. Some bands between 30 and 66 kDa

were slightly recognised by the sera. The band at 60 kDa was the only band recognised with sera FS3, from fish immunised with isolate R29. Kuzyk, Thorton & Kay (1996) observed a single protein of around 72 kDa and also several minor bands between 10 and 70 kDa, with anti *P. salmonis* serum from convalescent rainbow trout when they screened it against a variety of *P. salmonis* isolates obtained from Atlantic salmon and coho salmon. They did not observe any obvious differences between the immunoreactive bands of the isolates used. The authors suggested that the single protein band at around 72 kDa was an artifact that reacted with the mouse anti-salmon IgM second antibody. A doublet at 72 kDa was also observed in WB with sera raised against live *P. salmonis* in the challenge experimental in this study with bands observed at around 95, 60, 56, 51, 40, 36 and 20 kDa when screened against *P. salmonis*. No bands were stained with control fish sera tested using hybridoma cell line 4C10 containing monoclonal antibodies that recognised salmon IgM and ABP complex in this study. The 60, 56, 51, 40 and 36 kDa proteins appeared to be recognised with sera raised against live *P. salmonis*, unlike the sera raised against heat-killed *P. salmonis* in the immunisation assay, except for the strong reaction with a band at 60 kDa with sera FS3 from fish immunised with isolate R-29.

When the fish sera were used in IHC and IFAT against sections obtained from naturally infected Atlantic salmon, they gave only a slight reaction, indicating that even though the immunised fish serum did not have a strong humoral response against the bacterium, the antibodies were still able to react with *P. salmonis* present in the tissue sections. When the fish sera FS1, FS2 and FS3 were used in immunogold labeling of *P. salmonis* isolates, a similar pattern of reaction as obtained in ELISA was observed with the different isolates. Fish serum (FS3) against isolate R-29 gave the strongest reaction

in immunogold labelling. These sera appeared to recognise antigens on the cell surface of *P. salmonis*. Together with the results of the WB analysis with FS3, it suggests that the band recognised at 60 kDa may be a protein exposed on the surface of *P. salmonis*. In the PNA, sera obtained from fish immunised with isolate R-29 gave the lower number of plaques with diluted antisera than was seen with sera obtained from fish immunised with isolate LF-89 and SLGO-95. MAb and PAb used in the assay seem to completely block the cell invasion of rickettsiae, however, the amount of antibody of these sera would be higher than in fish sera used. The effect of antibody on the entry and intracellular survival of the rickettsiae in cultured host cells depends on the type of host cell and rickettsial isolate used. Targets for neutralising antibody present on the rickettsiae are currently unknown. Antibodies can block the entry of *Rickettsia tsutsugamushi* into chicken embryo cells, yet increases its rate of entry into guinea pig macrophages. In the same way the rate of entry of *Rickettsia typhi* into human macrophages is increased by antibody as is the entry of *Rickettsia prowazekii*. However, *R. typhi* coated with antibody also increases its rate of intracellular destruction, whereas antibody facilities the entry of *R. prowazekii* into human macrophages without enhancing subsequent intracellular destruction (Moulder 1985).

Isolate R-29 appeared to be the most immunogenic isolate among the isolates used in both challenge and immunisation experiments. These results suggest that future work should focus on the characterisation of immunoreactive proteins, found on the *P. salmonis* isolates, particularly on isolate R-29 in an attempt to identify potential vaccine candidates so as to produce an effective vaccine against piscirickettsiosis.

CHAPTER VII

IDENTIFICATION OF BACTERIOPHAGE ASSOCIATED WITH *PISCIRIKETTSIA SALMONIS*

7.1. INTRODUCTION

A century ago, Hankin (1896) reported that the waters of the Ganges and Jumna rivers in India had antibacterial properties, which appeared to stop cholera epidemics from spreading via these river systems. This anti-bactericidal activity was not examined any further until Twort (1915) and D'Hérelle (1917), independent of each other, observed a virus which appeared able to infect bacteria. D'Hérelle referred to them as bacteriophages (eaters of bacteria). The phages were initially thought to be ideal for controlling bacterial infections, but with the discovery of antibiotics, phage therapy was largely abandoned in the West. However, its use was still continued in the former Soviet Union and Eastern Europe. The modern age of bacteriophage research began in the late 1930s when physicist-turned-phage biologists, Max Delbruck and phage biologists Alfred Hershey and Salvador Luria used bacteriophages as model systems to study fundamental features of biological life (Kutter 1997; Abuelkhair 1998). Recently, there has been renewed interest in phage therapy in the West as antibiotic resistance in pathogenic bacteria has become a threat to public health (Casadevall 1996).

Although early phage therapy experiments in animals showed possible therapeutic effects as reported by Asheshov, Wilson and Topley (1937), Dubos, Straus and Pierce (1943), Ward (1943) and Lowburry and Hood (1953) cited by Barrow and Soothill (1997), phage therapy has only recently been carried out in Britain and the United States. In the 1980s, encouraging studies with phage therapy showed both prophylaxis

and treatment of mice and farm animals with phages to be possible and potentially more effective than antibiotics for combating full blown *Escherichia coli* infections (Smith & Huggins 1982, 1983; Smith, Huggins & Shaw 1987). In view of Smith's successes, Soothill (1992) carried out a study using phage therapy on antibiotic-resistant bacteria affecting humans, especially infections of burn victims. Levin and Bull (1996) later carried out detailed analysis of the population dynamics and tissue phage distributions of the 1982 study by Smith and Huggins which helped to explain why phages were more effective than antibiotics. This study was followed up by a series of experiments by Merril and co-workers who began to use modern scientific tools to improve phage therapy in 1996. They developed a method to isolate mutants of phage that persist in the blood of mice longer than the parental phage, using animals which had been injected simultaneously with the phage and lethal quantities of a laboratory strain of *E. coli* (Barrow & Soothill 1997).

Bacteriophages, or phages, have a head composed of protein, an inner core of single or double stranded nucleic acid, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), and a hollow protein tail. This 'head and tail' morphology, relating to their mode of cell penetration, is unique to phages and is not found among any other groups of viruses. (http://cbs.infoplease.com/ce5/CE004050.html; Abuelkhair 1998; Heng 1999). Bacteriophages, like bacteria, are ubiquitous in the environment and their presence is directly related to the numbers of bacteria that are present in a particular environment. There are currently at least 12 distinct groups of bacteriophages, which are very diverse, both structurally and genetically (http://www-micro.msb.le.ac.uk/224/Phages.htr).

Bacteriophages infect bacteria and destroy them through two main types of phage life cycle. With the first group of bacteriophages, virulent phages achieve 'bacterial lysis' via a process in which phage DNA enters the cytoplasm of the bacterial host and the metabolic pathways of the bacterium are used to produce viral enzymes and more phage nucleic acid. The viral proteins and nucleic acid molecules within the bacterial host assemble spontaneously to form up to a hundred new phage particles which eventually lyse the bacterium, thus releasing the newly made phage particles. The other group of bacteriophages, known as temperate phages, only lyse a small fraction of bacterial cells. After infection of its host, these phages integrate into the bacterial chromosome and replicate along with it; a process known as 'lysogeny'. The information of viral nucleic acid is not expressed, however. Under appropriate conditions, the lysogenic virus becomes virulent resulting in replication and bacterial lysis (Heng 1999; http://cbs.infoplease.com/ce5/CE004050.html).

The aquaculture industry is currently faced with a number of bacterial pathogens, which are resistant to antibiotics. Alternative methods of control, such as vaccine development, have made an important contribution in controlling disease. However, the use of bacteriophages as a biological control for disease in fish culture has aroused much interest in recent years, especially since no drug residues and drug toxicity are associated with this type of therapy. Furthermore the rate at which bacteriophages grow appears significantly faster than bacteria. Infection of bacteria with the phage occurs in the aqueous phase of the bacteria's life cycle and also infection by the phage is very host specific (Wu, Lin, Jan, Hsu & Chang 1981, Nakai, Sugimoto, Park, Matsuoka, Mori, Nishioka & Maruyama 1999).

A number of studies have helped to highlight the potential of phage therapy in controlling bacterial diseases in aquaculture. Wu *et al.* (1981) used bacteriophage AH1 as a biological control for the fish pathogen, *Aeromonas hydrophila.* When injected into a loach *Misgurnus anguillicaudatus,* this pathogen can cause inflammation and necrosis, resulting in 65% mortalities within 7 d post-injection. When the *A. hydrophila* culture was first infected with phage AH1 for 3 h prior to injection, no necrosis and lethality occurred in the infected fish. In similar experiments carried out by Wu and Chao (1982; 1984), bacteriophages effectively reduced the pathogenicity of twenty seven different strains of *Edwardsiella tarda* and eighteen strains of *Vibrio anguillarum,* respectively. Stevenson and Airdrie (1984) isolated eight bacteriophages effective against the causative agent of enteric red mouth disease in salmonids while Park, Matsuoka, Nakai and Murogo (1997) found a virulent bacteriophage against *Lactococcus garvieae,* a known pathogen of yellowtail *Seriola quinqueradiata.* Protective effects of anti- *L. garvieae* bacteriophages administered intraperitoneally or orally into yellowtail experimental infected with *L. garvieae* have been reported by Nakai *et al.* (1999). Ahne, Capousek and Popp (2000) carried out a study examining the source and spread of *Aeromonas salmonicida,* the causative agent of furunculosis, in a particular river system in Germany. They concluded that the presence of the phages in the water implied the presence of host bacterium which was released by *A. salmonicida* infected fish.

Morel, Veyrunes and Vago (1974), cited by Buchanan (1978), was the first to observe phages associated with the obligate parasitic bacterium *Porochlamydia buthi,* a chlamydial pathogen of the scorpion *Buthus occitanus,* and described them as short-tailed polyhedral particles. Although virus-like bodies have previously been associated

with or observed in rickettsia (Buchanan 1978; Wright, Sjöstrand, Portaro & Barr 1978; Shaw & Moloo 1993; Báo, Kitajima, Callaini & Dallai 1996), the first observation of viral particles associated with rickettsia isolated from fish was reported by Yuksel, Thompson, Ellis and Adams (2001).

The aim of this study is to identify and characterise phage particles associated with *P. salmonis* the etiological agent of piscirickettsiosis.

7.2. MATERIALS AND METHODS

7.2.1. Examination of *P. salmonis* for the presence of bacteriophages

P. salmonis isolates outlined in Table 2.1 were purified and examined under transmission electron microscopy (TEM) as described in the Sections 3.2.2 and 3.2.6 respectively. After experimental challenge of Atlantic salmon *Salmo salar* with *P. salmonis* isolates (Section 6.2.1), re-isolated *P. salmonis* isolates LF-89 and VQO13 were also examined for the presence of bacteriophages by TEM. A Philips 301electron microscopy (EM) was used for the analysis and samples were viewed at 80 kilovolt (kV) in transmission mode.

7.2.2. Trans-infection of one *P. salmonis* isolate to another with the bacteriophage

The experiment described here was designed to infect *P. salmonis* isolate R-29 with phage particles previously isolated from *P. salmonis* isolates LF-89 and VQO13. Isolate R-29 seemed to be bigger than the other isolates examined (see Section 3.3.1) and different from the other *P. salmonis* isolates examined in this thesis (see Sections 4.3.4). The analysis was based on the elimination of *P. salmonis* isolate LF-89 using Ultraviolet (UV) irradiation and recovery of free phage particles in a phage-infected

rickettsial culture. Supernatant from an identical flask, prepared from the same batch of *P. salmonis* as that used to prepare the UV irradiated LF-89, was examined under TEM to confirm the presence of phage particles. After an extensive Cytopathic effect (CPE) was established in the 75 cm^2 (Nunc A/S Roskilde, Denmark) tissue culture flask infected with isolate LF-89, the cell culture supernatant was collected and centrifuged at 20 000 x g for 60 minutes (min). The pellet was resuspended in approximately 10 ml of Phosphate buffer saline (PBS; Gibco) and this was divided into three aliquots of approximately 3 ml each. These were subsequently referred to as Samples A, B and C. Sample A was exposed to UV light (Bio-link ®, BLX-312) at an activity of 50 Joule sec^{-1} for 2 hours (h). The UV activity was based on the fact that microbial destruction is a product of both time and intensity, which is the amount of energy per unit area (Lupal 2000). This preparation was then mixed with cell culture supernatant infected with *P. salmonis* isolate R-29, which was collected after CPE development (approx. 3 ml). After incubation at 15°C overnight, approximately 500 microlitre (µl) of the *P. salmonis* mixture was removed and used in a plaque assay as described in Section 3.2.4. Wells containing only Chinook salmon, *Oncorhynchus tshawytscha* embryo cell line (CHSE-214) were used as a negative control while wells inoculated with *P. salmonis* isolate R-29 were used as a positive control. *P. salmonis* isolate R-29 used in the analysis was examined under TEM to confirm the absence of phage particles. Each sample was applied in two-fold dilutions to four replicates.

Sample B (LF-89 associated with phage particles) was not exposed to UV, but was then subsequently treated the same as Aliquot A by mixing with the same volume of cell culture supernatant infected with isolate R-29 (3 ml), which was collected after the development of a CPE. Again the culture was left overnight at 15°C to allow infection

of isolate R-29 with the phage particles. Sample C used as positive control had not been either treated with UV or mixed with R-29 infected supernatant. A supernatant from R-29 infected culture called Sample D was used as negative control. Samples from each of Sample A, B, C and D were added to two replicate 25 cm^2 flasks (Nunc A/S Roskilde, Denmark) of CHSE-214, cells seeded at a concentration of 5 x 10^4 cells ml^{-1} and incubated for 48 h at 22°C. The flasks were incubated at 15°C for 2 h to allow rickettsial absorption, after which the supernatant was removed and 10 ml of fresh antibiotic-free Eagle's minimal essential medium (MEM) with Earle's salts, supplemented with 2% (v/v) Foetal calf serum (FCS) and 2 milimolar (mM) L-glutamine was added. The cells were cultured at 15 to 17°C until an extensive CPE developed. Flasks with only CHSE-cells were used as negative control. After a CPE had developed, the *P. salmonis* were collected through centrifugation and prepared for TEM examination as described in Section 3.2.6.

7.2.3. Analysis of bacteriophage nucleic acids

Phage nucleic acids were examined using a protocol adapted from Storey, Lusher, Richmond and Bacon (1989) and Park *et al.* (1997). *P. salmonis* were harvested from CHSE cells cultured in a 150 cm^2 tissue culture flask (Nunc A/S Roskilde, Denmark) after an extensive CPE had developed within the cell monolayer. The supernatant was centrifuged at 210 x g for 10 min at 4°C to remove host cell debris, and then vortexed on a high setting. The rickettsia were collected and washed with PBS by centrifugation at 20 000 x g for 30 min at 4°C. The rickettsial pellet was resuspended in 9 ml PBS and this was distributed between 1.5 ml cryopreservation vials. The vials were subjected to 3 cycles of freeze/thawing, using liquid nitrogen to freeze the cells so as to rupture the cell walls and release the virus-like particles. The supernatant was then divided into

two and one part was passed through either a 0.2 or 0.4 micrometre (μm) filter to remove all cellular and bacterial debris. Virus in both the filtered and the unfiltered samples were pelleted by centrifugation at 100 000 x g for 2 h at 4°C in a Beckman SW28 Ultra centrifuge. The pellet was resuspended in 300 microlitre (μl) of a Proteinase K (Sigma) solution [200 microgram (μg) ml^{-1} diluted in Tris acetate EDTA buffer (40 mM Tris acetate, 1 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0)] and incubated for 60 min at 37 °C. The solution was then divided into three DNA/RNA-free sterile tubes. One tube was incubated with DNase I (20 μg ml^{-1}), one tube was treated with RNase A (20 μg ml^{-1}) (Sigma) and one tube was left untreated. The tubes were incubated at 37 °C for 60 min to remove free nucleic acids. After two phenol-chloroform extractions and one chloroform extraction, the samples were precipitated by adding a 1 in 10 volume of 3 Molar (M) sodium acetate, pH 6.0 and 2 volumes of ice-cold absolute ethanol. Each pellet was then dried and resuspended in 0.01 ml TAE buffer. These were then analysed by electrophoresis on a 0.8% (w/v) agarose gel, and nucleic acids present in the gel were stained with ethidium bromide. A λ DNA-*Hind* III Digest was used as DNA molecular weight markers (Amersham Pharmacia Biotech). The same analysis was also carried out on CHSE-214, and *P. salmonis* isolate R-29 with no apparent phage particle association using a 0.4 μm filter.

7.3. RESULTS

7.3.1. Identification of phage particles within *P. salmonis*

P. salmonis isolates, LF-89, AVG 5/268, VQO13, SRS-UACH, SRS-4, R-29, SLGO-95 and R980769 were examined by TEM for the presence of phage particles. Virus-like bodies were found to be associated with two of the *P. salmonis* isolates, LF-89 and VQ 013 (Figure 7.1 a and b). The phage-like particles were polyhedral in shape and had a

short rigid tailpiece attached to the vertex of the polyhedron (Figure 7.2) and the diameter of the phage particles was approximately 110-130 nanometre (nm). The phage particles were seen within *P. salmonis* and also attached by the tip of their tail to the cell walls of the bacterium (Figure 7.3 a, b and c). Eventually, the particles appeared to lyse the bacterial cells (Figure 7.3 d).

In some preparations of *P. salmonis* isolate R-29, a quadrilateral like shape (approximately 0.27 μm in diagonal length between the furthest edges) consisting of a paracrystalline array was present (Figure 7.4 a and b). In another sample of the same *P. salmonis* isolate (R-29), round shape particles 0.14-0.20 μm in diameter were observed (Figure 7.5 a and b). The origin of these structures and their relationship with phage or virus-like particles is at this time unknown. There was no evidence that the CHSE-214 cell line used to culture the *P. salmonis* was infected with any phage-like particles.

After the challenge experiment described in Section 6.2.1.2, the *P. salmonis* isolates were recovered from experimentally infected fish (Sections 6.2.1.4). The isolates previously associated with phage particles, LF-89 and VQ 013, were re-examined under TEM after re-isolation for the presence of the phage. However, no phage-like particles were evident in any of the bacteria examined after re-isolation.

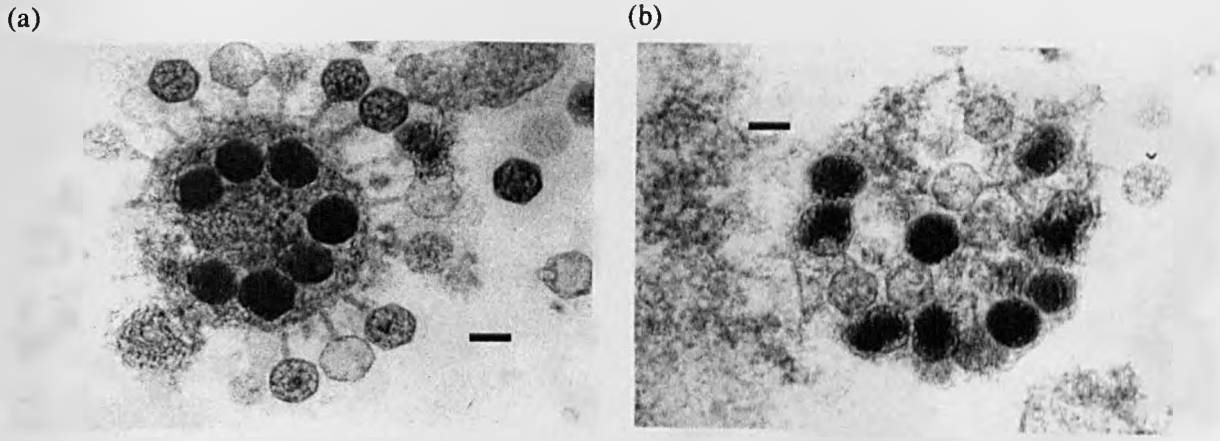


Figure 7.1. (a) Numerous bacteriophage particles attached to individual rickettsia-like bodies (strain LF-89) Bar: 0.1 μm; **(b)** Rickettsial organism (strain VQ 013) being lysed by virus-like bodies. A ghost phage after delivery of its genome into the rickettsia like organism's cytoplasm (arrow head) Bar: 0.1 μm.

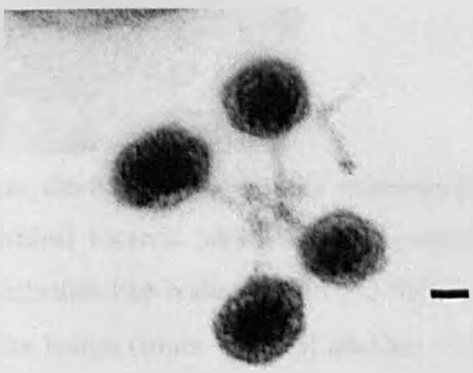


Figure 7.2. Higher magnification of the phage particles (Bar: 0.05 μm).

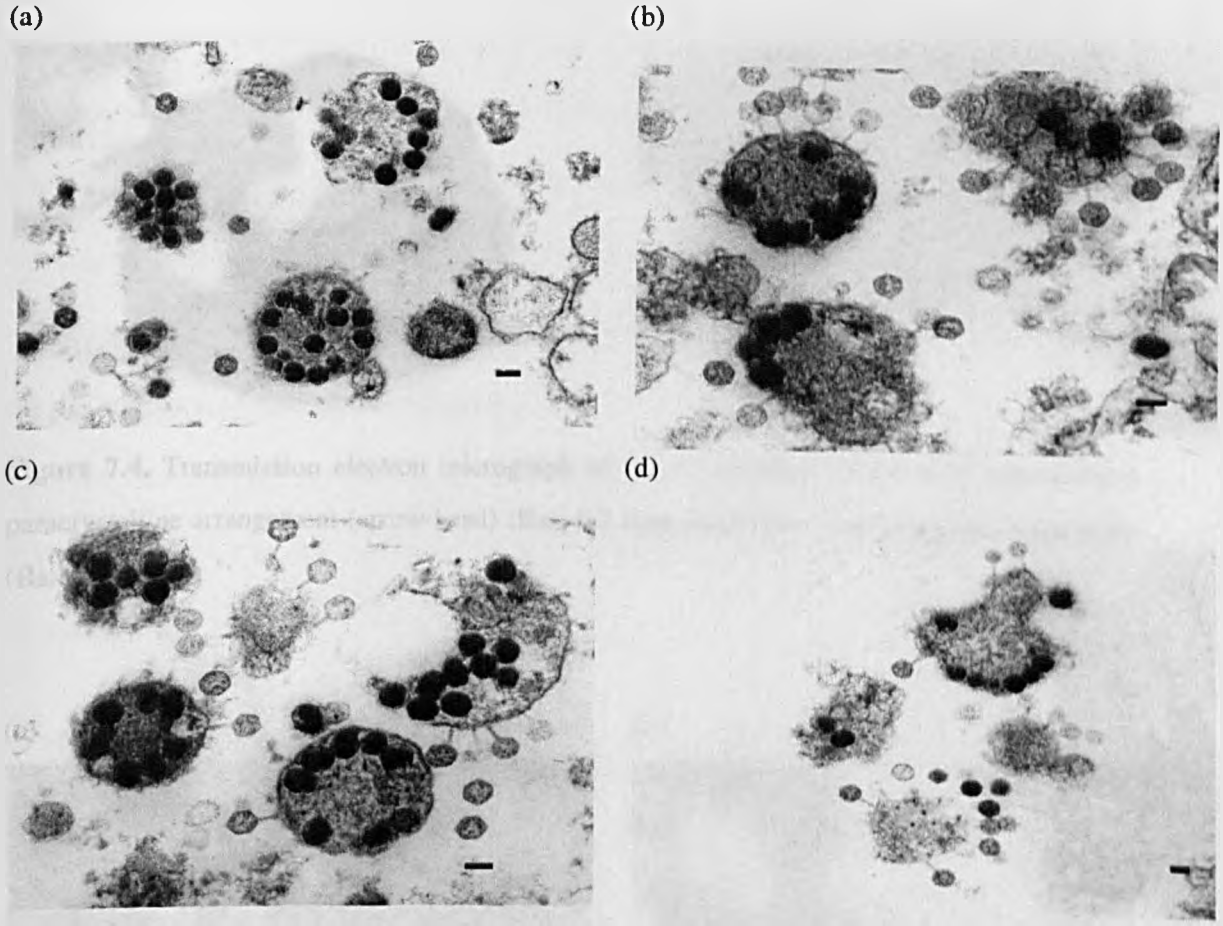


Figure 7.3. Transmission electron micrograph of rickettsia-like organisms associated with bacteriophages. **(a)** Individual bacteria (strain LF-89) containing phage particles (Bar: 0.15 μm); **(b)** Note swollen rickettsia-like bodies (strain VQ 013) containing phage particles (Bar: 0.1 μm); **(c)** Rickettsia-like bodies (strain VQ 013) attached to numerous phage particles (Bar: 0.1 μm); **(d)** Lysed rickettsia-like organisms (strain VQ 013) (Bar: 0.1 μm).

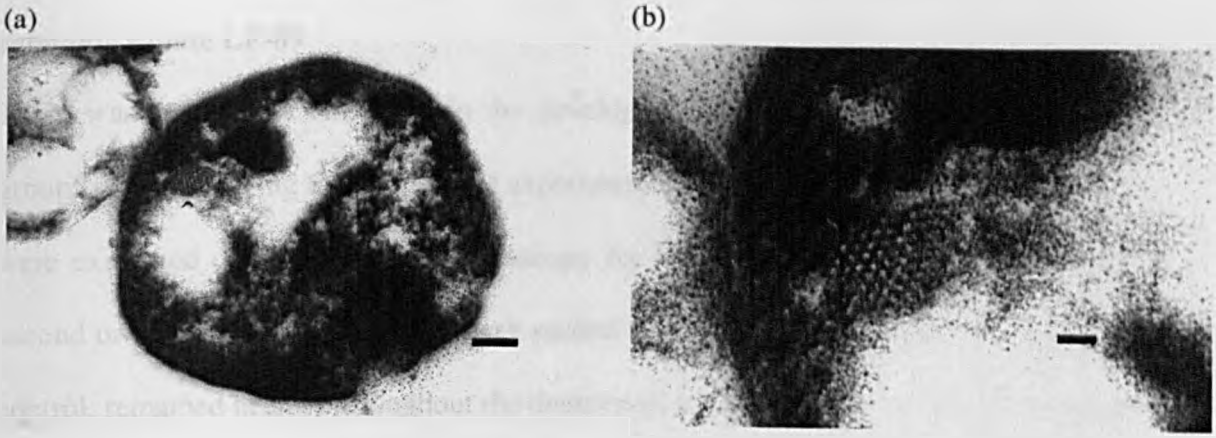


Figure 7.4. Transmission electron micrograph of **(a)** *P. salmonis* isolate R-29 containing a paracrystalline arrangement (arrow head) (Bar: 0.2 μm); **(b)** Higher magnification of the array (Bar: 0.05 μm).

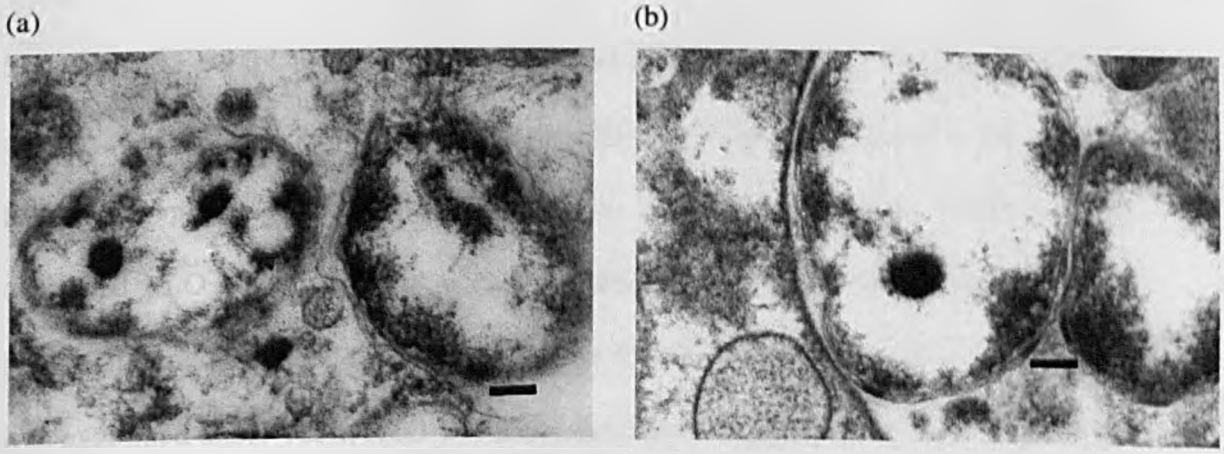


Figure 7.5. (a and b) Transmission electron micrograph of *P. salmonis* isolate R-29 containing a round shaped structure (Bar: 0.2 μm) under TEM.

7.3.2. Trans-infection of *P. salmonis* isolate R-29 with the bacteriophage from *P. salmonis* isolate LF-89

There was no obvious difference in the development of a CPE between the different groups of flasks during the 20 days (d) experiential period in which experimental flasks were examined daily under light microscopy for the first three days, and then every second or third day thereafter. The flask seeded with only CHSE-214 cells, as negative control, remained healthy throughout the duration of the experiment. After an extensive CPE had developed, the supernatants were collected and prepared for TEM to establish if the *P. salmonis* isolate not previously infected with the phage particle had become infected.

Sample A (LF-89 treated with UV + R-29) had an increased number of phage particles associated with the rickettsia like organisms (RLO). Phage particles could also be seen free in the media (Figure 7.6 a, b, c and d). Sample B (untreated LF-89 + R-29) contained RLO in various sizes with some phage particles attached to the rickettsial bodies (Figure 7.7 (a) and (b)). When Sample C (LF-89 not treated with UV radiation) was examined under TEM, RLO was seen associated with phage particles (Figure 7.8). Sample D, which contained only isolate R29, contained no phage particles, but for some reason the yield of the bacterium was very low in this sample. A possible differentiation of the size of the two isolates, LF-89 and R-29 could be observed when Sample C and Sample D were compared. The bacterial cell size of LF-89 seemed to be smaller than those of R-29. Although, RLO observed in Sample B with or without phage particles were in different size under TEM, it was not easy to decide for some of the RLO if it was LF-89 or R-29.

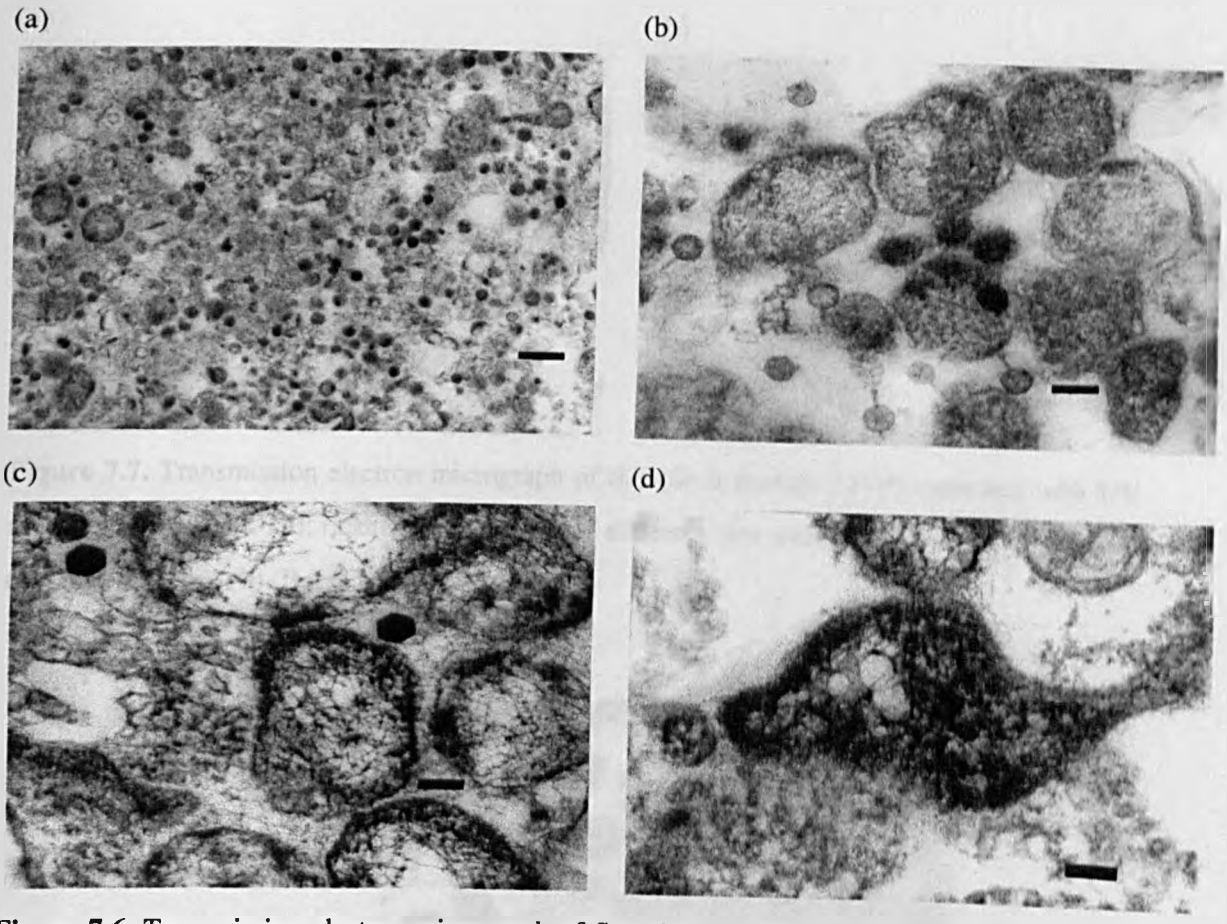


Figure 7.6. Transmission electron micrograph of Sample A (isolate LF-89 treated with UV + isolate R-29) (a) An increased number of phage particles were observed in the sample compared with sample C (Bar: 0.5 μm); (b) (Bar: 0.2 μm) and (c) (Bar: 0.1 μm): Rickettsial organisms associated with phage particles; (d) Individual rickettsial organisms associated with phage particles (Bar: 0.1 μm).

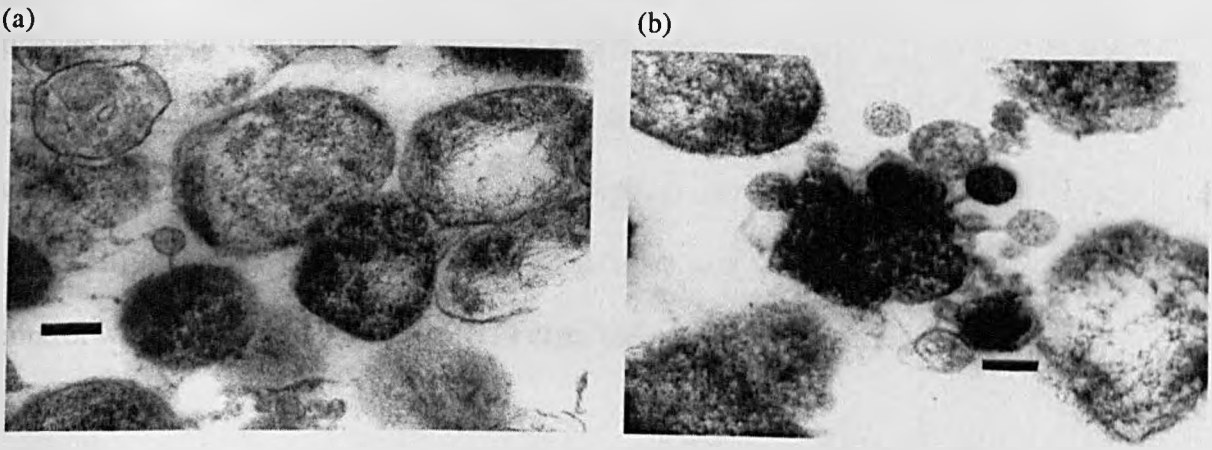


Figure 7.7. Transmission electron micrograph of Sample B (isolate LF-89 untreated with UV radiation + isolate R-29); rickettsial organisms of different size with associated phage particles **(a)** (Bar: 0.2 μm); **(b)** (Bar:0.1 μm).

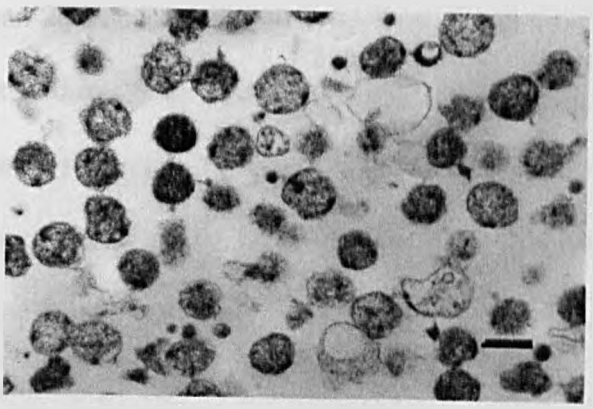


Figure 7.8. Transmission electron micrograph of Sample C; type strain LF-89 untreated with UV. (Bar: 0.5 μm).

In the plaque assay, wells containing only isolate R-29, used as a positive control, had 7 plaques per well (the mean of 4 wells) at a 10^{-6} dilution, which corresponds to a mean value of 7 $x10^{7}$ plaque forming units (PFU) ml^{-1} for four replicate wells. However, wells containing isolate R-29 infected with the phage particles had 6 plaques (the mean of 4 well) at the same dilution, giving a mean value of $6x10^{7}$ PFU ml^{-1}. The negative control wells containing only CHSE-214 cells had no plaques.

7.3.3. Analysis of the phage nucleic acids isolated from the phage particles

When the nucleic acids of the phage were analysed, in the samples filtered using a 0.4 μm filter, the nucleic acid appeared to be digested by RNase but not by DNase. The nucleic acids were larger than 20 kilo base pair (kbp) (Figure 7.9 a). However, when samples that had been filtered through the 0.2 μm filter were analysed, nothing was observed on the gel. The analysis was repeated on CHSE-214 cells and *P. salmonis* with no associated phage particles to establish that the nucleic acid bands obtained definitely originated from the phage particles and not from some other origin. No nucleic acid bands were obtained with these samples after filtration and treatment with either DNase or RNase (Figure 7.9 b).

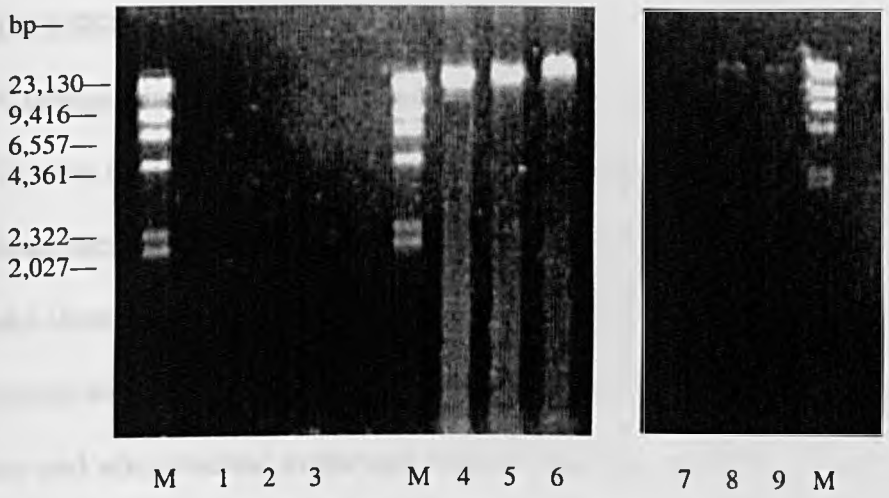


Figure 7.9. (a) Nucleic acid analysis of phage particles

(1) Filtrate from 0.2 μm filter treated with RNase; (2) Filtrate from 0.2 μm filter treated with DNase; (3) Filtrate from 0.2 μm filter untreated; (4) Unfiltered and untreated; (5) Unfiltered and treated with DNase; (6) Unfiltered and treated with RNase; (7) Filtrate from 0.4 μm filter treated with RNase; (8) Filtrate from 0.4 μm filter treated with DNase; (9) Filtrate from 0.4 μm filter untreated; (M) Marker (*Hind*III).

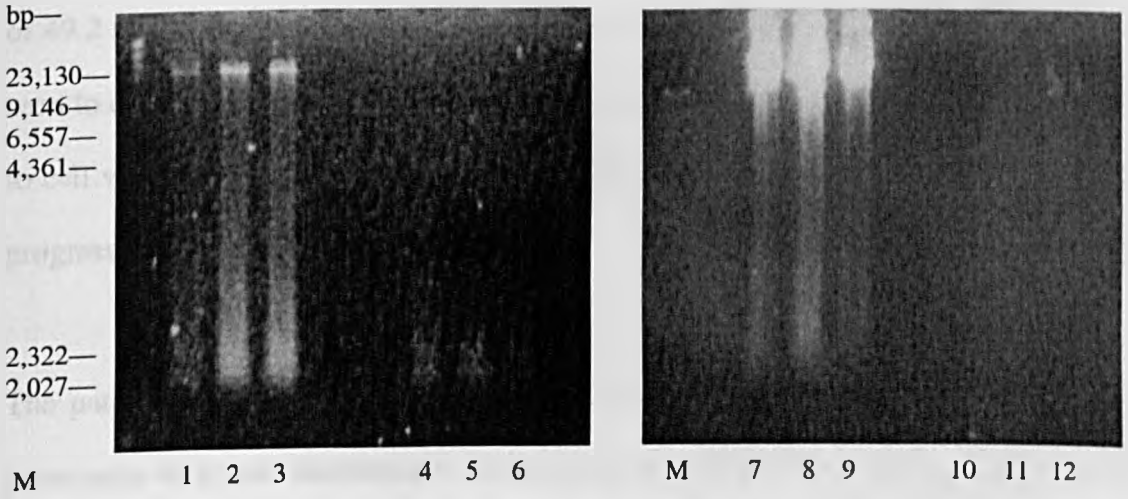


Figure 7.9. (b) Nucleic acid analysis of CHSE-214 and bacteria without phage particles. (1) Bacteria without phage, unfiltered and treated with RNase; (2) Bacteria without phage, unfiltered and treated with DNase; (3) Bacteria without phage, unfiltered and untreated (4) Bacteria without phage, filtrate from 0.4 μm filter treated with RNase; (5) Bacteria without phage particles, filtrate from 0.4 μm filter treated with DNase; (6) Bacteria without phage, filtrate from 0.4 μm untreated; (7) CHSE-214, unfiltered and treated with RNase; (8) CHSE-214, unfiltered and treated with DNase; (9) CHSE-214, unfiltered and untreated; (10) CHSE-214, filtrate from 0.4 μm filter treated with RNase; (11) CHSE-214, filtrate from 0.4 μm filter treated with DNase; (12) CHSE-214, unfiltered and untreated; (M) Marker*Hindu* III.

7.4. DISCUSSION

Two *P. salmonis* isolates originally isolated from fish, the type strain LF-89 and isolate VQO13, were found to have phage particles associated with them. The phage particles had a head were polyhedral in shape measuring approximately 110-130 nm in diameter, and had a short rigid tailpiece attached to the vertex of the polyhedron. From the TEM examination the virus-like particles could be seen throughout the cytoplasm of the *P. salmonis* and also attached to the cell walls of the bacterium by the tip of their tail. They appeared able to eventually lyse the bacterial cells they had infected.

Wright *et al.* (1978) reported the first observation of mature virus particles within the *Rickettsiales* associated with the genus *Wolbachia pipientis*. The particles of this virus had a short tailpiece and were spheroidal or polyhedral in shape with a mean diameter of 49.2 nm. They were usually, but not always, associated with swollen Wolbachia or seen to exhibit pyknotic patches. The authors suggested that the virions pass from cell to cell with the division of the prokaryote cell before the virus-induced pathology has progressed sufficiently to inhibit division.

The paracystalline array of virus associated with the bacteria in this study has been previously observed. Harshbarger, Chang and Otto (1976) observed phage particles, 50 nm in diameter, in a crystal lattice array within greatly distended initial bodies of chlamydia isolated from hard clams, *Mercenaria mercenaria*. Similar structures have also been associated with free virus infecting an Avian strain of *Chlamydia psittaci* (Storey *et al.* 1989) and in bacteria closely related to the genus *Coxiella* of the family *Rickettsiaceae* (Buchanan 1978). Buchanan (1978) concluded that phages are frequently arranged in dense paracrystalline arrays suggesting that many of the particles

were formed nearly simultaneously at a circumscribed place within the cytoplasm of the bacterium, allowing crystallisation to occur. Numerous small striated rod-like structures, were seen by the authors closely associated with the phage, and were only present in rickettsial cells containing phage particles, suggesting some sort of causal relationship between the two. The author suggested that the particles might be either viral components, cell products modified by the virus or merely a cellular reaction to the presence of virus. The diameter of the phages was 66-68 nm with a distinct hexagonal outline, and the electron dense core was surrounded by regularly repeating subunits which could be capsomers.

Shaw and Moloo (1993) observed virus-like particles (VLPs) in Rickettsia located within the cytoplasm of midgut epithelial cells of *Glossina morsistans centralis* and *Glossina brevipalpis*. The VLPs were also seen, but less frequently, within the cytoplasm of epithelial cells, invariably in close proximity to both infected and uninfected RLO. VLPs consisted mainly of a uniformly dense, spheroidal structure between 35 and 40 nm in diameter. However, some of them were composed of a dense core surrounded by a thin shell-like structure separated from the core by an electron-lucent zone, which may have been represented the assembly process of the VLPs. The authors also observed paracrystalline arrays consisting of empty, electron-lucent spheres 35-40 nm in diameter in the infected RLO. These lucent particles resembled empty nucleocapsids which seem to represent an early stage in the replication and/or maturation of the virus, individual or in paracrystalline arrays. The significant increase in the size and shape of the infected RLO that occurred was due in part to the presence of a large numbers of viral particles present within the bacterium. The authors did not observe the release of VLPs from the RLO, but suggested that this may happen as a

result of the lysosomal destruction of the RLO and/or that there is preferential destruction of virus-containing RLO by the host cells.

The paracrystalline array observed in this study with *P. salmonis* isolate R-29, seemed to be an early stage in the development of the virus particles. However, no mature virus particles were observed independently of the paracrystalline array with this isolate, except for the uncharacterised round shaped particles which were seen in the RLO.

The results of the trans-infection experiment described in this study were not completely clear. *P. salmonis* isolate LF-89 used as a source of phage infection unfortunately did not have many phage particles present to infect isolate R-29, but the amount of phage present seemed to increase after UV treatment. This together with the size difference observed between the isolates, LF-89 and R-29, as a criterion taken to evaluate trans-infection prior to TEM analysis, and the observation of phage particles associated with some of the RLO was not sufficient to confirm the trans-infection. Thus using purified phage particles in the analysis may have been more reliable.

Wu *et al.* (1981) indicated that most of the strains of *A. hydrophila* examined could not be infected by phage AH1 which had originally been isolated from *A. hydrophila* because of variations between strains of *A. hydrophila* and the high specificity of infection by this phage. *P. salmonis* may also have a similar strain variation in susceptibility to the phage, or the phage may have different specificities for its host. Nevertheless, the result of the plaque neutralisation assay gave some promising results in the trans-infection experiment. Trans-infection resulted in fewer plaques in wells

where isolate R-29 had been incubated with a solution containing phage compared with the positive control (i.e. wells containing *P. salmonis* isolate R-29 only).

Park *et al.* (1997) suggested that there was no correlation between phage sensitivity and antigenic variation when they analysed the sensitivity of a variety of *L. garvieae* strains to a virulent bacteriophage, PlgY. Kawaoka, Otsuki and Tsubokura (1983) described growth temperature-dependent differences in bacteriophage receptors of *Yersinia enterocolitica* and Stevenson and Airdrie (1984) claimed that cell-surface differences could account for the behaviour of some *Yersinia ruckeri* strains with respect to both growth temperature and phage sensitivity. Phage particles may potentially be of value to examine the correlation between the virulence and morphologic differences of the phage with respect to the sensitivity of *P. salmonis* isolates.

Analysis of the nucleic acids isolated from the phages which infected *P. salmonis* indicated the break down of phage nucleic acid by RNase when the sample was prepared by filtering through a 0.4 μm filter. However, the morphology of the phage particles did not resemble any of the known RNA phage families such as *Leviviridae* or *Cystoviridae* which have tail-less icosahedral heads and are smaller in size. It was not possible to confirm if the phage particles associated with *P. salmonis* are new phages, due to difficulties in culturing sufficient rickettsia itself or phage-infected rickettsia to obtain a significant amount of the phage to purify. The intracellular nature of the both rickettsia and phage made this work difficult to perform. Shaw and Moloo (1993) also attempted to characterise the VLPs mentioned above using a modified uranyl acetate/EDTA staining method for ribonucleoproteins (RNPs) together with the application of DNase-gold and RNase-gold complexes on thin glutaraldehyde-fixed,

Epon-embedded sections. The results were ambiguous, and whether the VLPs contained either DNA or RNA remains undetermined. No reaction was observed on the gel after filtration with a 0.2 μm filter with either digested or undigested samples despite the small size of the phage particles. They have a diameter of 110-130 nm and are filterable by the 0.2 μm filter. The reason for this is unknown at this time.

Phage therapy is based firstly, on the fact that growth of the bacteriophage is able to out-compete bacterial growth, and secondly infection by the bacteriophage is very specific for its host. However, potential difficulties associated with phage therapy need to be considered. For example, it is necessary to understand the heterogeneity and ecology of both the phage and the bacterium, and to be able to select highly virulent phages against target bacteria. It is also important to establish if bacterial strains have become resistant to the phage, and to understand which factors of the vertebrate host immune response are able to inactivate the phages and lyse bacteria (Barrow & Soothill 1997). These points are important when developing an effective phage therapy. It would appear from this study that the phage particles are able to grow successfully within the rickettsia, when the bacterium is grown *in vitro* within cell culture, while they disappeared when the rickettsia were cultured *in vivo* during experimental challenges performed in Section 6.2.1. It suggests that the phages may have been introduced during the cultivation of the bacterium. Differences in the culture environment of the rickettsia grown *in vivo* and *in vitro* may, in part, explain why the phages appear unable to grow within the rickettsia whilst in the host animal.

Vibrio bacteriophages have been shown to be able to lyse and genetically alter populations of *Vibrio* spp., particularly in environments where host organisms, Pacific

oysters, are present in high numbers and replicating (Baross, Liston & Morita, 1978). These organisms may be suited to environments, which favour bacteria-virus and bacteria-bacteria interactions. In another study, DePaola, McLeroy and McManus (1997) suggested a close ecological relationship between *Vibrio vulnificus* phages and host molluscan shellfish.

Phage-neutralising antibodies may cause complications in phage therapy (Smith & Huggins 1982, Smith *et al.* 1987). Therapeutic success was found to be due to high phage activity *in vivo* and the failure to produce phage-resistant mutants during treatment, whereas failure of phage therapy has not been shown to be due to reduced phage activity *in vivo* or due to rapid appearance of phage resistant bacteria (Smith & Huggins 1982). Phage therapy may provide an alternative to the problems associated with antibiotic therapy, however, the requirements of phages *in vivo* first needs to be elucidated. Further characterisation of the bacteriophages and establishing mutants with a broader spectrum of infection for the various strains of *P. salmonis* is necessary if phage therapy is to be considered as an approach to control pathogenic *P. salmonis* in fish culture.

CHAPTER VIII

GENERAL DISCUSSION

Members of rickettsiae, which are obligately intracellular organisms, cause a number of diseases in both man and animals (Buxton & Fraser 1977). *Piscirickettsia salmonis* is the first rickettsial pathogen to be recognised as the etiological agent of an epizootic in fish (Fryer & Lannan 1996). Since the first reports of piscirickettsiosis in the late 1980s, there have been numerous studies relating to the observation or isolation of the pathogen from infected fish tissue, as discussed in detail in Chapter 1.

During the course of evolution, organisms of wide ranging diversity, have adapted to the hostile environment of potential host cells. Here, they exploit the reservoir of nutrients available to them, with the result that they can reside and multiply within the host cell (Moulder 1985). Cell cultures infected with intracellular parasites are theoretically valid models for replicating the infection process. However, the parasite's virulence and host's resistance may only be modelled *in vitro* within the limits of the cell culture system (Moulder 1985). This is because the effect of an intracellular parasite on its multicellular host is a sum of the effects of the bacterium on all infected cells within its host, and this should therefore be taken into account when evaluating the effects seen in any particular cellular model (Moulder 1985).

Propagation of ten different isolates of *P. salmonis in vitro* obtained from three different geographical locations and from four different fish species, was carried out in six different fish cell lines (Chapter II). Culture and replication of the organism were successfully performed in this study, and it was possible to observe the growth cycle of

P. salmonis within the different fish cell lines by transmission electron microscopy (TEM) and light microscopy (LM) (Chapter III and Chapter IV). Although the susceptibility of fish cells to *P.* salmonis *in vitro* has already been demonstrated in a number of fish cell lines (Fryer, Lannan, Garcés, Larenas & Smith 1990; Cvitanich, Garate & Smith 1991), the development of the Cytopathic effect (CPE) within the various fish cell lines by various *P. salmonis* isolates has not previously been described until now.

The ability of the organism to invade and replicate within a cell is one of the most important properties of an intracellular organism. Two types of assays were used to measure the infectivity of viable organisms within the cell line in this study, 50% tissue culture infective dose ($TCID_{50}$) and a plaque assay. Plaques of different size and shape, caused by isolates LF-89, R-29 and SLGO-95, were observed within the CHSE-214 and EPC fish cells when examined in the plaque assay. The shape of the plaques varied from round to irregular, and some appeared as comet-tails. A higher plaque forming units (PFU) value and larger size plaques were observed in Chinook salmon, *Oncorhynchus tshawytscha* embryo cell line (CHSE-214) compared with Epithelioma papulosum cyprini *Cyprinus carpio* L. cell line (EPC), corresponding with the findings of Barnes (1996) who also observed higher numbers of plaques in CHSE-214 monolayers than in EPC monolayers. On the other hand, it was impossible to calculate the PFU in the remaining cell lines [Bluegill, *Lepomus macrochirus* Rafinesque fry cell line (BF-2), rainbow trout, *Oncorhynchus mykiss* gonad cell line (RTG-2), sea bass, *Cetropristis striata* larvae cell line (SBL) and Atlantic salmon, *Salmo salar* L. head kidney cell line (SHK-1)] examined because similar types of structures also appeared in the control wells. Weiss (1973) reported that under optimal conditions of the plaque

assay, differences were evident among the rickettsial species such as the required period for plaques to form or in the size of plaques which resulted. A number of factors can affect plaque formation within the plaque assay, such as the sensitivity of the cells to the intracellular organism, the condition of the host cells, the time of adsorption of the organism, the type of agar used in the overlay or the volume of the overlay, the type of serum used in the overlay medium which can inhibit or enhance plaque formation, the volume or type of medium used in the inoculum, the time of incubation needed for the development of plaques, the staining procedure used to observe the plaques, the interaction between the host cell and the intracellular organism, and the presence of a cytophatic effect by the organism for plaque formation to occur (Rovozzo & Burke 1973, Burleson, Chambers, & Wiedbrauk 1992, Nurtjahya 1995).

The $TCID_{50}$ assay was used to examine the growth conditions of *P. salmonis* within CHSE-214 cells, and to examine the susceptibility of the different fish cell lines to different *P. salmonis* isolates. The susceptibility of CHSE-214 cells to the different *P. salmonis* isolates was determined by calculating the $TCID_{50}$. It appears that *P. salmonis* has a 3-5 days (d) lag-phase and an 8 d log-phase of exponential growth in this particular cell line. This may be followed by a second log-phase of growth by the bacterium in when the remaining monolayer cells become infected by second generation *P. salmonis.*

Although the different fish cell lines used in this study showed variations in their sensitivity to the various *P. salmonis* isolates tested, it is difficult to assess host susceptibility *in vivo* by extrapolation of the results seen *in vitro*. The findings of this study suggest that *P. salmonis* can grow in both salmonid and non-salmonid cell lines,

and this may explain the wide distributions of hosts found to be susceptible to this bacterium. The general conclusion from the literature is that with some intracellular parasites, such as rickettsia and malaria, a good correlation can be obtained between the damage caused by the parasite in *vitro* and *in vivo* (Moulder 1985).

P. salmonis is more virulent in Atlantic salmon and coho salmon *Oncorhynchus kisutch* than it is in rainbow trout, and the trout appear to be more efficient than coho salmon in clearing the bacterium, although piscirickettsiosis can be experimentally reproduced in rainbow trout (Cvitanich *et al.* 1991; Garcés, Larenas, Smith, Sandino, Lannan & Fryer 1991; Smith, Contreras, Garcés, Larenas, Oyanadel, Caswell-Reno & Fryer 1996). When the susceptibility of six different fish cell lines to ten different *P. salmonis* isolates was examined based on the $TCID_{50}$ ml^{-1} of each isolate, the highest susceptibility to the bacterium was seen in CHSE-214 fish cells derived from chinook salmon, SHK-1 derived from Atlantic salmon and EPC derived from common carp. However, a CPE was not apparent in BF-2 cells derived from bluegill fry. The SHK-1 cells showed quite a high susceptibility to *P. salmonis*. The present study is the first report of culture of *P. salmonis* within SHK-1 and SBL cells, derived from sea bass. RTG-2 cell line, derived from rainbow trout, and SBL cells had the lowest susceptibility to all *P. salmonis* isolates examined. EPC cells have previously been reported to be susceptible to RLOs isolated from fish (Fryer *et al.* 1990; Cvitanich et al. 1991; Almendras, Jones, Fuentealba & Wright 1997), and they were also susceptible to all *P. salmonis* isolates tested in this study. However, Jones, Markham, Groman and Cusack (1998) reported that when *C. carpio* common carp (5 g) were injected intraperitoneally (IP) with 0.1 ml of a $10^{2.875}$ $TCID_{50}$ ml^{-1} RLO infected cell culture (CHSE-214), no carp died during the 21 d experimental period. However, 100% of

Atlantic salmon (10 g), 62% of coho salmon (10 g) and 22.5% of rainbow trout (25 g) died in the same experiment. All fish used in the experiment were held in fresh water at 18.0 ±0.5°C. It would be interesting to establish why a CPE was produced in EPC cell lines while no carp died in the described experiment. Smith *et al.* (1996) also reported that mortalities observed in rainbow trout were lower than those occurring in coho salmon during a piscirickettsia challenge. However, the mortalities in rainbow trout occurred earlier and in a shorter period of time, than seen in coho salmon and in addition the pathological findings in dead fish were consistently more severe in rainbow trout than seen in coho salmon. They suggested that the susceptibility of rainbow trout to *P. salmonis* was more variable than that seen in coho salmon. In the present study, isolate VQ013 recovered from rainbow trout was one of the isolates, to which CHSE-214 cells showed more susceptibility than the other isolates, and isolate VQ013 was also one of the isolates which varied mostly in its virulence to the different cell lines, especially in salmonid cell lines.

Both the intracellular nature of *P. salmonis* and the variations in virulence between different isolates make it difficult to standardise an effective experimental challenge model for the bacterium. An experimental challenge was performed in Atlantic salmon in Chapter VI in which fish were infected with one of nine different *P. salmonis* isolates. Although, the level of mortalities was low in the challenge experiment, the highest level of mortality was obtained with a coho salmon isolate SRS-4. The complex and constantly changing environment experienced by the bacterium during infection *in vivo* has a strong influence on the pathogenicity of the bacterium by affecting both its growth and the production of virulence factors. These factors may be over looked or not present during infection *in vitro* within fish cell lines, even though

they are effective during infections *in vivo* (Ellwood & Tempest 1972; Smith 1990; Turgut 2002).

Fish eliminate pathogens through both specific and nonspecific immune defence mechanisms (Ellis 1988); fish will either survive or die during this pathogen-host interaction. It was shown here that fish were able to develop an antibody response against the bacterium with continuous levels of *P.salmonis* during the experimental period of two months. Live attenuated vaccines generally provide better protection than killed vaccines as a consequence of immunogens present on the replicating bacteria stimulating a protective immune response in the fish (Turgut 2002). Immunisation with live bacteria may produce an antibody response against bacterial proteins that function as virulence factors within the macrophage where the bacteria are growing (Thorton, Garduño & Kay 1992; Marsden, Vaughan, Fitzpatrick, Foster & Secombes 1998; Gudding, Lillehaug & Evensen 1999; Turgut 2002). The antibodies produced against live bacteria recognised very distinct bands in Western blot (WB) analysis, compared with antibodies produced against killed bacteria when Atlantic salmon were immunised with a number of *P. salmonis* isolates (Chapter VI). *P. salmonis* isolate R-29 from Atlantic salmon appeared to be the most immunogenic isolate among those used in both the challenge and in the immunisation experiments. Although SHK-1 cells showed high susceptibility to R-29 *in vitro,* no fish died when they were injected with this isolate.

Garcés *et al.* (1991) reported that Atlantic salmon (10 g), held in filtered spring water at 9-12°C and injected IP with RLO at a dose of $10^{5.3}$ or $10^{4.3}$ $TCID_{50}$ $fish^{-1}$, showed 100% and 93% mortality at d 39 respectively. The mortalities which occurred had no signs of

disease. Jones, Markham, Groman and Cusack (1998) also experimentally infected Atlantic salmon (10 g) with 0.1 ml of $10^{2.875}$ $TCID_{50}$ RLO (IP) and held the fish in freshwater at 18 ±0.5°C, with 100% mortalities observed over 21 d. Environmental conditions present in the current study such as water temperature, salt water or the size of fish used in the challenge experiment, might have been responsible for the low level of mortalities obtained. There may have been a number of reasons why a low level of mortalities occurred in the challenge experiment. It may have been as a consequence of immunogens present on replicating bacteria stimulating a protective immune response as described with live attenuated vaccine above, or else the high passage number of the bacterium in culture may have led to a loss of virulence, or the dosage of the inoculum used in the challenge experiment may have been inadequate to kill the fish but sufficient to stimulate a protective immune response in infected animals during the two months challenge period. The bacterial inoculum used for the challenge experiment had been prepared at the Institute of Aquaculture in Stirling, prior to performing the experiment at the aquarium facilities of the FRS Marine Laboratory, Aberdeen. The bacterium had been kept in Phosphate buffer saline (PBS) for more than 24 hours (h) at 4°C before the fish were inoculated. The prolonged storage of the bacterial samples prior to the challenge may have affected the results obtained in the challenge experiment. It was not possible to repeat the challenge experiment or to perform a challenge experiment with the immunised fish before the end of the study because of limited time and the time required to produce sufficient quantities of rickettsiae for the challenge.

Good yields of viable rickettsia require a method for releasing the bacterium from the host cell and promptly harvesting it without losing its viability in the extracellular

environment (Weiss 1973). Purification of rickettsiae from host cells after culturing them *in vitro* is necessary for characterisation of the organism or for the development of a vaccine but it can be difficult to isolate the pathogen from the host cells because of its obligate intracellular nature (Kuzyk, Thorton & Kay 1996; Almendras & Fuentealba 1997; Yuksel, Thompson, Ellis & Adams 2000). *P. salmonis* was purified from CHSE-214 cells using a simple and effective method consisting of differential centrifugation and a Percoll gradient as described in Chapter III. When the purity of each preparation was assessed by TEM and LM, the appearance of the purified rickettsiae appeared intact with very little disruption to the morphology of the cell. Protein yields and viability of purified *P. salmonis* type strain LF-89 was also determined in this study. It was shown that both crude and purified preparations of rickettsiae retained their infectivity for fish cells after differential pelleting and Percoll gradient centrifugation. The purification protocol using the 30% Percoll gradient (v/v) had most infectious rickettsial particles and the highest protein content. They also had the lowest amount of fragmented material present compared with bacteria purified using the 40 % and 50 % Percoll gradients (v/v). However, the rabbit PAb (PAb-1) and the MAb probes (6A1F4C5, 6A1F4G3, 8G7, 7E2, 6A1G6 and 6A1G3) were produced against whole cell preparations of *P. salmonis* type strain LF-89 purified on a 40% Percoll gradient (v/v) (Chapter V). This was because, initially, a modified version of the method described by Kuzyk *et al.* (1996) using a 40% Percoll gradient (v/v) to purify the *P. salmonis* was used to produce those antibodies (Chapter V). However, as the study progressed, it was established that the purification method using 30% Percoll gradient (v/v) gave better yields of *P. salmonis*.

Following purification, the bacterium, type strain LF-89, was examined by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein profile of the purified *P. salmonis* had many bands with molecular weights at 157, 140, 115, 95, 72, 60, 56, 51, 40, 36, 32, 30, 28, 26, 23, 20, 16, 14 and 11 kilodalton (kDa). The lower molecular weight bands at 11 and 14 kDa were only seen when the gels were Silver stained. The SDS-PAGE analysis also revealed differences in the protein profiles between *P. salmonis* -infected CHSE-214 cell and uninfected CHSE-214 cells. The bands at 83, 64, 18 and 16 kDa were observed in the profiles of both CHSE-214 cells and the purified preparations of *P. salmonis*. This suggests that the purified *P. salmonis* might possess some proteins with the same molecular weight (MW) as the host cells, or else some of the bands originated from host cell contamination. The rabbit serum prepared against *P. salmonis* (PAb-1) in Chapter V reacted slightly with bands at 83 and 64 kDa in the profile of uninfected cells in WB analysis. The 64 kDa antigen, together with a 68 and a 70 kDa antigen, were strongly reactive in the profiles of uninfected CHSE-214 cells in a study of Barnes, Landolt, Powell and Winton (1998) using a polyclonal rabbit antiserum prepared against *P. salmonis* grown in CHSE-214 cell cultures without extensive purification. This suggests that it might be difficult to obtain *P. salmonis* completely uncontaminated by host cell antigens or that some antigenic cross reactivity exists.

The antigenic structure of the ten *P. salmonis* isolates was compared using SDS-PAGE (Chapter IV). The banding profile of the various *P. salmonis* isolates recovered from CHSE-214 cell appeared very similar to each other with silver staining, with only slight differences in the intensity of the staining between the isolates, especially in the low molecular weight region of the profiles. When the gels were stained with Coomassie

blue, similar banding patterns were again seen between the different *P. salmonis* isolates, but the staining was paler in intensity than observed with the silver staining. Even though the Silver stain is more sensitive than Coomassie blue, the difference in intensity of staining seen with the Silver stain could be due to the presence of a high quantity of carbohydrates in the cellular material. This was confirmed by digesting *P. salmonis* with proteinase-K. Most of the *P. salmonis* isolates treated with the enzyme showed considerable dark brown staining in the lower region of the gel when stained with Silver stain, particularly with bands below 30 kDa. The cell wall of *P. salmonis* was shown to be rich in glycoproteins with many carbohydrate moieties composed of mostly of α-D-galactosyl and N-acetyl-α-D-galactosaminyl residues, but also with D-galactose and α-D-mannosyl and α-D-glucosyl residues. Some of the material in the lower molecular weight regions of the gel was also recognised by the lectins used to screen the *P. salmonis*, possibly because they contained complex polysaccharides with different carbohydrates moieties (Chapter IV).

A band of approximately 10-16 kDa was recognised by MAb 6A1F4C5 in the WB analysis of proteinase-K digested *P. salmonis* (Chapter V). Kuzyk *et al.* (1996) reported similar results that rabbit antiserum developed against whole-cell *P. salmonis* which recognised two immunoreactive carbohydrate antigens at 16 and 11 kDa. The author claimed that the 11 kDa antigen is most likely the lipo-oligosaccharide (LOS) component of lipopolysaccharide (LPS) which is a main component of the outer membranes of rickettsiae and other Gram-negative bacteria, while the 16 kDa may represent the core region lipo-ologosaccharides (LOS) with one unit of O polysaccharide antigen. The high molecular weight carbohydrates generally represent core region LOS and O antigen with a declining number of repeating units (Amano,

Fujita & Suto 1993). The band between 10-16 kDa could be a repeated subunit of LOS with O antigens or proteolytic fragments of a protein antigen of *P. salmonis*. Bacterial cell surface polysaccharides i.e. capsular polysaccharides or LPS, are known to be involved in the pathogenicity and immunogenicity of bacterial pathogens (Barton, Bannister, Griffits & Lynch 1997; Sørum, Lendsottir & Robertson 1998b; Arakawa, Wacharotayankun, Nagatsuka, Ito, Kato & Ohta 1995). The serological specificity of Gram-negative bacteria is determined by the structure of different bacterial cell surface polysaccharides of the outer-membrane LPSs (Ziolkowski, Shashkov, Swierzko, Senchenkova, Toukach, Cedzynski, Amano, Kaca & Knirel 1997; Arakawa *et al.* 1995). The significance of carbohydrate in the pathogenicity of *P. salmonis* against host cells should be investigated further, the results of which may help in the development of an effective vaccine against the bacterium.

Although protein analysis of several members of *Rickettsiaceae* shows approximately 10 antigens between 20 to 120 kDa in size (Barnes 1996), the function and location of the proteins on the bacterium are still mainly unknown. However, the 120 kDa band is known to be the rickettsial outer membrane protein B (rOmpB) and the 32 kDa is a possible membrane anchor for the protein before cleavage. The 120 kDa rOmpB protein is strongly immunogenic and is believed to be a potential candidate for developing a subunit vaccine against rickettsial infections (Hackstadt, Messer, Cieplak & Peacock 1992; Barnes 1996). When an avirulant mutant of *Rickettsia rickettsii* was analysed, reduced amounts of the 120 and 32 kDa fragments were observed (Hackstadt *et al.* 1992).

Each species of rickettsia has its own species-specific antigens which stimulate the production of specific antibodies for that particular species. They also possess a soluble group of antigens, apart from *Coxiella burnetti*, and this is probably derived from the mucoid envelope of the organism (Buxton & Fraser 1977). Species-specific antigens are based on antigens localised on the surface of the rickettsial cell wall, such as LPSs or O antigens which are common to a particular rickettsial group (Teysseire & Raoult 1992). These surface proteins are highly immunogenic (Ching, Wang, Jan & Dasch 1996), and constitute between 10 and 15% of the total rickettsial cellular protein. These are believed to interact directly with components of the hosts immune system due to their association with the rickettsial cell envelop (Dasch 1981).

The 115 kDa protein of *Rickettsia conarii*, which belongs to the Spotted fever group (SFG) rickettsia, is a heat stable protein antigen and is also thought to be a surface antigen since it appears to be involved in the adhesion of rickettsia to the host cell surface (Winkler 1990). The 115 kDa protein recognised by the monoclonal antibodies (MAbs) and PAb-1 in this study may be involved in adhesion of the rickettsiae to the surface of the host cell. The disappearance of the 115 and 32 kDa proteins on *P. salmonis* isolate R-29 when it was passaged through BF-2 cell line may explain why this cell line is less susceptible to the bacterium as a result of reduction of rickettsial attachment to the host cell. The 115 kDa band was only slightly stained in the profile of R-29 when it was grown in RTG-2 cells compared to the other cell lines (CHSE-214, SHK-1 and EPC). On the other hand, the 32 kDa band disappeared completely from the profile of R-29 when it was grown in RTG-2 the same as occurred when it was grown in BF-2 cells. RTG-2 was the other fish cell line which had a lower susceptibility to *P. salmonis* isolates. The 32 kDa antigen recognised by PAb-1 and the

MAbs, was more evident when stained with MAb 8G7. The proteins of *P. salmonis* observed in Chapter V may be important in producing a protective immune response to piscirickettsiosis since they may have a role as potential virulence factors during intracellular growth. Therefore, identifying and characterising the proteins of *P. salmonis* expressed intracellularly may be useful in understanding the pathogenesis of the bacterium and help in the identification of candidate antigens for vaccine preparations.

The 60 kDa protein appears to be related to the highly conserved family of heat shock proteins (Hsp). This protein appears to be a structurally and immunologically common antigen, wide spread in eukaryotic and prokaryotic organisms, and is essential for bacterial growth and viability (Teysseire & Raoult 1992; Eremeeva, Ching, Wu, Silverman & Dasch 1998). The 60 kDa protein was intensively stained with the rabbit antiserum, slightly with some of the MAbs produced in Chapter V, and sera from fish immunised with *P. salmonis*, especially with sera from fish immunised with isolate R-29 (Chapter VI). The heat-shock protein antigen promotes translocation, folding and assembly of proteins in some forms of Mediterranean Spotted Fever Group (MSFG), which can cause malignancy (Teysseire & Raoult 1992). Hsp are also thought to provide effective protection for intracellular pathogens against the hostile environment within host phagocytic cells (Teysseire & Roult 1992; Eremeeva *et al.* 1998; Voet, Voet & Pratt 1999), but may be able to activate cell-mediated immunity.

Differences observed in the protein profile of the bacterium (isolate R-29) cultured within different cell lines were mostly in the lower MW region of the protein profile. Samples of the bacterium recovered from BF-2 cells had faint bands at 28 and 26 kDa,

while some lower molecular weight material between 14-20 kDa seemed to be missing when isolate R-29 was grown in this cell line (BF-2), compared to when it was grown in other fish cell lines tested. The differences in the banding profile of *P. salmonis* isolate R-29 when it was passaged through different fish cells needs to be examined in more detail to establish which are derived from the bacterium itself and which are from host cell components. The antigenicity of the different isolates needs to be examined further in view of developing a vaccine against *P. salmonis* which will be effective in different hosts.

The major antigens of *P. salmonis*, identified with rabbit polyclonal antibody (PAb-1), the mouse PAb, the MAbs produced in mice, and the fish sera produced against killed or live bacteria were located at 95, 72, 60, 36, 32 and 20 kDa. Additional bands at 115, 51, and 40 and between 10-16 kDa were also recognised by some of the sera (Chapter IV, Chapter V and Chapter VI).

The potential of using the rabbit PAb-1 and the MAbs produced here as diagnostic tools to detect the pathogen in infected fish tissue was also examined (Chapter V). PAbs, or MAbs with their greater specificity, have frequently been used in the rapid diagnosis of fish diseases (Johnson, Wobeser & Rouse 1974; Busch and Ling 1975; Adams, Thompson, Morris, Farias & Chen 1995; Austin and Austin 1999). As antibiotic treatment is expensive and often long term, diagnosis in the early stages of the infection is very important for effective control of the disease in aquaculture to prevent the spread of the pathogen to uninfected fish thus allowing effective farm management (Turgut 2002; Puttinaowarat 1999).

Preliminary diagnosis of piscirickettsiosis is usually made by detection of the pathogen in smears and tissue samples stained with either Gram, Giemsa, Acridine orange, Haematoxylin and Eosin (H&E), Macchiavello and Pinkerton's model for Basic fuchsin, or Castañeda, all of which were successfully used in this study (Chapter IV). However, ideally the identification of *P. salmonis* should be improved to make it more specific, more sensitive and more rapid (Almendras & Fuentealba 1997; Fryer & Lannan 1996). Isolation of *P. salmonis* from infected tissue is considered as the most specific method currently used to confirm the presence of the bacterium, although it is often complicated by contamination (Mauel & Miller 2002) and is time consuming to perform. Serological methods, such as Indirect fluorescent antibody technique (IFAT) (Lannan, Ewing & Fryer 1991); Immunohistochemistry (IHC) (Alday-Sanz, Rodger, Turnbull, Adams & Richards 1994) or Enzym-linked immunosorbent assay (ELISA) (Aguayo, Miquel, Aranki, Jamett, Valenzuela & Burzio 2002) have been used to detect *P. salmonis.* The antibody-based tests developed in this study were used either to identify *P. salmonis* in infected fish tissue (IHC and IFAT), to detect the pathogen grown in cell culture (Dot blot immunobinding assay (DBI), Line blot immunoassay (LBI), indirect ELISA and a sandwich ELISA) or to reveal if there were any antigenic differences between isolates (WB and Immuno gold staining (IGS)) with different antigen preparations techniques. Such tests with greater specificity and without limitations of cell culture techniques are useful for the detection of rickettsia in disease outbreaks and identification of new isolated *P.* salmonis isolates.

WB is a useful technique, particularly for characterising unknown antigens on the pathogen and examining antibody specificities. Although it is a qualitative method, not quantitative, it is a powerful serodiagnostic method for seroepidemiology and to

confirm the results from other conventional serological tests used in rickettsial diseases (La Scola & Raoult 1999). When biotin amplification was applied, the sensitivity of the reaction obtained was greatly increased when using MAb or fish sera, particularly in the lower MW region of the SDS-PAGE profile of the bacterium. However, when PAb-1 was used as the first antibody, biotin application resulted in a strong non-specific reaction. Jones, Mosley, Jeffrey & Stoddart (1987) reported that non-specific reactions sometimes occur as a result of biotin amplification.

The antibody probes prepared here were also used to develop some other antibody-based methods including DBI and LBI. This is the first report of LBI and DBI being used to detect *P. salmonis*. Large numbers of samples can be screened in these assays in a short space of time without the use of microscopes or incubators, and the technique has a quite high level of sensitivity. ELISA can also be used to examine many samples at the same time, and is both rapid and highly sensitive for measuring both fish pathogens and fish antibodies. The ELISA reaction can be read with the naked eye or with an ELISA reader for increased sensitivity.

IFAT and IHC were used here to examine samples from naturally infected fish. Although IFAT is widely used for the detection of antigen and antibody in fish, it needs specialised equipment. On the other hand, the use of microscope slides containing wells allows large numbers of rickettsial samples/antigens to be examined simultaneously in a microimmunofluorescence test (MIFAT) (La Scola & Raoult 1996, 1999). IHC has the advantage of allowing histopathological evaluation of lesions to be carried out, while confirming the presence of the pathogen (Alday-Sanz *et al.* 1994). IGS provides information on the location of the antibody-antigen reaction, although it is

expensive, time consuming and needs special equipment. Further examination of the reaction of the MAbs with *P. salmonis* in fish tissue with IGS would benefit our understandings of *P. salmonis* infections.

As well as the antibody based methods mentioned above, molecular techniques were also investigated as a means of detecting *P. salmonis* in infected tissue. Molecular techniques are highly specific, sensitive and rapid for detection of *P. salmonis.* A *P. salmonis*-specific nested polymerase chain reaction (N-PCR) was previously developed by Mauel, Giovannoni & Fryer (1996). Mauel *et al.* (1996) reported that the sensitivity of the N-PCR increased from 60 $TCID_{50}$ obtained with a single amplification step to less than 1 $TCID_{50}$ when a N-PCR was applied. It should allow the early detection of the rickettsial infection when the number of the bacteria in the tissue was low and the confirmation of negative results with replicate amplifications (Mauel *et al.* 1996). Mauel *et al.* (1996) suggested that the detection of less than 1 $TCID_{50}$ does not mean that less than 1 bacterium can be detected, but probably reflects the affinity of *P. salmonis* to host cell membranes, which leads to clumping. N-PCR procedures take 1 day to 2 d to perform, but because of the high sensitivity and specificity of the method, it can be used to detect very early stages of infections. It may also be possible to differentiate between *P. salmonis*-like organisms responsible for new disease outbreaks and also has the potential to define the modes of transmission of the disease. Although, this assay needs specialised equipment and can be costly for routine health screening programmes, it is both a valuable research and diagnostic tool (Mauel & Miller 2002).

Although, PAb-1 cross-reacted with some non-*P. salmonis* bacteria in the ELISA, it gave a very strong reaction with most of the *P. salmonis* isolates examined (Chapter V).

This cross-reactivity may possibly have been due to non-specific binding by the antiserum or else the other bacteria had antigenic epitopes in common with the *P. salmonis*. This needs to be investigated further using WB. On the other hand, PAb-1 can be used for the rapid detection of *P. salmonis* with high sensitivity in DBI and LBI analysis or in IFAT and IHC. The MAbs produced in this study also proved useful for the detection of *P. salmonis* since they did not cross-react with any non-rickettsial bacteria or CHSE host cells in any of the tests used. The MAbs are also useful in the development of a vaccine against *P. salmonis* as they identify potentially immunogenic antigens for using in the vaccine. Further studies to establish whether the anti-*P. salmonis* MAbs elicit any protection to fish against piscirickettsiosis in passive immunisation would be a worth while follow up to the present study. The reason for the slight differences in the reactivity of the antibodies against the different isolates is possibly due to differences in the virulence of the isolates reflected in their cell surface antigens or recognition of the antigens in the different techniques used. A combination of tests such as the antibody-based ELISA which will provide quantitative data and the PCR which provides better understanding of pathogenesis of *P. salmonis* in the fish, will help to elucidate how the pathogen is transmitted and, give an over all picture of the infectious process of the bacterium.

P. salmonis appears to contain species-specific antigens as well as other groups of antigens which also occur on other rickettsia species. Further work is needed to determine which are species-specific antigens and which are genus-specific antigens. Further establishing the antigenic similarity between *P. salmonis*, *Orientia tsutsagamushi* or any *Ehrlichia* species, will help in the taxonomical classification of *P. salmonis*.

The *P. salmonis* isolates analysed in this study were found to be antigenically very homogenous (Chapter V), however, only one isolate R-29 appeared to differ in the N-PCR. In an attempt to produce an effective vaccine against piscirickettsiosis, further studies should focus on the characterisation of immunoreactive proteins found on the different *P. salmonis* isolates, particularly on isolate R-29 with a view to identifying potential vaccine candidates. Identification and the inclusion of virulence factors in vaccines may be important as they appear to act as protective antigens in many cases (Evelyn 1997). Bacterial virulence is normally examined through a variety of biochemical, immunological or molecular genetics approaches, and often a combination of these is used (Jung 1999).

This is the first report of phage particles associated with rickettsiae in fish (Chapter VII). Additional studies are needed to characterise the phage particles associated with *P. salmonis* of fish and also to investigate if they have any potential for controlling *P. salmonis* infections in fish. Further studies using a systematic approach to examine Deoxyribonucleic acid (DNA) re-association, virulence, and phenotypic, biochemical and antigenic characteristics are required to establish the taxonomic diversity of *P. salmonis.* This may help to produce effective control strategies for piscirickettsiosis. However, problems highlighted and discussed here about *in vitro* growth characteristics, antigenicity, pathogenesis and virulence of various *P. salmonis* isolates indicates the need for further investigations which will help to resolve many unanswered questions on *P. salmonis* and control of the disease, piscirickettsiosis.

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APPENDICES

Appendix I

Buffers and Formulae

1.1. PBS

KH_2PO_4 (5.2 mM).....0.70 g/l

Na_2HPO_4 (8.1 mM) .....1.44 g/l

NaCl (116 mM) .....6.77 g/l

KCl (10 mM) .....0.70 g/l

Dissolved in 1 l distilled water and adjusted to pH 7.0; made freshly.

1.2. PBS (0.02 M Phosphate)

$NaH_2PO_4.2H_20$.....0.876 g/l

$Na_2HPO_4.2H_2O$.....2.56 g/l

NaCl (0.15 M) .....8.77 g/l

Dissolved in 1 l distilled water and adjusted to pH 7.2; made freshly.

2. Electrophoresis

2.1. Sample buffer (5 times concentration, 10 ml)

Tris-HCl (60 mM).....0.6 ml of 1M Tris-HCl, pH 6.8

Glycerol (25%).....5 ml of 50% glycerol

Sodium dodecyl sulphate (SDS) (2%).....2 ml of 10% SDS

Bromophenol blue (0.1%).....1 ml of 1% bromophenol blue

2-β-mercaptoethanol (14.4 M).....0.5 ml of 2-β-mercaptoethanol

Distilled water.....0.9ml

Stable for weeks in the refrigerator or for months at -20°.

2.2. Reservoir buffer

Glycine (192 mM).....4.4 g

SDS (0.1%).....1 g

Tris base (25 mM).....3 g

Dissolved in 1 l distilled water and adjusted pH 8.3.

Stable indefinitely at room temperature

3. Transblot and Immunoblotting buffers

3.1. Transblot buffer

Glycine (192 mM).....14.4 g/l

Tris base (25 mM).....3 g/l

Prepared in 100 ml of distilled water firstly and then added 200 ml of methanol and finally brought to 1 l and adjusted pH 8.3.

3.2. Tris buffered saline (TBS)

Tris base (10 mM).....2.42 g/l

NaCl (0.5 M).....29.24 g/l

Dissolved in 1 l distilled water and adjusted to pH 7.5.

3.3. Tris buffer saline with Tween (TTBS)

Add 0.1 ml Tween-20 to 1 l of TBS, pH 7.5.

3.4. Substrate

Stock solution: 4-chloro-naphthanol (0.33% w/v in methanol), store at -20°C

Working solution: 2 ml of stock solution mixed with 10 ml PBS and 10 µl of H_2O_2.

4. Buffers used in ELISA

4.1. Low salt washing buffer (LSWB)

Trisma base (0.02 M).....2.42 g/l

NaCl (0.38 M).....22.2 g/l

Tween-20 (0.05%).....0.5 ml/l

Dissolved in 1 l distilled water and adjusted to pH 7.3.

4.2. High salt washing buffer (HSWB)

Trisma base (0.02 M).....2.42 g/l

NaCl (0.5 M).....29.2 g/l

Tween-20 (0.1%).....1 ml/l

Dissolved in 1 l distilled water and adjusted to pH 7.7.

4.3. Coating buffer

Carbonate-bicarbonate buffer

Na_2CO_3 (15 mM).....1.59 g/l

$NaHCO_3$ (35 mM).....2.93 g/l

Dissolved in 1 l distilled water and adjusted to pH 9.6; made freshly.

Substrate

4.4.1. Substrate buffer

Citric acid (0.1 M).....21 g/l

Sodium acetate (0.06 M).....8.2 g/l

Dissolved in 1 l distilled water and adjusted to pH 5.4.

4.4.2. Stock chromogen solution

3'3'5'5'-Tetramethylbenidine dihydrochloride (TMB) (42mM)

in 1:2 acetic acid:distilled water; kept at 4°C.

4.4.3. Working chromogen solution

Substrate buffer.....15 ml

Stock chromogen solution.....150 µl

H_2O_2 (1 %).....5.0 µl

5. Solutions for Plaque assay

5.1. Methylcellulose overlay media

Methyl cellulose.....5.0 g

Distilled water.....150ml

Eagle's Minimum Essential Medium with Earle's salts (Gibco).....850 ml

[Supplemented with 2% (v/v) heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine (Gibco) and 1% (v/v) non-essential amino acids (NEAA) (100x-Sigma), referred to as antibiotic-free MEM-2].

Dissolve methyl cellulose in 150 ml distilled water using hot plate with a magnetic stirrer and autoclave immediately for 20 min and 15 p.s.i. After cooling the solution to 22°C add MEM-2 at the same temperature mixing thoroughly on a stir plate. Dispense the solution to a 100 ml sterile bottle and then cooled rapidly to 4°C with swirling. Allow 20 min for the solution to become clear and stored at 4°C.

5.2. Crystal violet solution

Crystal violate (Sigma-C-3886).....2 g

Ethanol (95%).....40 ml

Ammonium oxalate.....0.8 g

a. Dissolved Crystal violate in ethanol.

b. Dissolved ammonium oxalate in 300 ml distilled water.

Mixed solutions a and b and then filtered with No. 1 filter paper (Whatman, England) before used.

6. Histology

6.1. Fixation

Material for histological examination was placed in fixative solution (10% Neutral buffered formalin) for at least 24 hours prior to casetting.

6.2. Casetting

The allocated reference number was written on the cassette and tissue samples trimmed to a suitable size were placed into the cassettes. Small samples were wrapped in tissue paper before placing in the cassette. Casetted samples were not allowed to dry out and were left in a bowl of fixative until loading onto the processor. Soft and hard tissue were processed separately.

6.3. Tissue processing

The processing section was carried out by placing the casetted tissue into a basket and the basket was moved from stage to stage automatically at pre-set time intervals as follows:

1. 50% Methylated spirits.....30 min
2. 80% Methylated spirits.....90 min
3. 100% Methylated spirits.....90 min
4. 100% Methylated spirits.....90 min
5. 100% Methylated spirits.....90 min
6. 100% Alcohol.....105 min
7. 100% Alcohol.....90 min
8. Chloroform.....50 min
9. Chloroform.....50 min
10. Molten wax.....105 min
11. Molten wax.....90 min
12. Molten wax.....90 min

Casettes were removed from the tissue processor and placed in molten wax until ready to 'block out'. Embedded tissues were trimmed and cut into the sections using microtome.

6.4. Methylene blue solution for Casteñade staining

1. Buffer solutions

Solution A

$Na_2HPO_4.12H2O$.....23.86 g

Distilled water.....1000 ml

Solution B

NaH_2PO_4.....11.34 g

Distilled water.....1000 ml

Working solution-1

Solution A.....88 ml

Solution B.....12 ml

Formalin.....0.2 ml

2. Methylene blue solutions

Solution C

Methylene blue.....2.1 g

95% alcohol.....30 ml

Dissolve methylene blue in the 95% alcohol.

Solution D

Potassium hydroxide.....0.01 g

Distilled water.....100 ml

Dissolve potassium hydroxide in the distilled water.

Working solution-2

Mix Solutions C and D together and let stand for 24 h.

3. Staining solution

Mix Working solution-1 with 1 ml Working solution-2.

6.5. Acridine orange

Acridine orange powder.....20 mg

$CH_3COONa.3H_2O$ (1 M).....100 ml

HCl (1 N).....90 ml

Add Acridine orange powder to $CH_3COONa.3H_2O$ and HCl and adjusted pH to 3.5.

Stored in a brown bottle at 22 °C.

6.7. TBS

TBS used in immunohistochemistry assay was prepared as described in Appendix 3.2.

APPENDIX II

Method

Spearman-Karber Method

The formulae used for end-point infectivity titrations are as follows:

A) Mean log ID_{50} (m)=$x + d\,½ - d\Sigma\,(r/n)$

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

B) Variance of mean (V_m), when the same number of test subjects are used at each dilution level

$V_m = d^2/n^2\,(n-1)\,\Sigma\,[r\,(n-r)]$

C) Variance of mean (V_m), when a different number of test subjects are used at each dilution level

$V_m = d^2\,\Sigma\,[r\,(n-r)\,/\,n^2\,(n-10)]$

D) Standart error of mean (SE_m)

$SE_m = \sqrt{V_m}$

E) 95% confidence limits

95% confidence limits = $m \pm 2(SE_m)$