STUDIES ON MONOCLONAL ANTIBODIES CHARACTERIZATION AND IMMUNOHISTOCHEMICAL DETECTION OF *Lactococcus garvieae*

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To my grandfather and my mother,

The first person to show me how amazing fish are,

And the person that shows me everyday how amazing people can be.

DECLARATION

I hereby declare that this dissertation 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.

Signed: Jorge del Pozo MRCVS

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ABSTRACT

Lactococcus garvieae, the aetiological agent of Lactococcosis, has recently been responsible for significant disease outbreaks in a variety of economically important freshwater and seawater fish species cultured worldwide. The development of immunological diagnostic tests to use in the control strategies against *L. garvieae* has been limited due to a complicated global distribution of serotypes. It appears there are serotypic differences between *L. garvieae* recovered from different regions, and although perhaps a common Antigen (Ag) may be expressed by all serotypes, it has not been found yet. The identification of this Ag would enable the development of specific Monoclonal Antibodies (MAbs), which could be used, in conjunction with immunological techniques, for the detection of all isolates of the pathogen in infected fish and water.

In this study, nine MAbs produced in the Aquatic Vaccine Unit of the Institute of Aquaculture (IoA), University of Stirling against *L. garvieae* type strain NCIMB 702928 (3 MAbs) and against a *L. garvieae* Japanese clinical isolate (12-99)(6 MAbs), as well as two more MAbs, kindly donated by Professor Tae Sung Jung (Laboratory of Fish and Shellfish Diseases, Gyeongsang National University, Republic of Korea) against *L. garvieae* Korean isolates, were screened, using Enzyme-linked immunosorbent assay (ELISA), against a collection of 12 *L. garvieae* isolates from Europe and Asia, and *L. garvieae* type strains. None of the MAbs in the study recognized all the *L. garvieae* isolates tested, although some of the Japanese MAbs (specifically MAbs 3 G9, 8B12, 11F8 and 11B1) recognized a higher number of isolates than the rest of MAbs (including all the type strains in the study and several Japanese and Italian isolates). Differences in the intensity of the reaction has led to the idea that the Ag can be expressed at two different levels by different isolates, or that perhaps there are two different Ags displaying similar epitopes that are recognized at two levels. European MAbs and the rest of the Japanese MAbs were very specific to certain strains, including two of the type strains and one Japanese isolate but they did not recognize any of the Italian isolates.

Korean MAbs did not recognize any isolate, and this led to think about a possible absence of MAb in the supernatant. However, none of the two *L. garvieae* Korean isolates were recognized by any MAb.

The MAbs were also tested for cross-reactivity using ELISA with a collection of 32 isolates from other bacterial species (including *L. garvieae* related and unrelated species). Low levels of cross reactivity (ranging from 0.3% to 9.6%) were detected, with the exception of a Korean MAb (U99-33) that showed a significant cross reactivity with *Renibacterium salmoninarum*.

An Immunohistochemistry (IHC) test was developed with the MAbs studied. A preliminary IHC test was carried out with all the MAbs, and the results obtained correlated with those from the ELISA assay. Based upon the results obtained and availability of supernatants, two MAbs, (Japan) 3G9 and (Euro) 13, were used in IHC to screen tissue samples from a *L. garvieae* challenge with isolate NCIMB 702928 in rainbow trout, carried out during the study. Both MAbs were able to detect specifically *L. garvieae* in infected tissue sections of various organs.

The pathology observed in challenged fish is described. Most prominent features on clinical examination were exophthalmos, generalized congestion and haemorrhage, hepatomegaly, splenomegaly and serositis, which sometimes extended to the myocardium. Histopathological features included several inflammatory and degenerative processes in various organs (eye, swimbladder, spleen, liver, kidney and heart).

In conclusion, the findings of the study suggest that our knowledge on serotypes and antigenic profiles of the bacteria worldwide needs to be expanded, in order to acquire a level of knowledge that will allow the development of MAbs that recognize all *L.garvieae* isolates worldwide. If these are developed, it will be possible to use them in IHC to detect the bacteria in infected fish tissue, and will help to differentiate lactococcosis from other streptococcal diseases.

ABBREVIATIONS

µl	: Microlitre
µm	: Micrometre
16S rRNA	: 16 small subunit ribosomal Ribonucleic acid
ATCC	: American Type Collection
Ag	: Antigen
Ab	: Antibody
BA	: Blood Agar
BHIA	: Brain Heart Infusion Agar
BHIB	: Brain Heart Infusion Broth
CFU	: Colony forming Unit
cm	: Centimetre
DNA	: Deoxyribonucleic acid
DO	: Dissolved oxygen
E. seriolicida	: *Enterococcus seriolicida*
ECPs	: Extracellular products
ELISA	: Enzyme-Linked Immunosorbent Assay
G+C	: Guanine + Cytosine
g	: grams
h	: hour
H&E	: Haematoxylin and Eosin
HMDS	: Hexamethyldixilazane
HRP	: Horseradish Peroxidase
HSW	: High Salt Wash Buffer
IHC	: Immunohistochemistry
IoA	: Institute of Aquaculture, Stirling University
IgG	: Immunoglobulin G
Kg	: Kilogram
L	: litre
L. lactis	: *Lactococcus lactis*
L. garvieae	: *Lactococcus garvieae*
L. plantarum	: *Lactococcus plantarum*
Lab	: Laboratory
LSW	: Low Salt Wash Buffer
M. rosenbergii	: *Macrobrachium rosenbergii*
m	:Metre
MAb	: Monoclonal Antibody

mg	: Milligram
MIC	: Minimum Inhibitory Concentration
ml	: Millilitre
NCIMB	: National Collection of Industrial and Marine Bacteria
NCTC	: National Collection of Type Cultures
O. mykiss	: *Oncorhynchus mykiss*
OD	: Optical density
P. fluorescens	: *Pseudomonas fluorescens*
PAbs	: Polyclonal Antibodies
PBS	: Phosphate Buffered Saline
PCR	: Polymerase chain reaction
PFGE	: Pulsed-Field Gel Electrophoresis
PMEB	: Prawn Muscle Extract Broth
ppm	: Parts per million
RA	: Rogosa acetate agar
RAPDs	: Randomly Amplified Polymorphic DNA analysis
RBC	: Red blood cell
R. salmoninarum	: *Renibacterium salmoninarum*
RFLP	: Restriction Fragment Length Polymorphism Ribotyping
RPS	: Relative Percentages of Survival
RT-PCR	: Reverse transcriptase Polymerase Chain Reaction
S. quinqueradiata	: *Seriola quinqueradiata*
S. maximus	: *Scophthalmus maximus*
sec	: Second
SEM	: Scanning electron microscope
SKDM	: Specific Kidney Disease Medium
S. iniae	: *Streptococcus iniae*
SDS-PAGE	: Sodium dodecyl sulfate-polyacrylamide polyacrylamide gel electrophoresis
SLO	: Streptolysin O
TSA	: Tryptone Soya Agar
TMB	: 3,3',5,5'-Tetramethylbenzidine dihydrochloride
TBS	: Tryptone soya broth
TBS	: Tris Buffered Saline
UK	: United Kingdom
V. salmoninarum	: *Vagococcus salmoninarum*
(v/v)	: Volume/volume
(w/v)	: Weight/volume

TABLE OF CONTENTS

Pages

Declaration……………………………………………………………………………..I
Acknowledgements………………………………………………………………………II
Abstract…………………………………………………………………………...III
Abbreviations……………………………………………………………………..V
Table of contents……………………………………………………………………VII
List of tables…………………………………………………………………………XI
List of Figures……………………………………………………………………… XII

Chapter 1. Literature Review……………………………………………..1

1.1. THE PATHOGEN………………………………………………………………..1

1.1.1. Taxonomy……………………………………………………………………1
1.1.2. Morphology and biochemical profile………………………………………2
1.1.3. Culture requirements………………………………………………………..3
1.1.4. Biotypes……………………………………………………………………4
1.1.5. Serotypes……………………………………………………………………..5
1.1.6. Other classifications…………………………………………………………..7
1.1.7. Capacity of survival outside the host………………………………………..8
1.1.8. Virulence factors……………………………………………………………..9

1.2. THE HOSTS…………………………………………………………………...12

1.3 THE INFECTION……………………………………………………………...13

1.3.1. Incidence……………………………………………………………………..13
1.3.2. Epidemiology…………………………………………………………………14
1.3.3. Predisposing factors…………………………………………………………...15
1.3.4. Pathology……………………………………………………………………...16

1.4. DIAGNOSIS……………………………………………………………………20

1.4.1. Clinical and bacteriological methods………………………………………20
1.4.2. Immunological methods……………………………………………………..20

1.4.3. Polymerase Chain Reaction (PCR) methods……23
1.4.4. Other methods……24

1.5 CONTROL……25

1.5.1. Physical methods……25
1.5.2. Chemotherapeutic methods……25
1.5.3. Vaccination……26
1.5.4. Other methods……29

1.6 AIMS OF THE PROJECT……30

Chapter 2. Materials and Methods……31

2.1. MONOCLONAL ANTIBODIES……31

2.1.1. Antibodies used……31

2.2. BACTERIOLOGY……32

2.2.1. Bacterial strains……32
2.2.2. Bacteriology……32

2.3. ENZYME-LINKED IMMUNOSORBENT ASSAY……35

2.3.1. Bacterial preparation……35
2.3.2. Indirect Antibody Capture ELISA protocol……36
2.3.3. Characterization of the MAbs to L. garvieae……37
2.3.4. Cross-reactivity of MAbs……37

2.4. IMMUNOHISTOCHEMISTRY……38

2.4.1. Antibodies……38
2.4.2. Positive and Negative controls……38
2.4.3. Immunohistochemistry protocol……38

2.5. BACTERIAL CHALLENGE……40

2.5.1. Preparation of the bacteria……40
2.5.2. Quantification of bacteria injected to fish……40

2.5.3. Challenge procedure..41
2.5.4. Sampling protocol..42

2.6. HISTOLOGY..43

2.6.1. Preparation of histological sections..43
2.6.2. Haematoxilin and Eosin (H & E) staining...43
2.6.3. Gram staining..44

2.7. SCANNING ELECTRON MICROSCOPY..45

Chapter 3. Results...46

3.1. CHARACTERIZATION OF MAbs TO *Lactococcus garvieae*........................46

3.1.1. Specificity to L. garvieae strains...46
3.1.2. Cross reactivity with other bacterial species..48

3.2. IMMUNOHISTOCHEMISTRY..51

3.2.1. Preliminary tests...51
3.2.2. Immunohistochemistry on challenge samples..54

3.3. CHALLENGE RESULTS...56

3.3.1. Clinical examination...56
3.3.2. Histopathological description...59
3.3.3. Bacteriology...63
3.3.4. Scanning electron microscopy..63

Chapter 4. Discussion...64

4.1. MONOCLONAL ANTIBODIES..64

4.1.1. Characterization studies..64
4.1.2. Further studies relating to ELISA...67

4.2. IMMUNOHISTOCHEMISTRY..70

4.3. CHALLENGE...71

Chapter 5. Conclusions……..……………………………………………….72

REFERENCES…………………………………………………………...74

APPENDIX………………………………………………………………85

-Histology fixative……………………………………………………………85

-Stains and staining buffers…………………………………………………..85

-Immunohistochemistry buffer……………………………………………….88

-ELISA buffers………………………………………………………………88

-Histology: processing schedule……………………………………………...89

-Bacterial culture media preparation…………………………………………90

-CHEMICALS USED………………………………………………………..91

LIST OF TABLES

Pages

Table 2 1. Monoclonal antibodies raised against different isolates of *L. garvieae* and tested for cross-reactivity with related and unrelated bacterial species………………………………… 31

Table 2 2. *Lactococcus garvieae* isolates used in a characterization study to several Monoclonal Antibodies produced against *L. garvieae*…………………………………………….... 32

Table 2 3. Bacterial species used to screen the cross-reactivity of several Monoclonal Antibodies raised against *L. garvieae*…………… 33

Table 3 1. Response of anti-*L.garvieae* Monoclonal Antibodies against several strains of *L. garvieae* in an indirect ELISA for particulate antigens…………………………………………… 47

Table 3 2. Cross reactivity of anti-*L. garvieae* Monoclonal Antibodies with several bacterial species, determined by indirect ELISA for particulate Antigen………………………………………. 49

Table 3.3. Findings of clinical examination on *L. garvieae* NCIMB 702928 challenged rainbow trout…………………………………… 56

LIST OF FIGURES

Pages

Figure 1.1. Transmission electron microscopy (TEM) of *Lactococcus garvieae* strains (Ooyama *et al.*, 2002)……………………….. 10

Figure 1.2. *L. garvieae* infecting *M. rosenbergii*. (Chen *et al.*., 2001)…….. 17

Figure 1.3. Histopathological lesions observed in the Giant Freshwater prawn (*M. rosenbergii*) infected with *L. garvieae* (H&E, x200). (Chen *et al.*, 2001)……………………………………….……… 19

Figure 2.1. 100 litre tanks and thermostat used to keep the rainbow trout during the challenge with *L. garvieae* NCIMB 702928………… 41

Figure 3.1. Images of the spleen of a positive control section (D040013A), known to be infected with *L. garvieae* in IHC with the different MAbs of the study (x100, all images)…………………………… 52

Figure 3.2. Non-specific blue coloration due to endogenous peroxidase activity in several tissues of negative control rainbow trout, in IHC with MAb (EURO) 13; Gills (x100); B. Heart (x100); C-C' Tunica externa of the swim bladder (x100)……………….. 53

Figure 3.3. IHC positive reaction to *L. garvieae* NCIMB 702928 in spleen of rainbow trout.(IHC, x 400)……………………………………. 54

Figure 3.4. IHC with Euro 13, on tissue sections collected from *L. garvieae* NCIMB 702928 challenged rainbow trout. Eye (x200), Gills (x200), Kidney (x400), Liver (x400), Spleen (x100), Swim bladder(x400)……………………………………………… 54

Figure 3.5. Clinical signs of the eye in trout infected with *L. garvieae* NCIMB 702928: Unilateral Exophthalmos with severe clouding of the cornea, Intraocular hemorrhage……………………………. 57

Figure 3.6. Gross pathological appearance of a rainbow trout infected with *L. garvieae* NCIMB 702928……………………………………... 59

Figure 3.7. Gram stained blood smears of two Rainbow trout experimentally infected with *L. garvieae* NCIMB 702928………………………. 60

Figure 3.8. Diferent inflammatory processes in serosal surfaces in *L. garvieae* NCIMB 702928 infected trout: Liver capsulitis, pericarditis, spleen capsulitis, inflammation of the swim bladder, (All images, x200)………………………………………………… 61

Figure 3.9. Some of the histopathological signs observed in rainbow trout infected experimentally with *L. garvieae* NCIMB 702928: Islets of Langerhans of the pancreas (Gram, x 400), peritonitis (H&E, x 400), spleen (H&E, x400), Liver (H&E, x 400)………… 61

Figure 3.10. Microscopic images of Gram stained *L. garvieae* NCIMB 702928 (Gram, x400) and image of Petri dish with bacterial isolated from kidney of trout infected with *L. garvieae* NCIMB 702928……….. 63

Figure 3.11. Scanning electron microscopy images of two *L. garvieae* strains: Occtve and NCIMB 702928……………………………………….. 63

Chapter 1: LITERATURE REVIEW

1.1. THE PATHOGEN

1.1.1. Taxonomy

Lactococcus garvieae is a Gram-positive cocci bacterium, 0.5 to 0.7 µm long, short-chain-forming, facultatively anaerobic which is pathogenic to fish. It was first named *Enterococcus seriolicida* by Kusuda *et al.* (1991) who recovered twelve isolates of the bacterium from eels (*Anguilla japonica,* Temminck & Schlegel) and Yellowtail (*Seriola quinqueradiata*, Temminck & Schlegel). These isolates had originally been classified as *Streptococcus sp*. (Kusuda *et al.* 1978, Kusuda *et al.* 1982) and had been collected from diseased fish in Japan during the preceding twenty years.

Despite the adoption of the name *E. seriolicida* for these isolates, low DNA homology values where obtained with reference species of *Enterococcus*, with the greatest DNA homology to *E hirae* (i.e. 24% homology) (Kusuda *et al.* 1991).

Since this time the association of the organism with the genus *Enterococcus* has been challenged, and the organism has been finally classified as *Lactococcus garvieae* (Teixeira *et al.* 1996, Eldar *et al.* 1996).

This classification has been reinforced by results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole cell proteins (Pot *et al.* 1996), phenotypic characterization and DNA:DNA hybridization studies on type strains (Eldar *et al.* 1996). Hawkesford *et al.* (1997) employed Randomly Amplified Polymorphic DNA analysis (RAPDs) to compare Tasmanian isolates of *Streptococcus sp*. biovar I and demonstrated that they were, in fact, *L. garvieae*.

1.1.2. Morphology and biochemical profile

The characteristics of the bacteria in culture have been described as: non-motile; facultatively anaerobic; Gram positive cocci in short chains, which do not produce catalase, H_2S, indole or oxidase. It has α-haemolytic properties. Aesculin and arginine are hydrolysed, but not so casein, gelatin or sodium hippurate. Acid is produced from a wide range of carbohydrates, namely aesculin, cellobiose, D-fructose, galactose, D-glucose, maltose, mannitol, D-mannose, salicin, sorbitol and trehalose, but not from adonitol, D-arabinose, glycerol, glycogen, inositol, lactose, melezitose, melibiose, raffinose, L-rhamnose, starch, sucrose or D-xylose. The methyl red and tetrazolium reduction tests and the Vogues-Proskauer reaction are positive, but not nitrate reduction. Growth occurs between 10 and 45^0C but not at 50^0C (which includes the ability to grow at 37^0C, a necessary feature for a potential zoonotic agent), in 0.0 to 6.5% (w/v) sodium chloride, and at pH 4.5-9.6. The G+C ratio of the DNA is 44 % (Austin & Austin, 1999).

The organisms do not belong to Lancefield groups, A, B, C, D, E, F, G, H, K, L, M, N or O. (Austin and Austin, 1999). Eldar *et al.* (1999) reported a positive result for the Lancefield group N reaction. On the other hand, Teixeira *et al.* (1996) had reported it to be negative. Lancefield groups are a serological classification of haemolytic streptococci based on their specific carbohydrate Antigen (Ag) (Lancefield, 1933).

It is necessary to highlight the fact that *L. garvieae* may be biochemically indistinguishable from *Streptococcus parauberis* (Domenech *et al.* 1996) and in terms of phenotypic data, *L. garvieae* appears to be similar to *Lactococcus lactis*. These facts can lead to misidentification of fresh isolates (Zlotkin *et al.* 1998).

In an attempt to avoid this from happening, antibiotic sensitivity methodologies have been proposed as identification methods. *L. garvieae* is resistant to clindamycin, whereas *L. lactis* is sensitive (Elliot and Facklam, 1996). This method of differentiation between the two species has been recently challenged since the isolation of a clindamycin-sensitive isolate of *L. garvieae* from a diseased individual of wild Sea Wrasse (*Coris aygula*) by Colorni *et al.* (2003).

Phenotypic characterization of *Lactococcus* and *Enterococcus* was considered unreliable by Deasy *et al.* (2000), due to the existence of strains which do not conform to the traditional criteria for differentiating between these two genera. These workers proposed the use of polymerase chain reaction (PCR) to differentiate between them (discussed in more detail in section 1.4.3).

1.1.3. Culture requirements

With regards to isolation methods appropriate for this species, the use of Tryptone Soya Agar (TSA) or Brain Heart Infusion Agar (BHIA) incubated at 22-24^{0}C for 48 hours has been advocated by various workers for *Lactococcus* bacteria (Austin and Austin, 1999). A selective media for *Lactococcus* has been described, rogosa acetate agar (RA), which can be useful to differentiate *Lactobacilli* from other Gram positive bacteria such as *Carnobacterium, Arcanobacterium* and *Vagococcus* species (Buller, 2004). Buller (2004) also proposes Blood Agar (BA) as an appropriate medium for the culture of *Lactococcus* species.

1.1.4. Biotypes

Work by Vela *et al.* (1999) and Eldar *et al.* (1999) examines the phenotypic heterogeneity of *L. garvieae*. Both authors propose to divide the species into three different biotypes.

The biochemical characteristics proposed by Eldar *et al.* as means of differentiation of each serotype were the acidification of D-tagatose and sucrose. Twenty one strains, from different locations in Europe, Asia and Australia, were screened in the study.

There are some inconsistencies between these two studies, and further research has suggested that the strains are homogeneous regardless of the geographical location or host. However, to show homology it is necessary to standardize the density of the inoculum to OD_{580}=0.8, and all the inoculums to be tested come from BA plates (Ravelo *et al.* 2001). Slight variations for some parameters can occur, especially for β-galactosidase, hippurate, β-mannosidase, N-acetil-β-glucosaminidase, acid from melezitose, and acid from pullulan.

Further work by Vela *et al.* (2000) proposed a new classification scheme of *L. garvieae* containing 13 biotypes, on the basis of acidification of sucrose, tagatose, mannitol and cyclodextrin and the presence of enzymes pyroglutamic acid arylamidase and N-acetyl-β-glucosaminidase, although only six of the biotypes have been isolated from fish. More recently, the number of biotypes isolated from fish has increased to seven (namely biotypes 1, 2, 3, 4, 5, 6, 10) (Buller 2004).

1.1.5. Serotypes

Lactococcus garvieae strains isolated from diseased Yellowtail are believed to be divided into two serotypes (although these serotypes show identical biochemical profiles) (Kitao 1982).

These serotypes have been classified as KG+, which is agglutinated by rabbit serum raised against *L. garvieae* KG+ phenotype cells, and KG- which is not. Both these serotypes agglutinate with anti-KG- rabbit serum. Most isolates from diseased yellowtail are KG- (Kitao, 1982).

The *L. garvieae* KG- phenotype is more pathogenic for Yellowtail than the KG+ (Alim *et al.* 1996). Yoshida *et al.* (1996, 1997) reported that KG- cells are more hydrophilic than KG+ cells, more resistant to phagocytosis by yellowtail head kidney phagocytes, and have capsules. Immunoelectron microscopy has been used to localize the different capsular Ags of the pathogen (Okada *et al.* 2000), and further studies have been done in relation to the different capsule properties of *L. garvieae*. Some isolates have highly developed capsules and are highly immunogenic, others show micro-capsules with fimbriae like structures on the surface and others have no capsule. The latter are less immunogenic than the former (Ooyama *et al.* 2002; Hirokawa *et al.* 2004).

Barnes *et al.* (2002a), studying *L. garvieae* infection in rainbow trout, correlated the resistance to serum killing of the bacterium with capsules together with an increase in their pathogenicity. Hirono *et al.* (1999) worked on the identification of genes in *L. garvieae* KG- cells, cloning five different genes and describing the presence of a dihydropteroate synthase gene.

With regards to serotyping of isolates in Europe, antigenic characterization of *E. seriolicida* strains pathogenic for Turbot (*Scophthalmus maximus* (L.)) in Northwest Spain revealed serological homogeneity of the isolates, and thus the possibility of developing a vaccine for turbot farmed in this region of Spain (Toranzo *et al.* 1995a, b).

On the other hand, Romalde and Toranzo observed the presence of serological variability among strains isolated from rainbow trout in Spain (Cunningham, 2002).

More recently Eyngor *et al.* (2004), suggested the presence of two different serotypes in Europe, namely serotype I (including Italian and Israeli isolates), and serotype II (including Spanish, Greek and Bulgarian isolates). French isolates presented heterogeneous results, which the authors attribute to the absence of a process of evolution (evolution that would have led to a clonal distribution of strains in the case of all other isolates but not the French, which origin is still too recent to have gone through this evolutionary process).

The work relating to serotyping for *L. garvieae* indicates that further work is required to classify bacteria obtained from different geographical regions worldwide into different serotypes localized worldwide. This information is necessary to develop effective vaccines as well as Antibody (Ab) based diagnostic tests.

1.1.6. Other classifications

Restriction Fragment Length Polymorphism Ribotyping (RFLP) has been used to analyze a range of *L. garvieae* isolates from Europe, Asia and Australia. *Eco*RI and *Hind*III revealed two and seven ribotypes respectively. In both cases a close relationship has been detected between the Japanese and the Italian isolates. These results suggested that the appearance of this pathogen in Italy was linked to fish imported from Japan. (Eldar *et al.*1999).

To further complicate this issue, Vela *et al.* (1999, 2000) described 19 different pulsotypes using Pulsed-Field Gel Electrophoresis (PFGE), as well as the 13 biotypes for the aforementioned isolates. They reached this conclusion after examining 84 *L. garvieae* isolates from different species, although most of them came from Europe (some came from U.S.A., Brazil, and one isolate came from Japan). The results obtained suggested the existence of three genetically unrelated clones, one comprising Spanish and Portuguese isolates, another Italian isolates, and the third one comprising French isolates.

These results were not confirmed by RAPDs, which indicated that 9 of 11 French isolates clustered in the same genogroup as the Italian rainbow trout isolates (Ravelo *et al.* 2003). Previous work by Ravelo *et al.* (2000) had used RAPDs to examine European and Japanese strains of *L. garvieae* isolated from rainbow trout, catfish, and yellowtail. They were able to demonstrate at least three different genogroups, each of which was closely related to the host of origin.

Eyngor *et al.* (2004) compared RFLP data with serological data, from which they revealed that in endemic sites the bacterial population displays clonal structures; whereas bacterial diversity exists at sites where the infection is sporadic, such as France.

Bacteriophages of *L. garvieae* have been found in bacteria obtained from seawater and sediment, with 14 phage types defined from 111 isolates, but when studied, there was no correlation between phage type and geographical source of the isolates (Park *et al.* 1997, 1998).

1.1.7. Capacity of survival outside the host

There is evidence that these bacteria are abundant throughout the year in the aquatic environment, occurring in water, mud and in the vicinity of farmed yellowtail pens, as acknowledged by Kitao *et al.* (1979), and Kusuda and Kawai (1982). The first group of workers also observed a seasonality pattern in the numbers of bacteria. They were present in higher quantities in the seawater during the summer, but during autumn and winter the highest numbers of bacteria were found in the mud. This is a very interesting conclusion, but unfortunately, there is no further research to back up this phenomenon or why it occurs. Also, the fact that the taxonomic status of streptococci was still inconclusive at the time of the study made it impossible to carry out an accurate taxonomic description of the bacteria found. Further research in this field would perhaps clarify this theory.

The presence of streptococci in trash fish used for yellowtail diets (both fresh and frozen) has been reported (Minami, 1979). Minami discovered that these isolates were pathogenic and could survive for six months in a frozen state. The importance of food-borne infection was further highlighted by Taniguchi (1982a, b).

More recently, Manfrin *et al.* (2003) have isolated *L. garvieae* from rainbow trout in the pre-slaughtering phase in Italy. This group of workers also reported the potential risk to public health in this work.

1.1.8. Virulence factors

Studies on the extracellular products (ECPs) of *E. seriolicida* show they are correlated to the haemolytic activity of the bacterium and seem to induce specific clinical signs of the disease (Kusuda & Kimura, 1978; Kimura and Kusuda 1979, 1982). Furthermore, the haemolytic activity reported in the ECPs is similar to that of streptolysin O (SLO), because it is activated by cysteine. On the other hand, the intracellular products are correlated to the leucocidal activity, mainly affecting phagocytic cells, and seem to have cytotoxic properties. The lethal toxic factor(s) is probably of proteinaceous nature, because it is inactivated by protease treatment (Kusuda & Hamaguchi, 1988, 1989).

Studies by Alim *et al.* (1996) showed that the pathogenicity of *E. seriolicida* appears to be related to the agglutination pattern. They demonstrated, using a slide agglutination test, that the non-agglutinating strains of the bacteria (KG-) showed higher pathogenicity than agglutinating ones (KG+).

The capsule displayed by KG- cells (as opposed to KG+ cells) may play an antiphagocytic role in *E. seriolicida* infection in Yellowtail (Yoshida *et al.* 1996, 1997). This hypothesis was confirmed also in Rainbow trout (Barnes *et al.* 2002 a, b).

Surface appendages similar to fimbriae have also been described. In other bacteria, this type of structure is associated with the attachment and invasion of the host cells. However, strains in which fimbriae where identified appear to have a thinner capsule. These bacteria were more virulent than the bacteria with no capsule, but less virulent than the bacteria with a fully developed capsule (Ooyama *et al.* 2002; Hirokawa *et al.* 2004), as shown in Figure 1.1.

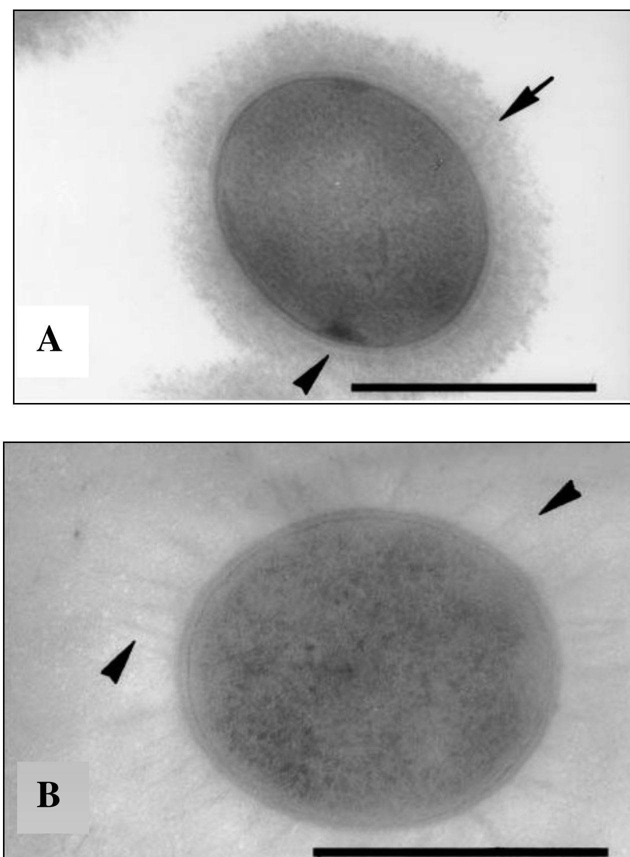

Figure 1.1. Transmission electron microscopy (TEM) of two *Lactococcus garvieae* strains:

A. KG9408 (KG- cells) showing a well-developed capsule (arrow) with faint inside layer (arrowhead). B. Fimbriae-like structures projecting from cell surface of MS93003 cells. (Photographs taken from Ooyama *et al.* 2002).

Siderophore production has been reported in *L. garvieae* under certain growth conditions, though the nature of these siderophores has not been determined.
Expression of the siderophore appears to be dependent on the nutrimental level rather than iron content, in that siderophores were detected in supernatants from iron-depleted medium, and hemo-enriched medium, but not from nutrient rich medium (Schmidtke *et al.* 2003). These authors also reported expression of novel proteins during growth *in vivo*, but the precise protein profiles could not be replicated in culture. The function of these proteins expressed *in vivo* remains to be determined.

The virulence of *L. garvieae* from freshwater prawn (*Macrobrachium rosenbergii*) appears to depend on the culture media as well as the number of passages on the agar: bacteria cultured in Brain Heart Infusion Broth (BHIB) are three and a half times more virulent than bacteria cultured in Prawn Muscle Extract Broth (PMEB). Additionally, bacteria cultured in BHIB have increased Lethal Dose sub (50) with every generation, whereas the opposite occurred when the medium used was PMEB (Sung & Sun, 2002).

1.2. THE HOSTS

The original description of the bacteria was made in 1984, when the organism was isolated from the mastitic udder of a bovine in the United Kingdom (Collins *et al.* 1984). Since then it has been isolated from diseased individuals of several fish species, both from freshwater and salt water, such as farmed Yellowtail (*S. quinqueradiata*) (Kusuda *et al.* 1991), cultured eels (*A. japonica*) (Kusuda *et al.* 1978), cultured Rainbow Trout (*Oncorhynchus mykiss*, Walbaum) (Ceschia *et al.* 1992; Eldar *et al.* 1996), cultured Turbot (*S. maximus*) (Nieto *et al.* 1995), cultured Adriatic Sturgeon (*Acipenser naccarii*) (Salati *et al.* 1996), farmed Grey Mullet (*Mugil cephalus* L.), (Chen *et al.* 2002), and several wild species, as well as sea water and mud (Colorni *et al.* 2003, Kusuda and Kawai, 1982). It is also a known pathogen of the freshwater prawn (*M. rosenbergii*) as described by Cheng and Chen (1998).

It has also been isolated from diseased humans and should therefore be considered a zoonotic agent (James *et al.* 2000). In the human host it has caused prosthetic valve arterial endocarditis (Elliot *et al.* 1991).

1.3. THE INFECTION

1.3.1. Incidence

The disease caused by *L. garvieae* is considered one of the most important diseases of cultured yellowtail in Japan (Kusuda *et al.* 1991), as it affects fish of a marketable size (Kusuda, 1992). It also affects the rainbow trout industry mainly in Europe and Australia, as well as having been reported from other locations (Chang *et al.* 2002; Diler *et al.* 2002; Eldar *et al.* 1996, 1999; Kusuda *et al.* 1991; Ravelo *et al.* 2003). Pereira *et al.* (2004) reported the presence of the pathogen in Portugal, with mortalities of up to 90% in the worst outbreaks, affecting rainbow trout of all sizes.

During the period from 1999 to 2000, epizootics with up to 10% of mortalities were recorded on some Taiwanese grey mullet farms with an average loss of approximately 5% on most farms with outbreaks of the disease (Chen *et al.* 2002).

An *Enterococcus*-like bacterium was first reported in farmed turbot (*S. maximus* L) in the Northwest of Spain on 1993 (Toranzo *et al.* 1994). Since that date, the disease has caused heavy economical losses to the turbot farming industry in Northwest Spain (Romalde *et al.* 1996a).

L. garvieae is also one of the disease factors involved in production decline of the freshwater prawn *M. rosenbergii* during the hot season (June to September) in Taiwan (Chen *et al.* 2001).

1.3.2. Epidemiology

In 1996, Robinson and Meyer carried out work on the transmission mechanisms of streptococcal diseases in fish using Golden shiners (*Notemigonus crysoleucas*) as the experimental model. They concluded that these diseases could be transmitted from diseased to healthy fish when placed in the same tank. In this study, healthy fish were exposed to 10^6 bacteria for 10 min.

Cooke and Lofton (1975) also reproduced the disease in healthy fish by intraperitoneal injection of 10^4 to 10^5 bacteria. Further experimental challenges carried out in Turbot showed that the *Enterococcus* isolates used in the experiment were only able to overcome the defense mechanisms of the surface of the turbot only if the skin was abraded prior to exposure (Romalde *et al.* 1996a).

As explained in Section 1.1.7., *L. garvieae* is able to survive in the environment, and has been isolated from mud samples. Bacteria were isolated from water from tanks containing fish challenged with *L. garvieae* (Muzquiz *et al.* 1999).

Taniguchi (1982a, 1982b) highlighted the importance of food-borne infection from contaminated fish feeds and his position was reinforced by Romalde *et al.* (1996a) who reported that the pathogen is able to overcome the adverse conditions in the stomach of fish when is associated with food or fecal material. It is also able to produce mortalities after 16 to 20 days post-ingestion.

All this evidence supports the idea that horizontal transmission occurs through water as well as the fecal-oral route and these may be the main avenues of infection of *L. garvieae*.

1.3.3. Predisposing factors

Several factors that favor both the infectivity and virulence of *L. garvieae* have been described. For instance, the stocking density of fish in net-pens has been found to be related to the level of mortality caused by enterococcal infection. Mizuno reported that net-pens with less than 1.6 kg m^{-3} had no mortality (cited by Kusuda, 1990). Poor water quality, low dissolved oxygen (DO) and water temperature above 17^0C have been linked to the occurrence of streptococcosis outbreaks in rainbow trout in Spain (Palacios, 1993). Other authors suggest temperatures above 15^0C are important (Ceschia *et al.* 1998; Eldar & Ghittino, 1999). Different ages of Rainbow trout show different susceptibilities to the disease. Fifty gram fish are more susceptible and suffer a longer acute period of disease than 100 g fish which are able to eliminate the bacterium from the organs (Muzquiz *et al.* 1999). A more recent study by Royo *et al.* (2001) has evaluated the relation between water temperature and the age of rainbow trout in the epidemiology of the disease. Low DO levels in the water appear to reduce the length of the incubation period of the disease, as well as increase the levels of cumulative mortalities which occur in cultured yellowtail infected with *L. garvieae* (Fukuda *et al.* 1997a). These authors also studied the effect of DO values on horizontal transmission of the disease in Yellowtail, concluding that hypoxic conditions favor the transmission of the disease (Fukuda *et al.* 1997b). The presence of blood flukes in cultured yellowtail has also been found to correlate with an increase the mortality levels associated *L. garvieae* infections (Kumon *et al.* 2002).

Different stressors have also been evaluated which increase the susceptibility to *L. garvieae* infection of the freshwater prawn (*M. rosenbergii*), namely the presence of copper sulfate, nitrite, benzalkonium chloride and potassium permanganate as well as the moult stage of the prawn. The moult stages A, D1/D2 and D3 increase susceptibility to the pathogen (Cheng & Wang 2001; Cheng *et al.* 2002, 2003 a, b, c).

Together these studies confirm that husbandry and management practices are important factors in the infection of fish with *L. garvieae*.

1.3.4. Pathology

The disease has been known by several names: streptococcal infection or streptococcosis; enterococcal infection or enterococcicosis; and more recently *Lactococcus* infection or lactococcosis. It is an infectious systemic disease characterized by haemorrhagic septicaemia (Eldar and Ghittino, 1999).

The typical gross pathology observed in most species is anorexia, loss of orientation, lethargy, reduced appetite (which appears early in the disease process) and erratic swimming. Uni- or bilateral exophthalmia is frequent, with intraocular hemorrhaging and clouding of the eye. In many cases, abdominal distension, darkening of the skin and hemorrhage around the opercula and anus are observed. (Chen *et al.* 2002; Eldar & Ghittino, 1999; Kusuda *et al.* 1991). Congestion of the pectoral and caudal fin vessels was observed in Rainbow trout infected with *L. garvieae* in Turkey (Diler *et al.* 2002).

Other gross signs described in turbot (*S. maximus* L) include ocular loss in severe cases of exopthalmos (Nieto *et al.* 1995). In this species, accumulation of purulent fluid is frequently observed in the periorbital tissues, as well as at the base of the dorsal and anal fins.

Most moribund infected freshwater prawn (*M. rosenbergii*) display anorexia, poor growth and a whitish body color. Gross patholological changes include whitish necrotic muscle (showing yellowish-white spots of a size from 0.5 to 2 cm) and a swollen, yellowish hepatopancreas (Chen *et al.* 2001). See Figure 1.2.

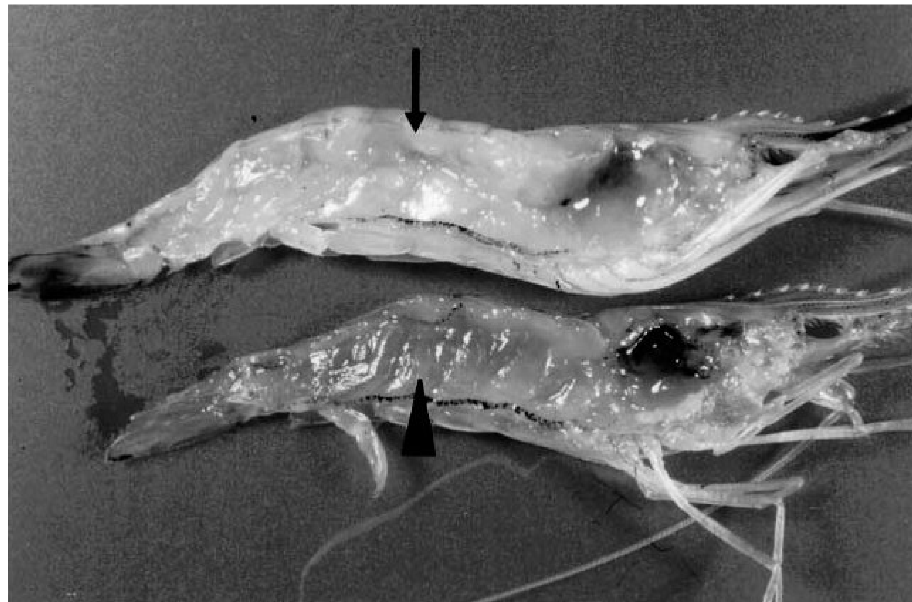

Figure 1.2. *L. garvieae* infecting *M. rosenbergii*. Giant freshwater prawn showing marked opaque and white muscle lesions on the top (arrow), compared to a normal prawn on the bottom (arrowhead). (Chen *et al.* 2001)

Internally, the clinical picture of the disease in most fish species is that of a hyper acute systemic disease. The abdominal cavity may contain varying amounts of exudates which may be purulent or contain blood. There can be hemorrhaging, congestion of the internal organs and enteritis (Eldar & Ghittino, 1999).

Eldar & Ghittino (1999) also described the pathology of the organs affected in Rainbow trout. The brain can present acute meningitis, with an exudate covering the brain surface that often contains large numbers of bacterial cells, with intracranial oedema also being frequently observed (Muzquiz *et al.* 1999). The eyes can present severe panophthalmitis, uni- or bilateral, with destruction of both the anterior and posterior chambers of the eye. The optic nerve papilla is usually affected (Eldar & Ghittino, 1999). The spleen may be enlarged and necrotic, as described by Romalde & Toranzo (Cunningham, 2002). The intestine usually contains fluid and focal areas of hemorrhaging, and histologically extensive superficial erosion with pseudo membrane-like formation can be observed (Eldar & Ghittino, 1999). The liver has diffuse blood filled spaces consistent with *peliosis hepatis*, as described by the same authors, who also observed a marked reactive hyperplasia of the haematopoietic tissue in the kidney and serositis, which sometimes extend into the myocardium, also generating a severe peritonitis where fat necrosis can be observed.

The signs described above for the intestine, liver, kidney as well as the serositis have been proposed by Eldar & Ghittino (1999) as features that can be used to differentiate between *Streptococcus iniae* infections, that do not present these signs, and *L. garvieae* infections, that do present them. The severe serositis observed during lactococcosis has been termed “oculo-splanchnic dissociation” by these authors.

More recently, Chang *et al.* (2002) described fibroplasia of the serosa in Rainbow trout infected with *L. garvieae*, reinforcing the presence of serosal inflammation as a characteristic sign of this disease.

Diffuse necrotic white spots on enlarged kidney and spleen, as well as inflammatory pericardial infiltrate and chronic meningitis have been described in *L. garvieae* infection in grey mullet (Chen *et al.* 2002).

Peritoneal, sub peritoneal and muscle hemorrhages have been observed in Turbot (*S. maximus*) by Nieto *et al.* (1995); they also observed acute bronchitis and dermal abscesses. The absence of lesions in the renal tubules also was noted, a finding that agrees with the position adopted by Eldar & Ghittino (1999).

The histopathology of the infection in freshwater prawn (*M. rosenbergii*) shows marked oedematous fluid accumulation between the cuticle and underlying muscle, with liquefaction necrosis of the later, as well as the hepatopancreas. Necrotic foci are also detected in the heart, stomach and other organs. Bacterial clumps can be detected at all these locations, as well as in the gill haemolymph (Chen *et al.* 2001). See Figure 1.3.

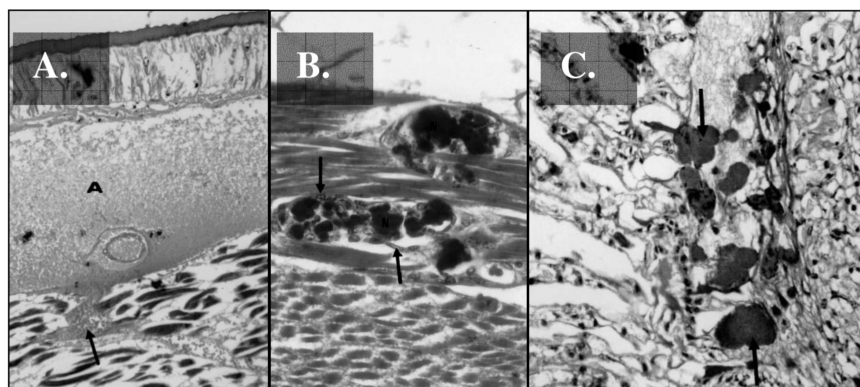

Figure 1.3. Histopathological lesions observed in the Giant Freshwater prawn (*M. rosenbergii*) infected with *L. garvieae*: A. edematous fluid accumulated between the cuticle and underlying muscle and muscle bundles (arrow). (H&E, x200). B. Necrotizing foci (N) surrounded by hemocytes (arrows) in muscle tissue. (H&E, x400). C. bacterial microcolonies (arrows) in the gill. (H&E, x200). (Photograph taken from Chen *et al.* 2001)

1.4. DIAGNOSIS

1.4.1. Clinical and bacteriological methods

Although clinical and bacteriological (including phenotypical) differences between diseases of fish caused by Gram positive cocci have been studied (Domenech *et al.* 1996, Zlotkin *et al.* 1998, Eldar & Ghittino, 1999), great confusion over the identity of these pathogens exists in recorded cases of the outbreaks. The application of sensitive and highly selective molecular and immunological techniques is now being used to complement diagnosis based on clinical examination (Leung, 2004).

1.4.2. Immunological methods

Immunological analysis can prove extremely useful in determining the streptococcal species involved in a particular outbreak, after preliminary bacteriological and biochemical identification of the agent has been made.

Immunological confirmation may be performed by a variety of methods such as slide agglutination (Kitao, 1982) or fluorescent Ab staining (Kawahara & Kusuda, 1987). The latter method has been used for differentiation of α- and β-haemolytic isolates. Kusuda and Kimura (1987) compared direct and indirect fluorescent Ab technique (IFAT) as methodologies for the identification of several yellowtail pathogens, concluding that the direct Ab technique (FAT) works better for this purpose. Both these studies use polyclonal antibodies (PAbs).

PAbs have also been used for the development of an agar gel immunodiffusion assay (Prearo *et al.* 2003). One hundred strains isolated from several countries (Italy, Spain, France, Greece and Israel) were used in the study, and the PAbs were raised against an Italian reference strain (ITP-2001/93). Only Italian and Israeli isolates gave a positive reaction, belonging to a single serotype (Serotype I, see Section 1.1.5.), while the rest gave no reaction. However, the possibility of cross- reactivity with other Gram positive bacilli and cocci using PAbs has been noted by Toranzo *et al.* (1995a).

Specific Monoclonal Antibodies (MAbs) have been used by Endo *et al.* (1998) directed against specific epitopes of *L. garvieae* in the development of a flow cytometry methodology for rapid detection of the bacteria in mixed cultures.

The method for obtaining MAbs was described by Kohler and Milstein in 1976. The general approach for developing MAbs is to use splenocytes from an immunized mouse, which are short lived, Ab producing B-cells. In order to create a continuous cell line, they are fused with a fast growing cell, such as a myeloma cell. The cell produced is called a hybridoma, and multiplies rapidly, creating a clone that produces large quantities of a single Ab. These antibodies are highly specific to an Ag, a fact that makes them extremely useful for the differential detection of specific pathogens, and they are currently extensively being applied for diagnostic purposes in aquaculture (Adams *et al.* 1995).

Monoclonal Antibodies have been used for the detection and characterization of a variety of fish pathogens such as *Mycobacterium* spp (Adams *et al.* 1996), *Renibacterium salmoninarum* (Adams *et al.* 1995), *Aeromonas* spp (Neelam *et al.* 1995) and yellowtail ascites virus (Nakajima & Sorimachi, 1996). These are only examples, and the number of pathogens detected with the use of MAbs is continually increasing, as new specific MAbs are developed.

Immunohistochemistry (IHC) is a rapid diagnostic test that 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 (Adams *et al.* 1994).

Enzyme-Linked Immunosorbent Assays (ELISA) rely on the ability of biological materials (i.e., antigens (soluble or particulate), antibodies) to adsorb to plastic surfaces such as polystyrene (solid phase). When antigens (or antigens bound to an Ab fixed onto the plate) bound to the solid phase are brought into contact with an Ab specific for the Ag, the Ab will bind to the antigen on the solid phase forming Ag-Ab complexes, which can then be detected through different procedures (e.g. Direct methods, when the detection Ab is labelled with an enzyme, or indirect detection, using an enzyme-labelled secondary Ab that recognizes the primary Ab used in the test). These usually involve chromogenic reactions that can be quantified using spectrophotometry. This method of detection of Ag was first described by Engvall & Perlman (1971).

1.4.3. Polymerase Chain Reaction (PCR) methods

Polymerase Chain Reaction (PCR) was developed in the 1980s, and has been the single most significant development for molecular diagnosis of disease in all organisms (Mullis & Faloona, 1987; Saiki *et al.* 1998). In this technique, short stretches of nucleic acid that are unique (or contain sequences that are unique to the target organism) are amplified. The presence of an amplification product can by itself be considered sufficient evidence that the target organism is present, although further analysis should be carried out to yield additional detail or confirmation of identity.

One inconvenience of PCR is that fish pathogens have not been studied in as much detail as other microorganisms, and this raises the possibility that PCR primers might cross-react with other closely related organisms, giving false positive results, which was the case with the first of the primers described below (Aoki *et al.* 2000).

As the genome of every single organism has not been sequenced, specificity cannot be guaranteed, but the chances of false positives can be reduced through judicious primer selection and appropriate validation tests (Leung, 2004).

In the case of *L. garvieae* different DNA sequences have been used as primers in the development of PCR tests, based on regions of the bacterial 16S rRNA gene (Zlotkin *et al.* 1998). Primers based on this sequence were able to detect *L. garvieae* from three different continents (Asia, Australia and Europe), and the test was sensitive enough to detect bacteria from 1µl of plasma although it was unable to detect the pathogen in water with actively infected fish. Several related and non-related bacteria were screened with the primers, as a proof of the specificity. The primers were used to differentiate between *Lactococcus* and *Enterococcus*, as well as in the development of a Multiplex PCR assay for detection of fish streptococcal pathogens (Deasy *et al.* 2000, Mata *et al.* 2004).

More recently, Aoki *et al.* (2000) have found that this primer sequence also amplifies DNA fragments from other species. These authors proposed another sequence that can be used as a specific primer for *L. garvieae*, based on the dihydropteroate gene (more specifically the SA1B10-1 of the dihydropteroate synthase gene), which was previously described in another study in which several genes of the KG- bacteria were sequenced (Hirono *et al.* 1999). In this study other related and non-related bacteria were screened for cross reactivity. Perhaps a higher number of bacterial species would have provided more confidence about the specificity of the test. Unfortunately, *L. garvieae* isolates from a variety of species were not included, and all the isolates came from Yellowtail.

Reverse PCR (RT-PCR) has been used in the development of a high throughput one tube RT-PCR-enzyme hybridization assay to detect selected bacterial fish pathogens, including *L. garvieae*. It is able to detect ribosomal RNA of the bacteria at 1-9 CFU per 200μl of sample: A very high sensitivity that provides the technique with a good potential to be used in the detection of bacteria in the water (Wilson & Carson, 2003).

1.4.4. Other methods

A simple procedure, based on SDS-PAGE was developed for comparing the whole cell protein patterns of *Lactococcus* spp., *L. lactis* and *L. garvieae*, from which it was possible to differentiate between them. This method was developed due to the difficulty of differentiate these bacteria using conventional tests. It was also developed for diagnosis of the disease in humans (Elliot *et al.* 1991).

1.5. CONTROL

1.5.1. Physical methods

Ozone has been used as a disinfecting agent against streptococcosis. A research study showed that the 99% inactivation point for *E. seriolicida* in sea water is achieved with concentrations of 0.111 mg/litre of ozone (Sugita *et al.* 1992).

A treatment rate ranging from 80 to 120 ppb of ozone with a dosage of approximately 0.3-0.5 ppm per min reduced the total bacterial figure more than three orders of magnitude in the logarithmic scale in fresh water (Beretta, 1999).

1.5.2. Chemotherapeutic methods

Historically, different chemotherapeutants have been used to treat lactococcosis with differing degrees of success. *Sodium nifurstyrenate*, proved to be more active (*in vivo* and *in vitro*) than aminobenzylpenicillin, chloramphenicol and tetracycline (Kashiwagi *et al.* 1977 a, b). *Erythromycin* and *Spiramycin* have produced resistance after ten serial passages *in vivo* (Kusuda & Onizaki, 1985). *Josamycin* is considered to be a particularly useful substance, as it does not present harmful side-effects in the fish and it does not affect the intestinal flora of the animal (Kusuda and Takemaru, 1987; Takemaru and Kusuda, 1988 a, b, c, 1990). More recently, *Tobicillin* has been evaluated as a useful drug for the treatment of enterococcicosis (Ooshima *et al.* 1997).

Although these substances have demonstrated their effectivity, improvement in stocking density and hygiene of the feed are also necessary, as well as avoiding overuse of chemotherapeutics in order to prevent the appearance of resistant strains.

Elliot and Facklam (1996) studied the Minimun Inhibitory Concentration (MIC) of *L. garvieae* to several antibiotics. *L. garvieae* showed higher MIC for penicillin, cephalotin and clyndamicin that *L. lactis*. Interestingly, for both these species, some isolates showed higher MICs for specific antibiotics than others. This suggests the presence of antibiotic resistant strains.

1.5.3. Vaccination

The limited usefulness of chemotherapeutants (lack of legally approved drugs, antibiotic resistance and anorectic condition of diseased fish), point towards the potential usefulness of immunoprophylaxis for the control of *L. garvieae*, and several attempts have been made in this respect (Iida *et al.* 1981; Carson & Munday, 1990; Ghittino *et al.* 1995, 2002; Toranzo *et al.* 1995b; Akhlagi *et al.* 1996; Romalde *et al.* 1996b, 1999, 2004). Considerable variability in the success of these vaccines has been observed, depending on the fish, the bacterial isolate used, the vaccine formulation, the route of administration, the age of the fish, as well as whether immunostimulants were or were not used.

Experiments on passive immunization of rainbow trout showed high levels of protection against the infection, achieved by injection of mammalian anti-*Streptococcus* antibodies. This protection lasts for one month after the injection (Akhlagi *et al.* 1996).

More recently, Barnes *et al.* (2002 a, b), reported an increase of rainbow trout resistance to *L. garvieae* infection after passive immunization, and attributed this effect to an increase of phagocytosis and killing of the bacteria by macrophages.

Barnes *et al.* (2002 a,b) also found that antibodies raised against capsulated isolates confer high protection, whereas antibodies against non-encapsulated isolates do not.

This did not seem to be the case with Yellowtail (cross protection against capsulated isolates was noted), which suggests that the protective Ags against *L. garvieae* in Yellowtail are not capsular in nature, and are located in the wall of both types of isolates (Ooyama *et al.* 1999).

Heat killed and formalin killed *L.* garvieae cells, detoxicated extra cellular products, administered by injection, oral and/or spray methods, provide only partial protection in marine fish (Iida *et al.* 1981, Kusuda *et al.* 1996).

Good levels of protection have been achieved in rainbow trout with formalin killed *L. garvieae* cells administered intraperitoneally, during a period of 2-3 months, although no protection was observed when the bacterins where administered by bath (Bercovier *et al.* 1997).

The intraperitoneal injection of bacterins was used in a farm trial on rainbow trout, achieving a reduction in mortalities of 16.7% for vaccinated fish compared with the 40.4% of the unvaccinated group (the control fish had to be treated with antibiotics).

A second trial was carried out six months later, and a certain degree of resistance could be noticed in the vaccinated group, showing mortalities of 18.6% (Ceschia *et al.* 1998).

More recently, two vaccination protocols where compared by Ghittino *et al.* (2002). The first consisted on administering bacterin twice, firstly by immersion (in January), and subsequently by intraperitoneal injection (0.2 ml per trout, with an oil-based adjuvant), in May. This protocol gave an excellent degree of protection (89% survival), and an Ab agglutination titre of 1:16 in October.

The second method compared consisted on a single injection of 0.2 ml trout $^{-1}$ of bacterin without the adjuvant. It also showed good protection (94% survival), but the Ab agglutination titre of 1:16 was found in July, followed by a fall in this response. This result suggests that the use of booster vaccination, instead of a single injection, achieves greater protection.

Vaccination trials have also been carried out against Enterococcosis in Turbot, using a toxoid-enriched whole cell bacterin (ET-2), administered by immersion and intraperitoneally. An immunostimulant containing β-glucan from yeast was also evaluated when administered with the vaccine. The vaccine proved to be very effective when administered intraperitoneally, achieving relative percentages of survival (RPS) of 89-100% (for 45 g fish) and of 67-86% (for 150 g fish), depending on the bacterial levels and time of the experimental challenge. The protection acquired, when administered intraperitoneally, lasted for one year. Very interestingly, no circulating antibodies were detected in the vaccinated Turbot, although a significant increase of the phagocytic activity of the spleen could be detected (Toranzo *et al.* 1995b).

An encapsulated oral vaccine has been tested in Rainbow trout, showing effectivity when used as a booster three months after intraperitoneal injection of the primary vaccine, but not generating sufficient protection enough to be used as the primary vaccine (Romalde *et al.* 2004).

1.5.4. Other methods

Probiotics have been used in an attempt to control the growth or invasion of *E. seriolicida* in fish, with little or no success. Examples of this type of work were conducted with *Lactobacillus plantarum* and/or *Bacillus coagulans* (Kusuda, 1984). On the other hand, oral administration of peptidoglycan derived from *Bifido-bacterium thermophilus* seems to enhance the resistance of yellowtail against *E. seriolicida* infection (Itami *et al.* 1996).

The use of bacteriophages as a biological control for disease of cultured fish has aroused much interest in recent years, especially since no drug residues and toxicity are associated with this type of therapy. Studies on bacteriophage protection against *L. garvieae* showed increased resistance to experimental infection in Yellowtail (Nakai *et al.* 1999), although no more studies have been found in this respect, particularly to commercial application.

1.6. AIMS OF THE PROJECT

As discussed in Section 1.1.4., no studies have been carried out on the global distribution of the serotypes of *L. garvieae*, more specifically the relation of the serotypes found in Asia with those found in Europe. It is also difficult to compare the research done in this field, due to the heterogeneity of the isolates used. Research to try and find an Ab specific for *L. garvieae* which binds to an Ag present on all pathogenic serotypes would be very useful for immunological diagnostics.

Several MAbs have been produced by the Aquatic Vaccine Unit, Institute of Aquaculture (IoA), University of Stirling, UK, against Japanese and European isolates of *L. garvieae*, originated from different species (Japanese and Korean isolates originate from marine species, whereas the European isolates come from a freshwater species). The aim of this project was to screen these MAbs against a collection of *L. garvieae* isolates, as well as other species of related and non-related bacteria. This would establish which MAbs recognize the greatest number of *L. garvieae* isolates, as well as examining the possible cross-reactivity with other bacterial species.

Having established the specificity of the MAbs, an IHC test was developed and used to locate the bacteria in sections of tissue from diseased fish, collected from a *L. garvieae* NCIMB 702928 (Type strain) challenge on Rainbow trout. This material was also be used to examine the pathology of the disease over the course of the experimental challenge.

Chapter 2. MATERIALS AND METHODS:

2.1. MONOCLONAL ANTIBODIES

2.1.1. Antibodies used

The MAbs used throughout this study were produced by immunizing mice with different strains of *L. garvieae*. The MAbs were provided by Kim Thompson as tissue culture supernatants (Unpublished data), with the exception of the Korean antibodies (U99-33 and GC 246), which were provided by Professor Tae Sung Jung (Laboratory of Fish and Shellfish Diseases, Gyeongsang National University, Republic of Korea). A list of the MAbs provided and used in the study is shown in Table 2.1.

Table 2.1. Monoclonal antibodies raised against different isolates of *Lactococcus garvieae* and tested for cross-reactivity with related and unrelated bacterial species.

MonoclonalAntibody	*Lactococcus garvieae Isolate*	*Region of origin*(+)
MAb 2	NCIMB 702928	Europe
MAb 10	NCIMB 702928	Europe
MAb 13	NCIMB 702928	Europe
MAb U 99-33	Unknown(*)	Korea
MAb GC 2-4-6	Unknown(*)	Korea
MAb 8B12	12-99 (IoA)	Japan
MAb 3G9	12-99 (IoA)	Japan
MAb 11F8	12-99 (IoA)	Japan
MAb 11B1	12-99 (IoA)	Japan
MAb 1B3	12-99 (IoA)	Japan
MAb 1A2	12-99 (IoA)	Japan

NCIMB: National Collection of Industrial and Marine Bacteria (Aberdeen UK). IoA: Institute of Aquaculture. (*): MAbs provided by Professor Tae Sung Jung (Laboratory of Fish and Shellfish Diseases, Gyeongsang National University, Republic of Korea) to be evaluated in this study. (+): Region of origin of isolates used for the mice immunization protocol.

NOTE: *The recipes for all the buffers and stains used in all procedures can be found in the Appendix.*

2.2. BACTERIOLOGY

2.2.1 Bacterial strains

Information about all the bacteria used in this study can be found in Tables 2.2 and 2.3.

Table 2.2. *Lactococcus garvieae* isolates used in a characterization study to several Monoclonal Antibodies produced against *L. garvieae*

Designation	*Genus/species*	*Host*	*Source*
NCIMB 702155	*L. garvieae*	Bovine	NCIMB, Aberdeen, UK
NCIMB 702927	*L. garvieae*	Unknown fish species	NCIMB, Aberdeen, UK
NCIMB 702928	*L. garvieae*	Unknown fish species	NCIMB, Aberdeen, UK
12-99	*L. garvieae*	Yellowtail/Japan	Clinical isolate
Milze 2	*L. garvieae*	Rainbow trout/Italy	Clinical isolate
Milze 4	*L. garvieae*	Rainbow trout/Italy	Clinical isolate
Occtve	*L. garvieae*	Rainbow trout/Italy	Clinical isolate
Reve 2	*L. garvieae*	Rainbow trout/Italy	Clinical isolate
Reve 3	*L. garvieae*	Rainbow trout/Italy	Clinical isolate
Reve 4	*L. garvieae*	Rainbow trout/Italy	Clinical isolate
Namhe 7	*L. garvieae*	Yellowtail/Japan	Clinical isolate
Namhe 13	*L. garvieae*	Yellowtail/Japan	Clinical isolate

NCIMB: National Collection of industrial and marine bacteria; Italian isolates, courtesy of Professor Manfrin (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy); Japanese isolates courtesy of Professor Yoshida, O.(Miyazaki University, Japan).

2.2.2. Bacteriology

(a) Bacterial culture: Strains of *L. garvieae*, as well as related and unrelated bacteria (see Table 2.2 and Table 2.3) were grown on Tryptone Soya Agar (TSA), with the exceptions of *Vibrio* spp. and *Listonella* spp., grown in TSA with 2.5% NaCl. *R. salmoninarum* was grown in specific kidney disease medium (SKDM), and *Flavobacterium psychrophilum,* grown in Cytophaga Agar. Incubation took place at 22^0C (With the exception of *Flavobacterium psychrophilum*, grown at 17^0C) for as long as necessary (48-72 h for most species), on Petri dishes.

Table 2.3. Bacterial species used to screen the cross-reactivity of several Monoclonal Antibodies raised against *Lactococcus garvieae*

Designation	*Genus/species*	*Source*
NCIMB 2264	*Carnobacterium piscicola*	NCIMB, Aberdeen, UK
NCIMB 775	*Enterococcus faecalis*	NCIMB, Aberdeen, UK
NCIMB 1406	*Lactobacillus spp (plantarum-like isolate)*	NCIMB, Aberdeen, UK
NCIMB 1113	*Renibacterium salmoninarum*	NCIMB, Aberdeen, UK
K0101-5	*Streptococcus agalactiae*	Kuwait isolate
NCIMB 13196	*Lactococcus piscium*	NCIMB, Aberdeen, UK
ATCC 29178	*Streptococcus iniae*	ATCC, Rockville, USA
NCIMB 700637	*Streptococcus parauberis*	NCIMB, Aberdeen, UK
NCIMB 13133	*Vagococcus salmoninarum*	NCIMB, Aberdeen, UK
NCIMB 13205	*Aeromonas ichthyosma*	NCIMB, Aberdeen, UK
NCIMB 12065	*Aeromonas sobria*	NCIMB, Aberdeen, UK
ATCC 49657	*Aeromonas trota*	ATCC, Rockville, USA
ATCC 35024	*Aeromonas veronii*	NCIMB, Aberdeen, UK
NCIMB 9240	*Aeromonas hydrophila*	NCIMB, Aberdeen, UK
NCIMB 1109	*Aeromonas salmonicida achromogenes*	NCIMB, Aberdeen, UK
NCIMB 1102	*Aeromonas salmonicida*	NCIMB, Aberdeen, UK
NCIMB 2237	*Aeromonas media*	NCIMB, Aberdeen, UK
NCIMB 13016	*Aeromonas caviae*	NCIMB, Aberdeen, UK
NCIMB 74	*Aeromonas eucrenophila*	NCIMB, Aberdeen, UK
NCIMB 1953	*Pseudomonas fluorescens*	NCIMB, Aberdeen, UK
ATCC 11778	*Bacillus mycoides (cereus)*	ATCC, Rockville, USA
NCTC 8241	*Bacillus pumilus*	NCTC, London, UK
NCIMB 3610	*Bacillus subtilis*	NCIMB, Aberdeen, UK
B 96221	*Citrobacter freundi*	IoA, Stirling, UK
NCIMB 2084	*Edwardsiella tarda*	NCIMB, Aberdeen, UK
0130	*Edwardsiella ictalurii*	IoA, Stirling, UK
NCIMB 6	*Escherichia coli*	NCIMB, Aberdeen, UK
NCIMB 10	*Listonella anguillarum*	NCIMB, Aberdeen, UK
ATCC 27853	*Pseudomonas aeruginosa*	ATCC, Rockville, USA
NCIMB 1920	*Nocardia asteroides*	NCIMB, Aberdeen, UK
NCIMB 2057	*Nocardia kampachi*	NCIMB, Aberdeen, UK
B08008	*Flavobacterium psychrophyllum*	IoA, Stirling, UK

ATCC: American Type Collection; NCIMB: National Collection of industrial and marine bacteria; IoA: Institute of Aquaculture; NCTC: National Collection of Type Cultures, London, UK. All bacterial species above the line are bacterial species related to *L. garvieae*, whereas the ones under the line are unrelated.

(b) Gram staining: The following procedure was used to stain bacterial suspensions and blood. Using a sterile loop, a drop of sterile saline was aseptically placed onto a clean glass slide. Afterwards, 1-2 colonies of bacteria were taken from an agar plate and resuspended in the saline on the slide. The slide was allowed to air dry and bacteria were heat-fixed by slowly passing the slide through a Bunsen flame three times. The blood smears were fixed to the slide using an absolute ethanol bath for 5 min. The slides were immersed in crystal violet solution for 1 min, and residual stain was washed off with water before immersing the slide in iodine, and leaving it for 1 min. The slides were differentiated using Acetone for 2-3 sec. The reaction was stopped with tap water, and the slide counter stained with a safranin solution for 2 min, and then washed off with plenty of tap water. The slides were then examined under light microscopy.

2.3. ENZYME LINKED IMMUNOSORBENT ASSAY

(Indirect Antibody capture ELISA)

The reasons why an ELISA assay was used are that it offers the possibility to screen a large number of bacteria and Ab in a single assay (each plate has got 96 wells), it is quantifiable (using spectrophotometry), reliable, relatively fast, and small amounts of reagents are necessary to carry out the assay.

2.3.1. Bacterial preparation

Strains of *L. garvieae*, as well as related and unrelated bacteria (see Table 2.2 and Table 2.3) were cultured in Petri dishes, as described in Section 2.2.2. After 48-72 h of growth (or as long as necessary for slow growing bacteria), several colonies were inoculated in 40 ml of Tryptone Soya Broth (TBS), or the media for the particular bacteria in question, and incubated at 22^0C for 48 to 72 h. The bacteria were then centrifuged at 1,200 g for 15 min. The supernatant was discarded, and the bacteria resuspended in 10ml of Phosphate Buffered Saline (PBS) and centrifuged again at the same settings in order to wash the pellet. Finally the bacteria were resuspended in PBS at an OD_{610} of 1.00.

Following this procedure, the bacteria were placed in crystal universals and heat killed, by placing the universals in a water at 60^0C for a period of 1 h, or 2 h in the case of *L. garvieae*, as some of the isolates showed resistance to heat killing.

2.3.2. Indirect Antibody capture ELISA protocol

The following procedure was the one described by Adams & Thompson (1990), with modifications. A 96-well ELISA plate was coated with 1% (w/v) poly-L-lysine in coating buffer (50µl per well) for 60 min. The plate was washed twice with Low Salt Wash Buffer (LSW). A 100µl aliquot of the bacteria suspended in PBS at OD_{610}=1 was added to the wells, and incubated overnight at 4^{0}C, after which time 50µl of 0.05%(v/v) of glutaraldehyde in PBS was added, in order to fix the bacteria to the plate.

The plate was incubated for 20 min, after which the plate was washed three times with LSW. Two hundred and fifty µl $well^{-1}$ of 3% (v/v) casein was added to the wells and incubated for 2 h, in order to block non-specific binding sites. The casein was removed from the plate by washing three times with LSW. Afterwards 100µl of neat hybridoma supernatant (containing antibodies) was added to each well, and incubated for 60 min at room temperature. Excess Ab was removed by washing thoroughly five times with High Salt Wash Buffer (HSW), incubating for 5 min in the last wash.

After washing, conjugate Ab was added at 100µl $well^{-1}$ (in this case anti-mouse IgG-HRP (Diagnostic Scotland, UK), diluted 1/1000 in conjugate buffer), and incubated for 30 min. The plates were washed as before at the end of the incubation, to make sure that excess conjugate was removed. The chromogen was then added (100µl of chromogen in substrate buffer), and incubated for 10 min, after which the reaction was stopped with 50µl per well of stop solution. The absorbance of the wells was read at 450 nm (ELISA microplate reader, Dynatech MR5000). Positive wells showed a yellow coloration whereas negative wells remained clear.

2.3.3. Characterization of the MAbs to L. garvieae

All the MAbs examined in the study (Table 3.1) were tested (in duplicate) against all the available strains of *L. garvieae* (Table 3.3). Cross-reactivity was the mean of duplicate wells, expressed as a percentage of the optical density obtained by wells containing *L. garvieae* NCIMB 702928, which was used as a positive control.

2.3.4. Cross reactivity of MAbs

A number of related and unrelated bacterial species (Table 3.4) were screened against the MAbs with the ELISA described above, in order to establish the specificity of the MAbs. Cross reactivity was again the mean of duplicate wells, expressed as a percentage of the optical density obtained by wells containing *L. garvieae* NCIMB 702928, which was used as a positive control.

2.4. IMMUNOHISTOCHEMISTRY

2.4.1. Antibodies

A preliminary IHC test was carried out with all the MAbs in order to determine which of the MAbs produced the strongest staining with this technique (See Section 3.2.1.).

Then IHC was used to screen all the samples collected from a *L. garvieae* experimental challenge. MAbs 13 and 3G9, which where originally developed using European and Japanese isolates respectively, were used in the IHC.

2.4.2 Positive and Negative controls

The tissue section used as a positive control was from an archival histology block from a previous rainbow trout (*O. mykiss)* challenge with *L. garvieae* NCIMB 702928. This section had a very infected spleen, and was used as a positive control for all the IHC tests in this study [Reference number: D040013A (IoA)]. All the negative controls were the same section with PBS instead of MAbs.

2.4.3. Immunohistochemistry protocol

The procedure used was based on the method described by Adams & de Mateo (1994), with modifications. The slides were dewaxed in two successive xylene baths of 5 min each and rehydrated as follows: 100% ethanol bath (5 min), 70% ethanol bath (3 min), and distilled water (30 secs). Afterwards, the tissues were encircled with wax using a ImmEdge™ pen (Vector Labs. California, USA), to allow small quantities of fluid to be retained on the tissue. Endogenous peroxidase activity in the tissues was blocked by incubating them at room temperature (20^0C) for 10 min with 3% (v/v) hydrogen peroxide in methanol.

This incubation, as well as all the following incubations, took place in a humid chamber, in order to minimize desiccation of the samples. Following this, the slides were washed three times using Tris buffered saline (TBS), and incubated with goat serum, diluted 1/10 in TBS, for 10 min at room temperature. This incubation has the effect of blocking non-specific binding sites, in an attempt to minimize unwanted background staining. After the incubation, the serum was poured off the slides and the excess liquid removed by tapping the edge of the slides onto a paper towel. Supernatants of the MAbs were added to the slides and incubated for 1 h.

Afterwards, the slides were washed three times again with TBS. After this step, goat anti-mouse HRP Abs, diluted 1/50 in TBS were incubated on the slides for 30 min at room temperature, followed by three washes with TBS.

The slides were then incubated with 3,3',5,5'-Tetramethylbenzidine [True Blue™ (KPL, Maryland, USA], which turns blue in presence of peroxidase. This reaction was stopped after 10 min with distilled water. This step allows the visualization of the reaction, if present. The slides were counterstained with Red contrast™ (KPL, Maryland, USA) for 4 min, to allow visualization of the rest of the tissue. After this, the slides were washed thoroughly with distilled water to eliminate the excess stain.

The slides were then dehydrated as follows: 70% ethanol (3 min), 100% ethanol (5 min) and 2[xylene (5 min)]. Finally, the slides were mounted using Pertex™ and observed under a light microscope.

2.5. BACTERIAL CHALLENGE

2.5.1. Preparation of the bacteria

An inoculum consisting on a suspension of *L. garvieae* NCIMB 702928 in PBS was prepared as follows: The bacteria were cultured in TSA (Tryptone Soya Agar) in order to carry out a purity check, and incubated for 48 h at 22^0C prior to the challenge. Afterwards, 6-8 colonies were inoculated in 20 ml of Tryptone Soya Broth (TSB) in a glass universal and incubated overnight at 22^0C to produce a log phase bacterial suspension.

The bacteria were then washed with PBS by centrifugation at 1500 g for 10 minutes, the supernatant discarded, and the pellet resuspended in 10 ml of sterile PBS. They were again centrifuged and finally resuspended in 5 ml PBS.

The absorbance of the suspension was adjusted to an OD_{610}= 1.00, using PBS. This suspension was used to challenge the fish. The procedure for establishing the number of bacteria in the inoculum was carried out retrospectively.

2.5.2. Quantification of bacteria injected to fish

The procedure used was the Miles/Misra method. Seven tenfold dilutions were successively made in seven bijoux, using PBS as the diluent. An aliquot (20µl) from each dilution was dropped onto a TSA plate which had been divided into six sections, one drop per section. The plates were then incubated overnight at 22^0C. The following day, the dilution showing between 20 and 200 colonies was counted.

These values were used to calculate the number of bacteria in 1 ml of suspension, which was $1.07 \cdot 10^9$ CFU's ml^{-1} of suspension.

2.5.3. Challenge procedure

Thirty three rainbow trout of one and a half years of age with an average weight of 57 g were provided by the Aquarium Research Facility at the Institute of Aquaculture, Stirling University. Three of these fish were sampled before starting the experiment as a negative control (Day 0 samples). (See section 2.5.4).

Thirty rainbow trout were injected intraperitoneally with 0.1 ml of the inoculum ($1.07 \cdot 10^8$ CFU's per fish). Fish were first anesthetized by placing them in 5 l of water containing 10 ml of 2-phenoxiethanol. After injection, the fish were placed in oxygenated water for recovery, and then transferred into the challenge tank. No mortalities were recorded during the process. All the fish were placed in a cylindrical 100 l tank, with a constant water flow of 1 l min^{-1}, and a constant temperature of 14^0C, regulated by a thermostat (Figure 2.1). The fish were not fed during the experiment, and were checked twice daily.

Figure 2.1. One hundred litre tank used to keep the rainbow trout during the challenge with *L. garvieae* NCIMB 702928, with a constant flow of water of approximately 1 l min^{-1}. An image of the thermostat used to keep a constant temperature of 14^0C is also shown.

2.5.4. Sampling protocol

The challenge lasted 25 days and fish were sampled on Days 0 (Control), 1, 2, 3, 5, 10, 14, 21 and 25. During the challenge, the fish were observed at least two times a day. On each sampling day, three trout were humanly killed by an overdose of benzocaine and sampled. When mortalities occurred, the trout affected were sampled as soon as possible, in order to preserve tissue morphology and prevent autolytic changes. The last day of the challenge, all remaining fish (4, in this case) were sampled, the tank emptied, cleaned and disinfected.

A clinical examination of the fish was carried out on each trout sampled, and the same tissues were taken from each one, namely:

- Blood smears.
- Histology samples: Eye, gills, Brain, Spleen, Heart, Liver, Kidney, Swim bladder.
- A kidney smear was inoculated on a TSA plate.

Spleen smears on a slide were taken from the two mortalities found during the challenge. All histology samples were stained with Haematoxylin & Eosin and Gram stain. They were also screened with the antibodies, using IHC.

2.6. HISTOLOGY

2.6.1. Preparation of histological sections

For the preparation of the histological sections, tissue samples were placed in 10% buffered formalin for at least 24 h. These were placed in plastic cassettes for processing. Processing was performed in an automatic tissue processor (CITADEL ™ Tissue processor, Shandon Inc, Pittsburgh, Pennsylvania, USA) according to the processing schedule outlined in the Appendix. Cassettes were removed from the tissue processor and embedded in wax. The tissue sections were cut using a manual rotary microtome (LEICA™ Microsystems GmbH) at 5µm per section. No decalcification was carried out in any of the samples. Racked slides were left to dry in an oven at 60^0C overnight, to allow fixation of the tissue to the slide.

2.6.2. Haematoxylin and Eosin (H&E) staining

The method used was the one described by Stevens and Wilson (1996), with modifications. Before staining, sections were dewaxed by placing them in two sequential xylene baths, each for a period of 5 min. The slides were rehydrated in 100% ethanol for 2 mins, followed by a methylated spirits bath for 1 min, and finally tap water bath for 30 to 60 sec. Afterwards, the slides were placed in Mayer's Haematoxylin (Appendix) for 5 min. Slides were again washed in running tap water prior to being quickly dipped three times in acid alcohol (1%). Afterwards the slides were washed with running tap water (30 sec), and then immersed in Scott's tap water substitute for 1 min.

Following another bath with running tap water, the slides were stained in eosin (Appendix) for 5 min and washed again in tap water. The slides were dehydrated by placing in methylated spirits (1 min), absolute alcohol (x2, 2 min), and xylene for 5 min. The racks were then transferred to a further xylene bath, and immediately coverslipped using Pertex© mounting medium. Slides were left to dry in a fume hood for at least 10-15 min prior to microscopy.

2.6.3. Gram staining

The protocol followed for Gram staining in paraffin embedded tissues was different from the protocol used for bacterial suspensions and blood smears. Initially the sections were dewaxed, using two consecutive baths of xylene (3 and 2 min, respectively). It was followed by rehydration (absolute ethanol bath for 2 min, followed by methylated spirits bath for 1 min, and a final rinse with water of 3 sec). The slides were flooded with Crystal Violet stain for 3 min. They were washed with tap water and then flooded with Gram's Iodine for a further 3 min. Afterwards, the slides were washed again and then differentiated using pure acetone until the section was colorless. The reaction was stopped with water, and the tissue sections counterstained with Neutral Red (1%) for 2 min. After rinsing with tap water and blotting dry using filter paper, the slides were dehydrated and differentiated through three quick dips in each of two successive absolute methanol baths. The slides were cleared in xylene for 5 min and then coversliped using Pertex™ mounting medium. Gram positive bacteria appear blue/purple, and Gram negative appear pink/red.

2.7. SCANNING ELECTRON MICROSCOPY

The specific bacterial isolates used for scanning electron microscopy were *L. garvieae* NCIMB 702928 and Occtve. The bacteria was suspended at an OD_{610}=1.00, in PBS, measured in a spectrophotometer, and then prepared for scanning electron microscopy following the method proposed by Nation (1983), which is particularly useful for samples collected on membrane filters. The membrane used was a Cyclopore© track etch membrane (0.4μm) from Whatman. Samples were allowed on the membrane for 30 min and then fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate at 4^0C during 1 h. After this period, the solution was substituted to a 3% glutaraldehyde in 0.1 M sodium cacodylate, which was left to incubate for three days at 4^0C. The samples were post fixed in 1% osmium tetroxide in buffer for 2 h, and then dehydrated with successive baths of ethanol (30%, 60%, 90%, and 100%, 30 min each). Afterwards, ethanol was gradually substituted with hexamethyldixilazane (HMDS) in two stages (first at 50% in ethanol for 30 min, second two consecutive baths with pure HMDS for 30 min each). Afterwards the samples where air dried in a fume cupboard, and then mounted on stubs and sputter coated (Edwards sputter coater S158B, UK). The samples were examined using a scanning electron microscope (JEOL JSM-6460-LV).

Chapter 3. RESULTS

3.1. CHARACTERIZATION OF THE MONOCLONAL ANTIBODIES TO *Lactococcus garvieae*

3.1.1. Specificity to L. garvieae strains

Eleven MAbs had been produced against different strains of *L. garvieae*, from different origins (Europe, Japan and Korea). The nomenclature of these antibodies is given in Table 2.1. An indirect Ab capture ELISA was set up to test these MAbs against different strains of *L. garvieae*, available from the bacterial collection at the IoA, Stirling University. All of the strains tested and information relating to them can be found in Table 2.2. The MAbs tested had a different response to each of the strains. The percentages of reaction obtained with each MAb against each bacterial strain examined can be observed in Table 3.1.

Table 3.1. Response of anti-*Lactococcus garvieae* Monoclonal Antibodies against several strains of *L. garvieae* in an indirect ELISA for particulate Antigens.

L. garvieae strains	Monoclonal Antibodies										
	(Euro) 2	*(Euro) 10*	*(Euro) 13*	*(Japan) 8B12*	*(Japan) 3G9*	*(Japan) 11F8*	*(Japan) 11B1*	*(Japan) 1B3*	*(Japan) 1A2*	*(Korea) U99-33*	*(Korea) GC246*
NCIMB 702927	75.1	101.5	85.0	93.5	102.7	101.8	89.3	130.2	100.6	-	-
NCIMB 702155	-	-	2.6	29.0	27.5	37.8	30.8	5.1	4.3	-	-
12-99 (IoA)	87.9	96.7	103.4	103.7	112.9	95.9	78.7	121.3	108.0	-	-
Reve 2 (IoA)	-	0.7	2.3	15.5	22.1	14.9	18.4	4.8	9.7	-	-
Reve 3 (IoA)	-	-	1.5	14.4	7.5	13.0	11.7	-	1.7	-	-
Reve 4 (IoA)	-	-	-	38.3	18.2	51.7	42.8	-	1.7	-	-
Milze 2 (IoA)	-	-	-	39.0	16.0	42.0	28.3	-	0.0	-	-
Milze 4 (IoA)	-	-	-	11.0	13.0	17.6	14.7	-	1.3	-	-
Occtve (IoA)	-	-	-	15.8	27.9	21.4	29.9	-	0.9	-	-
Namhe 7	0.9	4.2	3.6	6.8	NT	-	9.5	2.2	1.5	-	-
Namhe 13	3.0	5.9	3.5	10.2	NT	4.6	14.1	3.8	5.1	-	-

NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen UK; IoA: Institute of Aquaculture, Stirling, UK. -: Values equal to 0% and lower. Response is the mean of duplicate wells, expressed as a percentage of the optical density obtained by type strain NCIMB 702928 (OD_{450}=0.89-1.33) in the indirect ELISA for particulate Ag; the mean OD_{450} of negative control (PBS) was 0.07-0.17.

The results in Table 3.1 show that all the European MAbs strongly recognise both of the type strains isolated from fish, as well as a clinical isolate from Japan (12-99), but do not recognise any of the clinical isolates from Italy, the clinical isolates from Korea nor the type strain isolated from bovine mastitis (NCIMB 702155). The Japanese MAbs gave similar results to those obtained with the European MAbs, with regards to the detection of both the type strains isolated from fish and the Japanese clinical isolate (12-99). Some of these MAbs also show cross-reaction with other *L. garvieae* isolates, although at a lower level. MAbs 8B12, 3G9, 11F8 and 11B1 all had a reaction of about 30% when compared to the type strain isolated from a case of bovine mastitis (NCIMB 702155). Regarding reaction with the Italian isolates, MAbs 8B12, 11F8 and 11B1 showed a reaction of between 28.3% and 51.7% to Reve 4 and Milze 2, MAbs 3G9, 11F8 and 11B1 showed a reaction of between 21.4% and 29.9% to Occtve, and all these MAbs gave a reaction between 14.9% and 22.1% with Reve 2. MAb 3G9 was not included in the screening of the Korean isolates, due to limited supply of supernatants.

In contrast, the Korean MAbs did not recognize any of the *L. garvieae* tested, not even the type strains. This made it impossible to compare them to the rest of MAbs in the study.

3.1.2. Cross-reactivity with other bacterial species

Complementing the studies on the specificity of the MAbs to different *L. garvieae* isolates, the MAbs were also screened against thirty two non-*L.garvieae* bacterial species (shown in Table 2.3), in an attempt to rule out the possibility of cross-reactivity and confirm the specificity of the MAbs to only *L. garvieae*. The results obtained are shown in Table 3.2.

Table 3.2. Cross reactivity of anti-*Lactococcus garvieae* Monoclonal Antibodies with several bacterial species, determined by indirect ELISA for particulate Antigen.

Bacterial species	Monoclonal Antibodies						
	(Euro) 2	*(Euro) 10*	*(Euro) 13*	*(Japan) 1B3*	*(Japan) 1A2*	*(Korea) U99-33*	*(Korea) GC236*
Aeromonas caviae NCIMB 13016	-	3.2	2.4	-	6.0	-	-
Aeromonas eucrenophila NCIMB 74	-	-	5.3	-	-	-	-
Aeromonas hydrophilla NCIMB 9240	-	-	2.1	-	-	-	-
Aeromonas ichthyosma NCIMB 13205	-	-	-	-	-	-	-
Aeromonas media NCIMB 2237	-	-	-	-	-	-	-
Aeromonas salmonicida achromogenes NCIMB 1109	-	4.0	-	-	-	-	-
Aeromonas salmonicida NCIMB 1102	-	-	5.0	-	-	-	-
Aeromonas sobria NCIMB 12065	-	2.0	4.1	-	-	-	-
Aeromonas trota ATCC 49657	-	-	5.3	-	-	-	-
Aeromonas veronii ATCC 35024	-	-	-	-	-	-	-
Bacillus subtilis NCIMB 3610	-	-	1.8	-	-	-	-
Bacillus mycoides ATCC 11778	-	2.4	1.6	2.7	4.0	-	-
Bacillus pumilus NCTC 8241	1.1	2.7	3.1	0.5	5.4	-	-
Citrobacter freundi B96221 (IoA)	-	1.1	-	2.7	3.7	-	-
Edwardsiella ictalurii 0130-66 (IoA)	-	2.5	0.3	-	-	-	-
Edwardsiella tarda NCIMB 2084	-	0.6	-	-	2.2	-	-
Escherichia coli NCIMB 6	-	-	-	-	1.1	-	-
Flavobacterium psychrophilum B08008 (IoA)	-	0.7	1.5	-	-	-	-
Listonella anguillarum NCIMB 10	-	1.4	-	-	-	-	-
Nocardia kampachi NCIMB 2057	1.3	2.3	-	-	2.5	-	-
Nocardia asteroides NCIMB 1290	-	1.1	1.1	2.9	0.5	-	-
Pseudomonas aeruginosa ATCC 27853	0.8	1.7	2.7	2.0	3.7	-	-
Pseudomonas fluorescens NCIMB 1953	2.9	6.1	5.9	1.5	5.7	-	-
Carnobacterium piscicola NCIMB 2264	3.0	1.5	1.5	-	2.1	-	-
Enterococcus faecalis 775-V32 (IoA)	0.9	2.6	-	-	-	-	-
Lactobacillus plantarum NCIMB 1406	4.3	5.3	3.0	0.6	5.2	-	-
Lactococcus piscium NCIMB 13196	1.1	2.3	-	-	1.5	-	-
Renibacterium salmoninarum NCIMB 1113	-	1.6	1.5	-	-	-	-
Streptococcus agalactiae K0101 (IoA)	0.8	2.1	2.4	-	-	-	-
Streptococcus iniae V64 (IoA)	1.5	5.6	5.4	1.3	2.4	-	-
Streptococcus parauberis NCIMB 700637	0.5	2.1	0.9	-	-	-	-
Vagococcus salmoninarum NCIMB 13133	3.8	9.6	4.2	1.4	10.1	-	-

NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen UK; ATCC: American Type Collection, Rockville, USA. -: Values equal to 0% and lower. Response is the mean of duplicate wells, expressed as a percentage of the optical density obtained by type strain NCIMB 702928 in the indirect ELISA for particulate Ag (OD_{450}=0.42-0.93); The mean OD_{450} of negative control (Phosphate Buffered Saline) was 0.05-0.11. Unrelated species: Above the line; Related species: Under the line.

The results in Table 3.2 show low percentage cross-reactivities of the anti-*L. garvieae* MAbs with the non-related bacteria. Some cross-reactivities were, however, obtained with values over 5%, as was the case of (Euro) 10 with *Pseudomonas fluorescens* (6.1%); (Euro) 13 with *P. fluorescens* (5.9%), *Aeromonas trota* (5.3%), *Aeromonas eucrenophila* (5.3%) and *Aeromonas salmonicida* (5.0%), and (Japan) 1A2 with *Aeromonas caviae* (6%), *Pseudomonas fluorescens* (5.7%) and *Bacillus pumilus* (5.4%).

The related species generally showed low percentages of cross-reactivity, although there were some cross-reactivities over 5%, by (Euro) 10 with *Lactococcus plantarum* (5.3%) and *Vagococcus salmoninarum* (9.6%), by (Euro) 13 with *S. iniae* (5.4%), and by (Japan) 1A2 with both *V. salmoninarum* (10.1%) and *L plantarum* (5.2%).

It should be noted that one of the Korean antibodies, more specifically U99-33, gave an OD_{450} of 0.247 against *R. salmoninarum* in an ELISA assay in which the background (PBS) was of OD_{450}=0.07. This is a significant finding, but this reaction is not included since the MAbs gave no reaction against the positive control (type strain *L. garvieae* NCIMB 702928) which was used as 100% reactivity index.

The Korean MAbs gave a zero percentage of cross-reactivity with the rest of bacterial species.

3.2. IMMUNOHISTOCHEMISTRY

3.2.1. Preliminary tests

The results of the IHC trial (which included all the European MAbs, Japanese MAbs 11F8, 8B12, 3G9 and Korean MAbs U9933 and GC246) showed that all of the European MAbs (2, 10 and 13) worked very well, especially (Euro) 13, which gave a very strong and specific positive reaction. This was also the case with some of the MAbs against the Japanese isolate, more specifically MAbs 11F8, 8B12, and, in a lesser degree, 3G9. On the other hand, the MAbs against the Korean isolates did not react with the bacteria on the sections. These findings correlate with the results obtained in the ELISA. The (Euro) MAbs had been produced using the same bacterial strain used for the challenge (NCIMB 702928). Images of the results obtained in the IHC can be seen in Figure 3.1.

An (IHC) test was developed for the detection of *L. garvieae* in infected tissue sections. The main problem found was due to non-specific positive reactions due to endogenous peroxidase activity in the tissues. Examples of this type of activity can be observed in Figure 3.2. This non-specific activity ceased to be evident after incubating the tissues with 3% (v/v) hydrogen peroxide in methanol for 10 min (the solution had to be made fresh in order to be fully effective).

With these results in mind, one European MAb (EURO 13) and one Japanese MAb (3G9, due to the lack of supernatant of the other two antibodies) were used in an IHC protocol to screen all the tissue sections from the challenge.

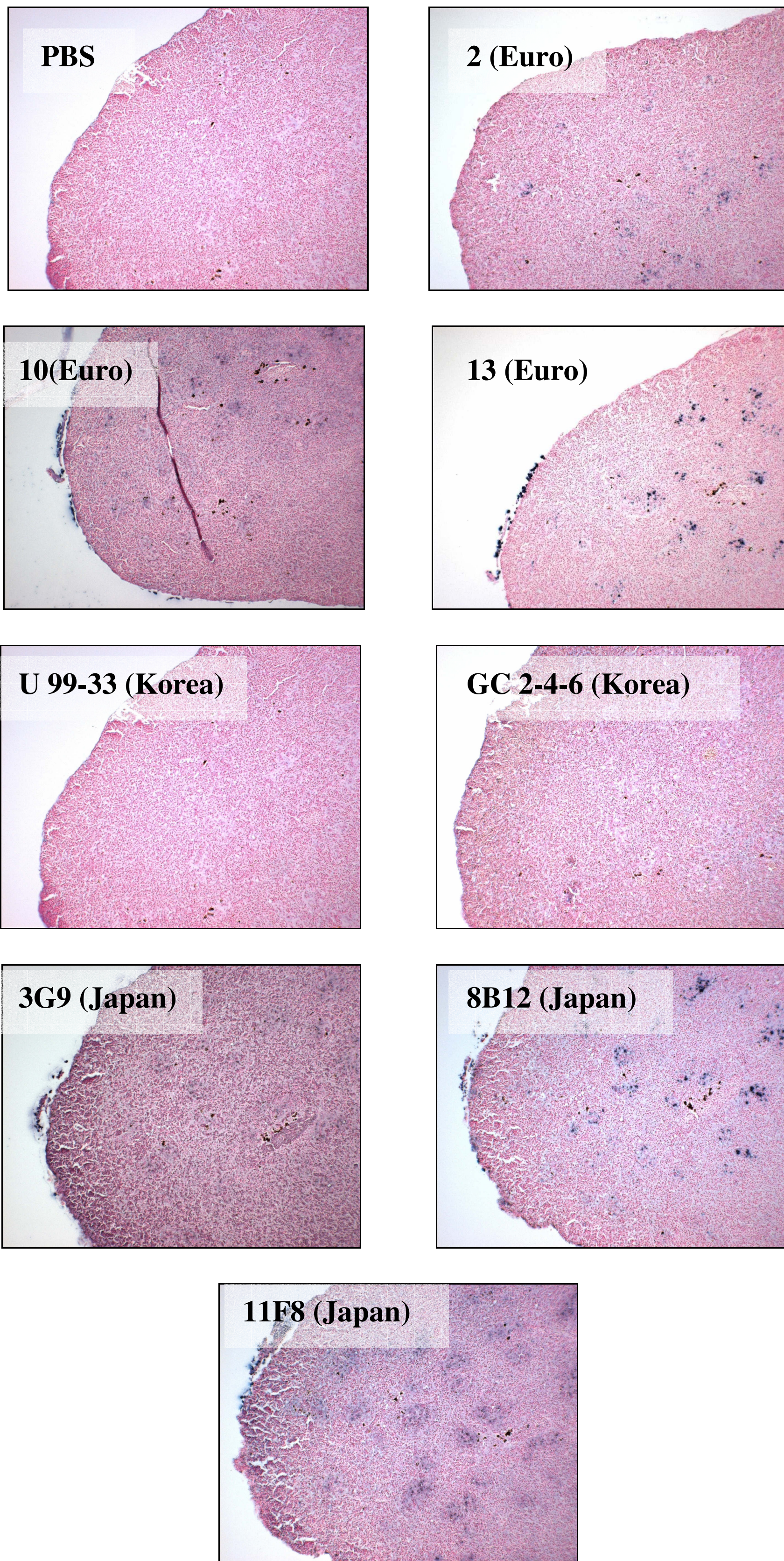

Figure 3.1. Image of the spleen of a positive control section (D040013A), known to be infected with *Lactococcus garvieae* in IHC with all the MAbs studied, as well as the negative control (PBS). Note the differences in the reaction obtained with each different MAbs. (x100, all images)

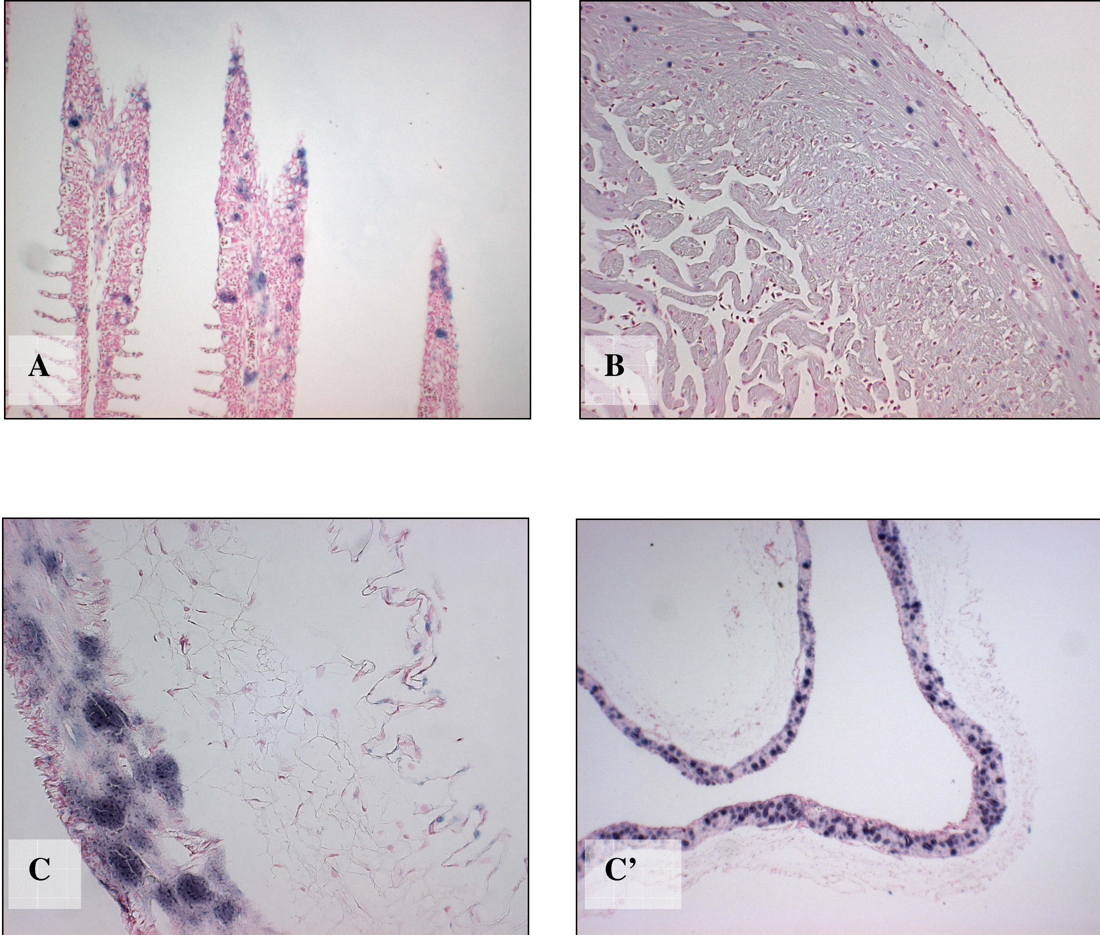

Figure 3.2. Non-specific blue coloration due to endogenous peroxidase activity in several tissues of negative control rainbow trout, in IHC with MAb (EURO) 13: A. Gills (x100); B. Heart (note the non-specific blue coloration of several red blood cells (RBC) (x100); C-C'. *Tunica externa* of the swim bladder (x100).

3.2.2. Immunohistochemistry on challenge samples

Two MAbs [(Euro) 13 and (Japan) 1B3] were used in an IHC test to screen the tissue sections obtained from the bacterial challenge carried out during this study (section 2.5). Examples of the staining obtained with these MAbs in IHC with different organs can be observed in Figure 3.4. It was possible to detect the bacteria in different organs, such as the brain, connective tissue surrounding the pyloric cecae, eye, gills, kidney, liver, spleen, and swim bladder.

Some of the sections presented an apparent diffusion of the blue staining. This could be related to the MAbs recognizing ECP of the bacteria, rather than Ags located on the bacterial surface. An example of this is shown in Figure 3.3

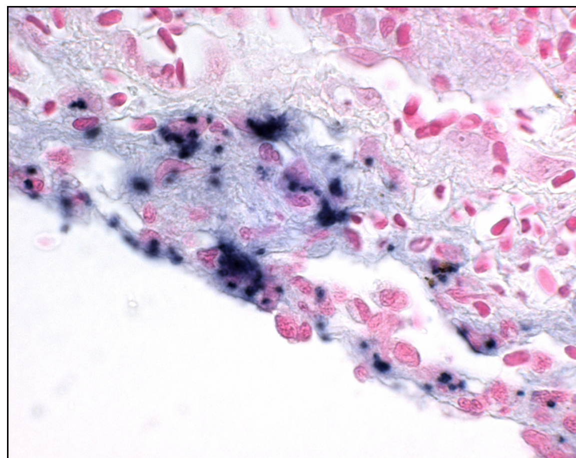

Figure 3.3. IHC positive reaction to *Lactococcus garvieae* NCIMB 702928 in spleen of rainbow trout. (Note that the blue colour tends to be diffused, rather than outlining the contour of the bacteria) (IHC, x 400).

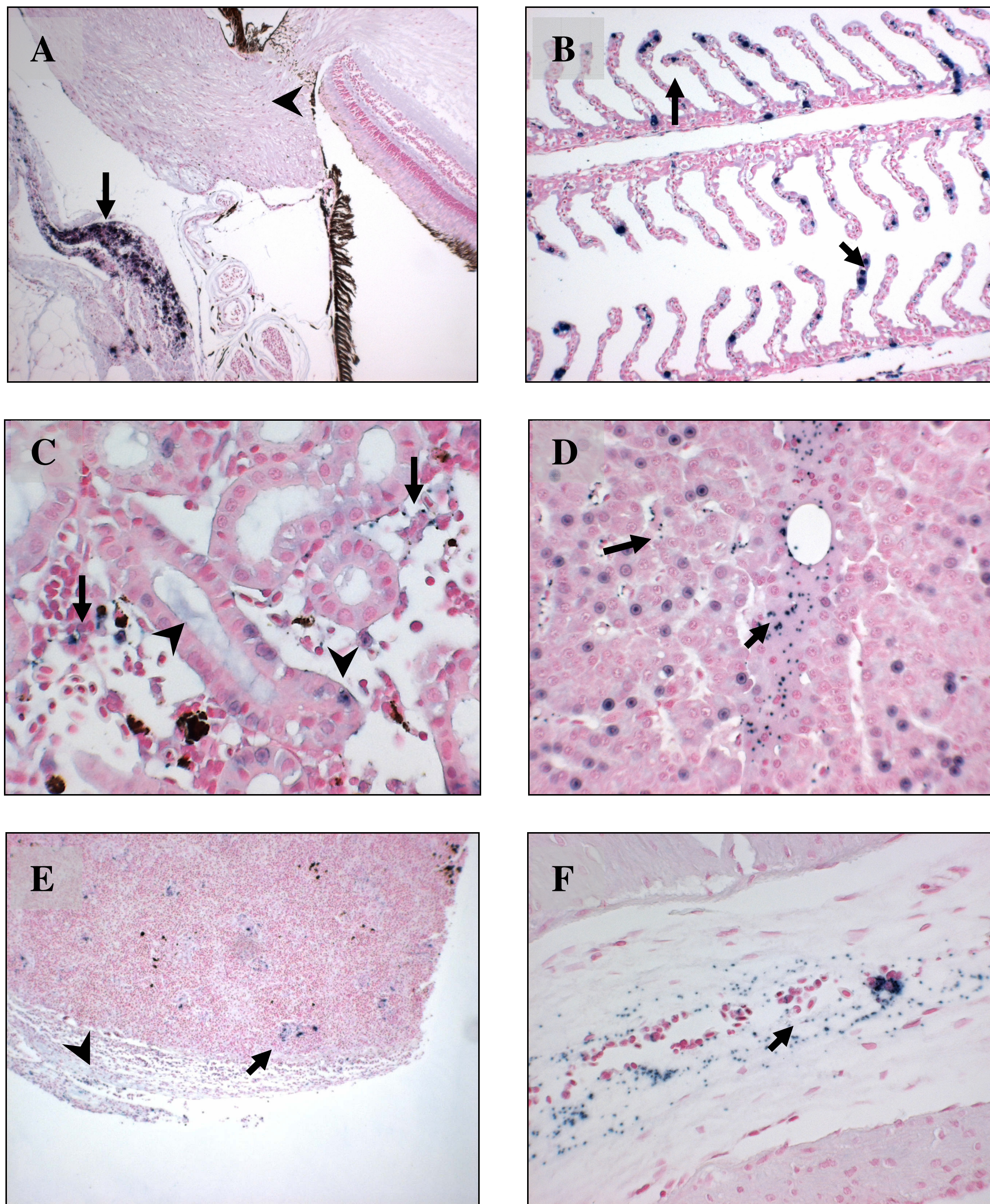

Figure 3.4. IHC with Euro 13, on tissue sections from a challenge with *Lactococcus garvieae* NCIMB 702928 on rainbow trout:

A. Bacteria located in inflammation area (arrow) adjacent to neural papilla of eye (arrowhead)(x200)

B. Bacteria located in lamellar blood sinuses of a trout in a septicemic state (arrow) (x200)

C. Bacteria in interenal tissue (arrow). Note the unspecific coloration displayed by some tubular lumen and tubular cells nuclei (arrow head) (x400)

D. Bacteria in liver parenchyma and sinusoids (arrow) (x400).

E. Bacteria in spleen parenchyma (arrow), and capsule (arrowhead) (x100)

F. Bacteria colonizing the *tunica interna* of the swim bladder (arrow) (x400).

3.3. CHALLENGE RESULTS

3.3.1. Clinical examination.

During the course of the challenge, the clinical examination carried out on each of the sampled fish was recorded (Table 3.3).

Table 3.3. Findings of clinical examination on *Lactococcus garvieae* NCIMB 702928 challenged rainbow trout.

Apparently asymptomatic:	11/30 *
Darkening of the skin (not reliable)	2/30
Lightening of skin (not reliable)	7/30
Lethargy	7/30
Clear nervous signs	1/30
Poor body condition	1/30
Intracranial oedema	7/30
Bilateral exopthalmos	1/30
Unilateral exopthalmos	7/30
Clouding of the cornea	12/30
Intraocular haemorrhage	4/30
Gill pallor	6/30
Haemorrhagic cavity content	4/30
Fluid in cavity	4/30
Distended abdomen	4/30
Anus inflammation	3/30
Congestion	7/30
Watery gut contents	5/30
Splenomegaly	10/30
Hepatomegaly	5/30
Liver lesions	3/30
White multifocal areas in kidney	4/30
Pericardial lesions	2/30
White multifocal areas in heart	2/30
Density of blood very reduced	3/30
Swim bladder hyperplasia	4/30
Slight adhesions	1/30
Gram positive chain forming cocci isolated from kidney	27/30

* No of fish displaying sign/total number of fish

Clinical signs were first detected on the fifth day of the challenge, consisting on unilateral exophthalmos, generalized congestion of the internal organs and mild hepatomegaly and splenomegaly.

A consistent feature found in infected trout was the presence of exophthalmos, which was unilateral for a higher number of trout. This finding was usually accompanied with clouding of the cornea, which became more frequent and severe as the days progressed, becoming ulcerated in some cases. Also, intraocular haemorrhage was observed in some cases (all shown in figure 3.5).

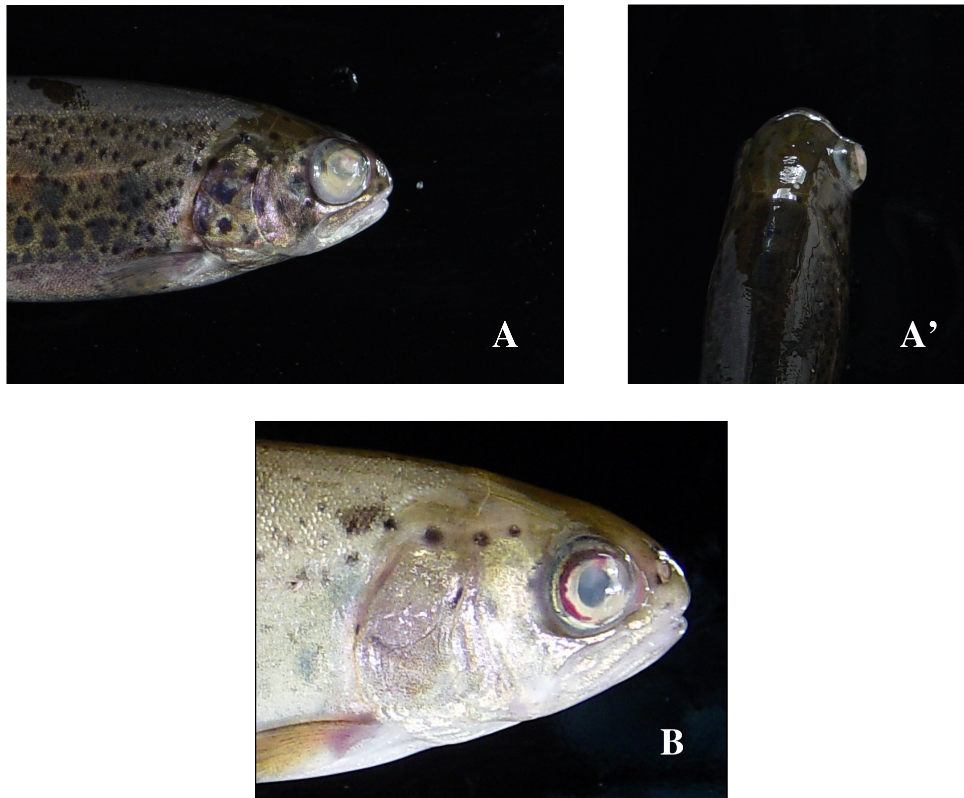

Figure 3.5. Clinical signs of the eye in trout infected with *Lactococcus garvieae* NCIMB 702928: A. Unilateral Exophthalmos with severe clouding of the cornea. [(A) Lateral and (A’) dorsal view]. B. Rainbow trout showing intraocular hemorrhage, a typical sign of Lactococcosis.

Darkening or lightening of the skin was difficult to observe, due to the trout being kept in a room with lighting that did not allow an adequate observation of the fish in the tank. Only in cases where the change in skin became very apparent it was recorded. Lightening of the skin, as well as lethargy, seemed to correlate with moribund fish.

The nervous signs observed consisted of disorientation and unability to maintain a normal posture. The trout affected adopted a sinusoidal posture that made normal swimming very difficult, tending to lie on one side. This observation was recorded on the last day of the challenge (Day 25).

Gill pallor was observed, correlated with a decrease of the number of red blood cells (RBC) observed in the blood smears. Increased number of erythroblasts could be observed.

Internally, most affected trout had an accumulation of fluid in the abdominal cavity, which ranged from clear to blood tinged. When this accumulation was severe, abdominal distension was observed. Generalised congestion was a consistent feature, frequently combined with haemorrhaging in various locations with different severities, mainly petechiae in the abdominal fat surrounding the pyloric cecae and multifocal haemorrhages in the liver. Hepatomegaly and splenomegaly were also frequent. Some of the signs described can be observed in Figure 3.6.

Some of the trout presented dilation of the intestine, with a yellowish content.

Infection of the serosal surfaces, including swim bladder and pericardium was frequently observed, first detection being on day 10. On day 21 of the challenge, one of the trout sampled presented slight adhesions of the viscera to the abdominal wall in the hepatic region.

Only two moribund trout were obtained during the course of the trial (on day 8 and day 14 of the challenge. Both moribund trout showed all the signs described above.

Figure 3.6. Gross pathological appearance of a rainbow trout infected with *Lactococcus garvieae* NCIMB 702928. Features that can be observed are hepatomegaly, splenomegaly, generalized congestion and haemorrhage (especially petechiae on digestive system and hemorrhagic areas in liver), with presence of hemorrhagic ascitic fluid. Exudate around heart can also be observed.

3.3.2. Histopathological description

The blood smears taken from the challenged trout were Gram stained. Most of the trout affected showed an apparent increased number of erythroblasts, and some individuals had drastically reduced number of RBC, a finding that was correlated with gill pallor (haemolytic anaemia, supported by the increased number of erythroblasts and hyaline accumulations in renal tubules). Gram positive streptococci were also detected in blood smears of several infected trout, at low numbers and forming very short chains (diplococci), with a tendency to attach to the RBC surface. High numbers of bacteria were only detected in moribund trout and very infected individuals (Figure 3.7).

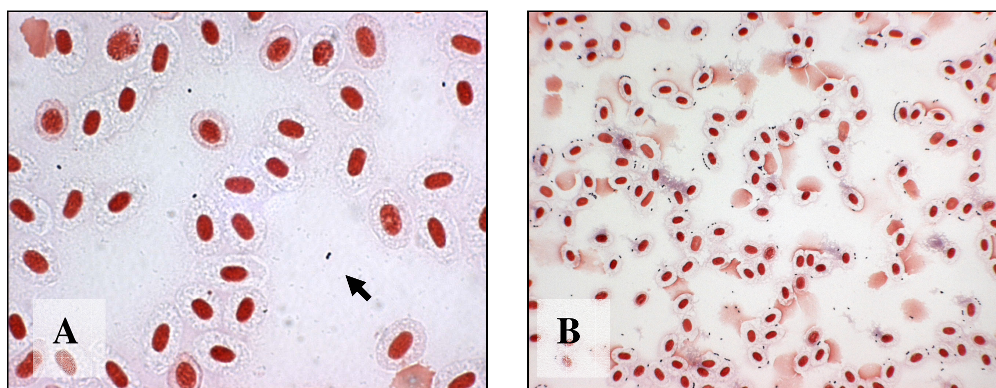

Figure 3.7. Gram stained blood smears from a Rainbow trout experimentally infected with *Lactococcus garvieae* NCIMB 702928: A. Trout sampled on Day 2 post-injection. The smear shows sparse diplococci (arrow) amongst RBC (Gram, x1000). B. Mortality 1 (Day 8). Severe septicemic state is observed. In both cases, note the tendency of the bacteria to adhere to the RBC membrane (Gram, x400).

The eye of infected trout presented a severe panophthalmitis, with destruction of the anterior and posterior chambers, in the most severely affected individuals. In these cases, an eosinofilic exudate could be observed in both eye chambers, heavily infiltrated with a mixed inflammatory response. In some cases the cornea was affected by this inflammation in its periphery. Gram stain and IHC showed large quantities of bacteria. The start of this inflammatory process was observed in earlier cases, with inflammation of the connective tissue around the neural papilla and iris base.

Gills were largely unaffected throughout the study, with the exception of trout in a septicemic state, in which a lamellar epithelium mild hyperplasic reaction was observed. Gram and IHC stains showed vast numbers of bacteria within the lamellar blood sinuses.

The brain meninges showed a mild mixed inflammatory response in only a few individuals (3 trout), from Day 10 of the challenge onwards.

Congestion of hepatic sinusoids was a common feature, and brown granules were observed in hepatocytes (perhaps haemosiderin from the hemolysis caused by the bacteria). Degenerated RBC was observed outside the blood vessels, suggesting endothelial damage. Differing degrees of capsulitis could also be observed (Figure 3.8).

Different degrees of inflammatory infiltration could be observed in the swim bladder *tunica interna*, in which bacteria could be detected (Gram and IHC). The most severe cases showed necrosis and loss of structure, with the *tunica externa* being affected as well. Fibrous tissue could often be seen (Figure 3.8).

Both anterior and posterior kidney showed congestion, and in severe cases haemorrhaging. A mobilization of melano-macrophages towards the blood vessels, consistent with a toxemic condition was observed, and a left shift (increase in neutrophil proliferation, Prof. Hugh Ferguson, personal communication) could be noted in localized areas of the interenal tissue. This is consistent with an organic active infection.

In the most severe cases, the spleen showed generalised loss of structure and necrosis of the elipsoids, with a massive bacterial invasion (Gram and IHC). In less affected trout, congestion and isolated bacterial colonies could be observed. Differing degrees of capsulitis were also noted (Figure 3.8).

Severe pericarditis was another feature of severely affected trout, with sporadic myocardium necrosis (compact and spongy layer), although in most cases, the heart looked normal. An increased macrophagic activity of endocardial cells was a feature in septicemic trout (Figure 3.9).

Peritonitis was usually correlated with congestion and petechial haemorrhages in the peritoneal area. Bacteria were spotted in the Islets of Langerhans (Prof. Hugh Ferguson, personal communication). Destruction of the pyloric cecae mucosa was also observed. Some of these signs are shown in Figure 3.9.

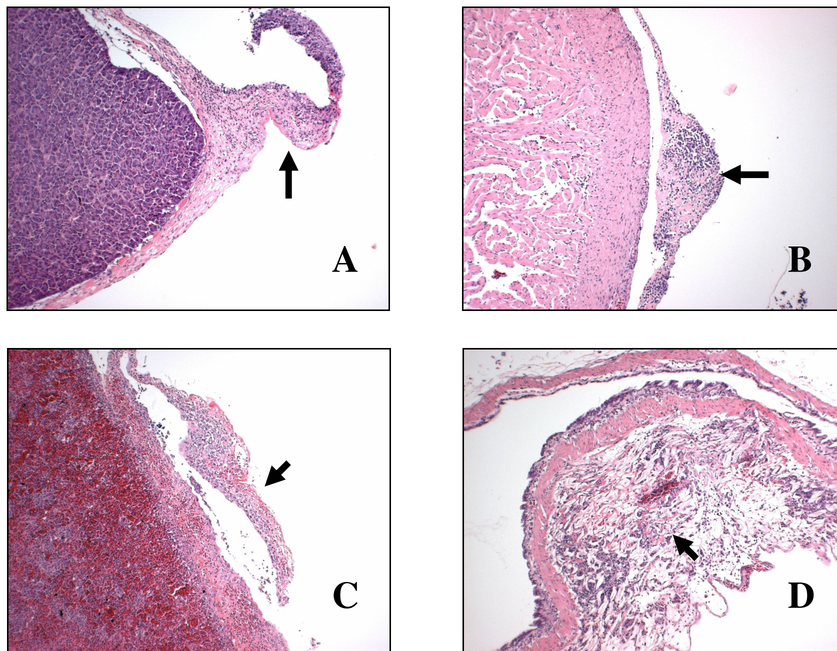

Figure 3.8. Diferent inflammatory processes in serosal surfaces in *Lactococccus garvieae* NCIMB 702928 infected trout (arrow): A, Hepatic capsulitis; B, Pericarditis; C, Spleen capsulitis; D, Inflammation of the *tunica interna* of the swim bladder (All images, x200).

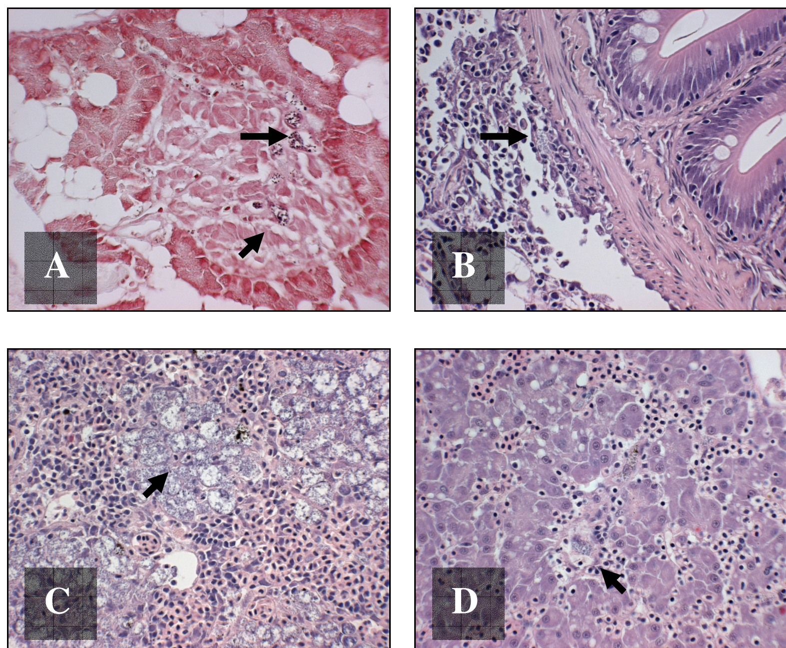

Figure 3.9. Some of the histopathological signs observed in rainbow trout infected experimentally with *Lactococcus garvieae* NCIMB 702928:

A. **Bacterial colonies in islets of Langerhans of the pancreas (arrow) (Gram, x 400).**
B. **Peritonitis and bacterial colonies in the surface of pyloric cecae (arrow) (H&E, x 400).**
C. **Necrosis and destruction of ellipsoid structure in spleen, with uncontrolled bacterial proliferation (arrow) (H&E, x400).**
D. **Congestion of hepatic sinusoids, vacuolar degeneration of hepatocytes, and bacterial colonies (arrow) (H&E, x 400).**

The Gram stains, as well as the IHC techniques applied to the slide samples, helped in many occasions to locate the bacteria within the tissue sections. Bacteria were first localized in Day 2 post-injection, using IHC with (Euro) 13, at very small numbers.

3.3.3. Bacteriology

Gram positive streptococci were recovered from kidney smears of 27 fish of 30. Appearance of the colonies on the Agar plate, as well as a microscopic image of the bacteria can be observed in Image 3.10.

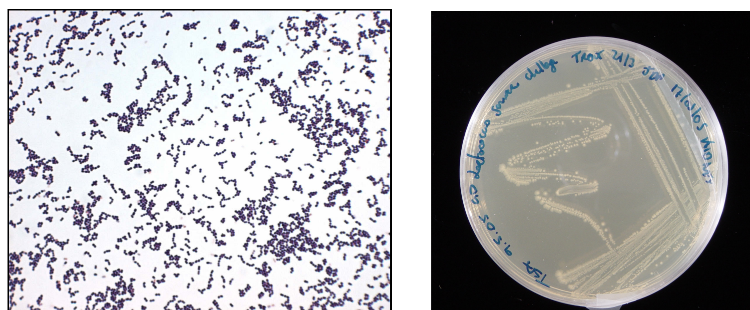

Figure 3.10. A. Microscopic isolate of Gram positive streptococci isolated from kidney of trout infected with *Lactococcus garvieae* NCIMB 702928 (Gram, x400); B. Image of Petri dish with bacterial colonies of streptococci isolated from kidney of trout infected with *L. garvieae* NCIMB 702928.

3.3.4. Scanning electron microscopy (SEM)

No fimbriae- like structures could be observed in the images obtained by SEM. Some of the images obtained can are shown in figure 3.11.

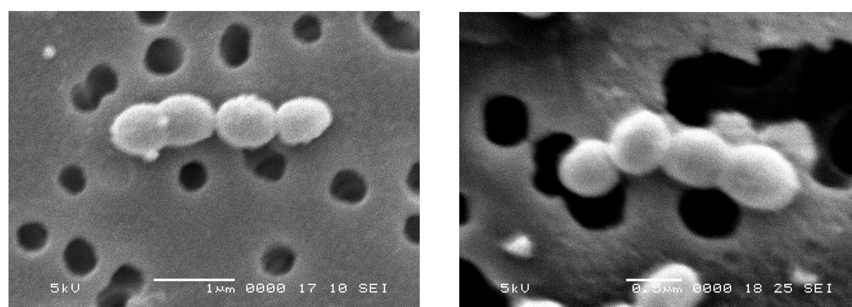

Figure 3.11. Scanning electron microscopy images (SEM) from two *Lactococcus garvieae* strains: A. Occtve (clinical isolate); B. NCIMB 702928.

Chapter 4 DISCUSSION

4.1. MONOCLONAL ANTIBODIES

4.1.1. Characterization studies

The specificity of the MAbs produced in the Aquatic Vaccine Unit, Stirling University, was examined by screening them against various isolates of *L. garvieae*. For this purpose, neat tissue culture supernatant from the hybridoma cell lines producing the MAbs was used. The supernatant was collected after growing the cells for 10 days in culture, and no purification process was carried out, only the hybridoma cells were removed by centrifugation. This fact makes it difficult to effectively compare magnitude of response of one MAb with another, as the MAbs could have been at different concentrations. But this should not have impeded the characterization studies.

All the European MAbs 2, 10, 13, produced against the type strain NCIMB 702928 had very similar patterns of reactivity. They all strongly recognized the same *L. garvieae* isolates, namely NCIMB 702927, 12-99 (Japanese clinical isolate), and the isolate which they were produced against (NCIMB 702928). They did not recognize any of the other *L. garvieae* isolates tested, however. This suggests that the antigen recognized by the MAbs is present on the isolates they were developed to, but not present in the other isolates examined, or that the Ag is not expressed or somehow masked in the strains that are not recognized by these MAbs.

The MAbs provided by Professor Tae Sung Jung (U99-33, GC 2-4-6), were produced against Korean clinical isolates of the bacterium. These MAbs did not recognize any of the *L. garvieae* isolates examined in the study, not even the Korean bacterial strains.

This suggests that either the Ag recognized by these MAbs were not present in the *L. garvieae* isolates examined (or hidden or not expressed), or that perhaps the hybridoma cell lines stopped producing MAbs, and there is no MAb in the supernatant used, although there was evidently some cross-reaction of MAb U99-33 with *R. salmoninarum*, suggesting that this is probably not the case. Purification and determination of the MAbs concentration would help to clarify this issue.

The MAbs produced against Japanese isolates, on the other hand, offered very interesting results. All of them behaved very similarly to the European MAbs recognizing the type strains NCIMB 702928 and 702927 and isolate 12-99, but some of them also recognized other bacterial strains at a lower level of reactivity. This was the case with MAbs 8B12, 3G9, 11F8 and 11B1, which recognised the type strain NCIMB 702155, as well as isolate Reve 2, at a lower level. MAbs 8B12, 11F8 and 11B1 also reacted with Reve 4 and Milze 2, and MAbs 3G9, 11F8 and 11B1 recognized the Occtve isolate. This is an interesting and complicated picture; the pattern of reactivity is not homogeneous. MAb 3G9 does not seem to fit in the pattern of reactivity expressed by MAbs 8B12, 11F8 and 11B1, as it does not recognise the Italian clinical isolates Reve 4 and Milze 2. Perhaps the reason behind the heterogeneity shown by MAb (Japan) 3G9 is that it recognizes a completely different Ag to the one that MAbs 8B12, 11F8 and 11B1 recognize, or perhaps a lower concentration of MAb 3G9 in the supernatant is the reason for the differences observed. Japanese MAbs that recognize Occtve do so at very low levels; this may be due to a lower level of expression of the Ag by this isolate.

The differences observed between the Japanese MAbs which recognized a larger number of strains, and the European MAbs, could be due to the Japanese MAbs recognizing completely different Ags in comparison to the ones recognized by the European MAbs. This Ag may be expressed strongly in isolates NCIMB 702928, NCIMB 702927, and 19-99 (Japan) and also be present on the Italian isolates at a lower level of expression or masked.

Another possibility is that the Japanese MAbs specifically recognise an epitope expressed by NCIMB 702928, NCIMB 702927, and 19-99 (Japan), but also recognise another epitope present in the Italian strains and NCIMB 702155 which is similar to the first epitope, but is not so strongly recognised (the antibody presents a lower avidity for this epitope, but recognises it due to its similarity to the first one). This type of interaction in which a single Ab express a lower avidity for other epitopes that present a similar but not identical determinant to the one presented for their specific epitope has been previously described (Roitt, 1997)

None of the MAbs recognized the Korean isolates (Namhe 7 and Namhe 13). These Korean isolates could present a different antigenic profile altogether, although at the present moment it is not possible to know.

The cross-reactivity results with other non-*L. garvieae* species were satisfactory for all the MAbs with the exception of MAb (Korea) U99-33. This MAb cross reacts with *R. salmoninarum*, and therefore, its use as a tool for specific detection of *L. garvieae* is not recommended.

In conclusion, none of the MAbs studied reacted with all the *L. garvieae* isolates, although some of the Japanese MAbs studied (more specifically MAbs 11B1, 11F8, 8B12, and 3G9) recognized a larger number of *L. garvieae* isolates than the rest of the MAbs.

More research on the bacteria, MAbs and nature of the Ags is necessary if we want to expand our knowledge of *L. garvieae* to a level that allow us to develop a globally effective immunologically based diagnostic test.

4.1.2. Further studies relating to the ELISA

Firstly, the identity of all the *L. garvieae* strains included in further study should be confirmed, as there is still much confusion over the identification and classification of *L. garvieae*. To do this, species specific primers (Aoki *et al.* 2000) could be used in PCR, and the results compared with the biochemical profiles of the bacteria. Perhaps some of the field isolates used in this study have been mistyped, as biochemical analysis can lead to confusion with other bacteria (Domenech *et al.* 1996, Zlotkin *et al.* 1998). This could be potentially be the case with all the clinical isolates used in the study, and perhaps they are not recognized by the MAbs because they are from different bacterial species.

Different culture conditions should also be evaluated, as *L. garvieae* isolates can present different biochemical profiles, when cultured in different media. Blood Agar (BA) has been proposed to reduce biochemical variation (Ravelo *et al.* 2001). Perhaps a specific culture medium may allow more bacterial strains to express the Ag specific to the MAb. The influence of the number of passages after isolation should also be evaluated, as perhaps the antigenicity of the bacterium is reduced with each passage.

The pathogenicity of *L. garvieae* to giant freshwater prawn is reduced with the number of passages and varies with the culture medium (Sung & Sun, 2002). Perhaps the Ag recognized by the MAbs is related to the pathogenicity of *L. garvieae* and its expression varies depending on the aforementioned factors. This influence could be acknowledged by challenging fish after the culture *in vitro* of the bacteria and checking for any changes in antigenicity.

Transmission electron microscopy studies using specific immunological staining procedures, such as immunogold staining (Morris *et al.* 1997) would allow us to learn about the surface properties of the strains involved in the study. This could provide useful information about the nature of the Ag. These methodologies succesfully have been used to locate capsular and non-capsular antigen in *L. garvieae* isolated from Yellowtail (Okada, 2000), and would allow us to know what the location of the antigens recognized by the MAbs is and the possibility of them being masked by other structures (i.e. the capsule). Perhaps the Ag is located on the fimbriae-like structures described for this species (Ooyama *et al.* 2002; Hirokawa *et al.* 2004). These structures are not displayed by all the isolates. Perhaps they are in fact capsular Ags, not being present in non-capsulated strains. These Ag could be present on the bacterial cell surface or could be part of the bacterial ECPs. This is not incompatible with the observation of diffuse staining in the IHC sections of this study (Section 3.2.2). *L. garvieae* has been reported to have proteins that are only expressed *in vivo*, and not *in vitro* (Schmidtke *et al.* 2003). This could have an influence in the study if one of these proteins is antigenic for the MAbs, which would recognize the bacterium only if the protein was present.

Perhaps a competition ELISA between the Japanese and the European MAbs would clarify if they are recognizing the same or a different Ag. This assay is based on the use of two MAbs in the same well, in a way that one will block the other if both react with the same Ag, and no blocking will occur if each MAbs recognizes a different Ag.

The purification and a standardization of the concentration of MAbs used would enable the MAbs to be directly compared. Western blot studies combined with SDS-PAGE would elucidate which Ags are being recognized by each MAb.

There is also a lack of knowledge about the relation between the serotypes described for European isolates of *L. garvieae* (Eyngor, 2004) and the described serotypes in Asia (Kitao, 1982). Perhaps a correlation can be made between both serotyping systems and a connection might be found. RFLP studies suggested a close relationship between Japanese and Italian isolates (Eldar *et al.* 1999), and this could be reflected in the antigenic profile of these two populations, although further immunological studies of these two populations are required in order to understand the mechanisms behind this possible connection.

Further studies on the antigenic profile of the bacteria would perhaps lead to the discovery of a common Ag to all *L. garvieae* isolates that can be recognized by a single MAb specific to *L. garvieae*. This MAb could be used globally for the detection of this bacterial species. If, in the other hand, there is a heterogeneous distribution of the antigenic profile of this species with the inexistence of a common specific Ag, an Ag common to a group of isolates would have to be found for each one of all existing groups of *L. garvieae* isolates. MAbs could be developed against each one of these Ags, and a MAb cocktail used that would recognize all the groups, therefore detecting all *L. garvieae* isolates worldwide.

4.2 IMMUNOHISTOCHEMISTRY

The results observed in the IHC protocol between the different Abs correlated with the results obtained in the ELISA. Therefore, a MAb that recognizes all *L. garvieae* isolates in the ELISA assay would very probably also recognize these different isolates of *L. garvieae* in tissue sections from diseased fish. On the other hand, a MAb that does not recognize a specific *L. garvieae* isolate in the ELISA assay will be unlikely to detect this isolate in infected fish tissue sections. The differences observed in intensity of the signal obtained could be due to different concentration of the MAbs in the supernatant.

There are various other chromogen substances that can be used in IHC such as 3, 3' di-amino benzidine (DAB), for example. Each of the different chromogens exhibit different properties, which should be evaluated. This would also be the case of the counterstains which differentiate the tissue from the stained pathogen.

Direct IHC, avoiding the use of a conjugate Ab, by labelling the primary Ab with an enzyme, could be also considered, as it decreases the time necessary to do the assay. Also, different concentrations of purified MAb should be evaluated, in order to find the optimal concentration of MAb. Non-specific background reactions due to endogenous peroxidase activity should always be blocked using 3% (v/v) hydrogen peroxide in methanol for a minimum period of 10 min. This time should be extended if non-specific background was obtained. The results obtained suggest that IHC could be used successfully as a diagnostic tool for *L. garvieae* if a MAb that recognizes all *L. garvieae* isolates was developed, as the results obtained with the ELISA correlate with those ones obtained in IHC. In the absence of cross-reactivity with related species, it would be useful to differentiate lactococcosis from other streptococcal infections.

4.3 CHALLENGE

The results of the challenge were satisfactory, and it provided a good source of histological material. Perhaps the signs would have been more acute and higher mortalities would have been registered if the water temperature was 5^0C higher than the temperature used of 14^0C, according to Palacios (1993), Ceschia *et al.* (1998) and Eldar & Ghittino (1999). Trout of 100 g have been reported to be able of eliminating the bacteria (Muzquiz *et al.* 1999). In this case, fish of 57 g of average weight were not able to eliminate the bacteria in 25 days, as Gram positive streptococci were isolated from the kidney of challenged trout from Day 2 until the end of the challenge.

Most of the pathological findings correlated with those described by Eldar & Ghittino (1999), with some differences. The most predominant difference was perhaps the low incidence of meningitis observed in the challenged trout, which was always consistent of a mild mixed inflammatory response. This finding contrasts with the observations by Eldar & Ghittino, who describe "acute meningitis, consisting on an exudate covering the brain surface. Within the exudate, colonies of Gram positive cocci were widely distributed". In this case very few bacteria were observed. Perhaps there is a factor (e.g. temperature?) that modulates the presentation of this clinical sign.

An interesting finding was the tendency of *L. garvieae* to attach to the cell wall of RBC. This finding is probably related to hemoagglutinating properties of the bacteria.

Other observation of interest is the presence of bacterial colonies on the Islets of Langerhans. Perhaps this bacterial infection could have endocrine repercussions on the fish.

Chapter 5. CONCLUSIONS

At the present moment, our knowledge of the antigenic profile of this bacterium, on a world wide level, is insufficient to enable us to produce a MAb or a MAb cocktail that recognizes all *L. garvieae* isolates. Further work on the antigenic profiles and possible antigenic similarities between Asian and European isolates is necessary. This study has shown that MAbs produced against a Japanese isolate were able to recognize Italian isolates at a lower level. The mechanisms behind these differences should be studied.

The differences observed between Japanese and European MAbs of the study, with regards to what Ag is being recognized should also be studied. Korean MAb GC99-33 is not recommended for future studies, as it shows cross-reactivity with other bacterial species. Perhaps further studies could be done with Korea GC 236, as it did not record any cross-reactivity in the present study, although the fact that Korean isolates were not recognized by Korean MAbs, raises doubts about the specificity of these MAbs, the identity of the Korean isolates, or the presence of MAbs in the hybridoma culture supernatant used in the study. With the exception of GC 99-33, the rests of the MAbs in the study did not show significant cross-reactivities with any of the other bacterial species in the study. Therefore they should be considered specific to *L. garvieae*

The IHC test developed in this study worked successfully in the detection *L. garvieae* in tissue sections from infected fish. The results obtained correlated with the ones obtained in the ELISA assay. This suggests that the IHC will recognize only the strains that are positive in the ELISA assay with a specific MAb, and the opposite. It will be only as specific as the MAb is, but will work successfully in the detection of *L. garvieae* isolates for which the MAb is specific.

The challenge carried out in this study allowed the collection of histological samples to use with IHC, and also to describe briefly the pathology produced by *L .garvieae* in experimental conditions. Perhaps future challenges should be carried out at higher temperatures (17^0C or more), in order to mimic more effectively the farm conditions present when this type of infection is common.

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APPENDIX

Histology fixative

- 10% neutral buffered formaline

Sodium dihydrogen phosphate (monohydrate)	$4.0g \cdot l.^{-1}$
Di-sodium hydrogen phosphate (anhydrous)	$6.5g \cdot l.^{-1}$
Formaldehyde 40%	100 ml
Distilled water	900 ml

Stains and staining buffers

- Mayer's Haematoxilin

Haematoxylin	1 gr
Sodium Iodate	0.2 g
Potassium alum.	50 g
Citric acid	1 gr
Chloral hydrate	50 g
Distilled water	1000 ml

Allow the haematoxylin, alum. and sodium iodate to dissolve overnight in the distilled water (using a pyrex flask). Add chloral hydrate and citric acid.
Place in a stopper flask with cotton wool, then heat to boiling in a fume cupboard. Boil for 5 min. Ready for immediate use.

- 1% Eosin

Eosin yellowish	10 g
Distilled water	1000 ml

Dissolve the eosin in the water. Leave overnight before use.

- Putt's Eosin

Eosin	4 g
Potassium dichromate	2 g
Saturated aqueous picric acid	40 ml
Absolute alcohol	40 ml
Distilled water	320 ml

Dissolve the eosin in the distilled water, add the potassium dichromate, and add the absolute alcohol. Finally, add the priced acid carefully.

- 1% Acid alcohol

Methylated spirit	1000 ml
Hydrochloric acid	10 ml

Measure the methylated spirits into a Winchester bottle. Carefully add the hydrochloric acid.

- Scott's tap water substitute

Sodium bicarbonate	3-5 g
Magnesium sulphate	20 g
Distilled water	

Dissolve by heating if necessary. Add several crystals of thymol in order to preserve.

- Crystal violet solution

Crystal violet	2 g
95% Ethanol	20 ml
Ammonium Oxalate	0.8 g
Distilled water	300 ml

Dissolve the dye in ethanol, the ammonium oxalate in the water, and mix the two solutions together. Filter before use. The stain is sTable for 2-3 years.

- Gram's iodine

Iodine crystals	1 gr
Potassium Iodide	2 g
Distilled Water	300 ml

As Iodine is more readily soluble in a strong solution of potassium iodide, dissolve the potassium iodide in 5ml of water, and dissolve the iodine in this. Dilute to 300ml for Gram's iodine.

- 1% aqueous neutral red

Neutral red	1 gr
Distilled Water	100 ml

Dissolve the neutral red in the water and filter before use.

- Safranin

Safranin	2.5 g
Absolute ethanol	100 ml
Distilled water	900 ml

Dissolve the safranin in the ethanol and add distilled water.

Immunohistochemistry buffer

- Tris buffered saline

0.05M trisma base	2.42 g · $l.^{-1}$
0.15M Na Cl	29.24 g · $l.^{-1}$
Distilled water	1000 ml

Adjust pH to 7.2 using concurrent HCl.

ELISA buffers

- Coating buffer

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled water	1 l

Adjusted to pH 9.6. Made fresh.

- Low Salt Wash buffer (LSW)

Trisma base	24.2 g
NaCl	222.2 g
Tween 20	5 ml
Distilled water	1 l

Adjusted to pH 7.3 with concurrent HCl. Dilute 1/10 before use.

- Phosphate Buffered Saline (PBS)

$NaH_2PO_42H_2O$	0.876 g · $l.^{-1}$
$Na_2HPO_42H_2O$	2.56 g · $l.^{-1}$
Na Cl	8.77 g
Distilled water	1 l

Adjusted to pH 7.2 using concurrent HCl

- High Salt Wash buffer (HSW)

Trisma base	24.2 g
NaCl	292.2 g
Tween 20	10 ml
Distilled water	1 l

Adjusted to pH 7.7 with concurrent HCl. Dilute 1/10 before use.

- <u>Conjugate Ab buffer</u>

Add 1 g of Bovine Serum Albumine to 100 ml of PBS

- <u>Substrate buffer</u>

Citric acid	21.0 g
Sodium Acetate	8.2 g
Distilled water	1 l

Adjust to pH 5.4 using 1M NaOH. Store 4^0C.

- <u>Conjugate buffer</u>

Add 1 g of BSA to 100ml of Low Salt Wash buffer.

- <u>Substrate</u>

3'3'5'5'-Tetramethylbenzidine dihydrochloride (TMB) (42mM) was added to 1:2 acetic acid: distilled water.
Prepare Acetic acid solution by adding 2 ml of glacial acetic acid to 4 ml water
42mM TMB dihydrochloride-0.07896g/6ml (0.01316/ml).
Cover in foil and store at 4^0C.
150µl of this solution was added to 15 ml substrate buffer and 5µl H_2O_2.

- <u>Stop reagent</u>

2M H_2SO_4 in distilled water- Add 10ml of concurrent H_2SO_4 to approximately 70 ml of water, once cooled make up to 90 ml. (Never add water to acid, always the opposite)

<u>Histology: Processing schedule</u>

• *Dehydration*	50% Methylated spirit, 30 min 80% Methylated spirit, 1 hr 30 min 3· (100% Methylated spirit, 1 hr 30 min) 100% Ethanol, 1hr 45 min 100% Ethanol, 1hr 30 min
• *Clearing*	Chloroform, 50 min Chloroform, 50 min
• *Wax impregnation*	Molten wax, 1 hr 45 min 2· (Molten wax, 1 hr 30 min)

Bacterial culture media preparation

- Selective Kidney Disease Broth (Austin *et al.* 1983).

Distilled water	450 ml
Tryptone T (OXOID, LTD., Basingstoke, Hampshire, England)	5 g
Yeast Extract (OXOID)	0.25 g
L-Cysteine Hydrochloride (SIGMA)	0.5 g
Cyclohexamide(SIGMA)	0.025 g.

Adjust pH to 6.8. Autoclave at 120^0C for 20 minutes. Add sterile solutions shown bellow after autoclaving, and ensure solutions are at room temperature.

Heat inactivated foetal calf serum(FCS) (SIGMA)	50 ml
Polymyxin B sulphate (SIGMA)	0.0025% w/v
Oxolinic acid (SIGMA)	0.00025% w/v
D-cycloserine (SIGMA)	0.00124 % w/v

Antibiotics are dissolved in distilled water and filter sterilised through 0.45 µm pore size filter.

- Cytophaga Broth (Modified from Anaker & Ordal)

Distilled water	1 ltr
Tryptone	5.0 g
Yeast extract	0.5 g
Sodium acetate	0.2 g
Beef extract	0.5 g

Adjust to pH 7.2 with concurrent NaOH. Autoclave at 120^0C for 20 minutes

- Tryptone soya agar (TSA):

Add 40 grams of already prepared media (Oxoid CM 0131) to one litre of distilled water, dissolve and autoclave at 120^0C for 20 minutes. Allow to cool down in a water bath at 45^0C and pour onto Petri dishes.

- Tryptone Soya Broth (TSB):

Add 30 grams of already prepared media (Oxoid CM 129) to one litre of distilled water, dissolve and autoclave at 120^0C for 20 minutes.

CHEMICALS USED:

Acetone	Fisher Scientifics A/0560/21
Casein (Skimmed milk)	CADBURY'S Marvel
Citric Acid	BDH 100813M
Congugate Ab (goat anti-mouse) Ig G HRP	SAPU S254-201
Contrast red ™	Kirkegaard Perry Laboratories
Ethanol	FisherScientificsM /0650DF/21
Glutaraldehyde	SIGMA G6403
Hydrogen Peroxide	SIGMA H1009
H_2SO_4	Fisher Scientifics BDH 10276
Methanol	Fisher Scientifics M/3950/21
Na_2CO_3	SIGMA S-2127
$Na_2HPO_42H_2O$	BDH 4575K
$NaH_2PO_42\ H_2O$	BDH 310324
$NaHCO_3$	SIGMA S-6014
Pertex™	
Poly-L-Lysine	SIGMA P8920
Serum (goat)	SAPU S028-220
Sodium Acetate	BDH 30104
Sodium Chloride	SIGMA S9625
3'3'5'5'-Tetramethylbenidine dihydrochloride (TMB)	SIGMA T-8768
Trisma base	SIGMA T1503
True Blue™	Kirkegaard Perry Laboratories
Tween 20	SIGMA P-1379
Xylene	Fisher Scientifics X/0200/21

Figure 3 1. Response of anti-*L.garvieae* Monoclonal Antibodies against several strains of *L. garvieae* in an indirect ELISA for particulate Antigens.

***L. garvieae* strains**	**Monoclonal Antibodies**										
	(Euro) 2	*(Euro) 10*	*(Euro) 13*	*(Japan) 8B12*	*(Japan) 3G9*	*(Japan) 11F8*	*(Japan) 11B1*	*(Japan) 1B3*	*(Japan) 1A2*	*(Korea) U99-33*	*(Korea) GC246*
NCIMB 702927	75.1	101.5	85.0	93.5	102.7	101.8	89.3	130.2	100.6	-	-
NCIMB 702155	-	-	2.6	29.0	27.5	37.8	30.8	5.1	4.3	-	-
12-99 (IoA)	87.9	96.7	103.4	103.7	112.9	95.9	78.7	121.3	108.0	-	-
Reve 2 (IoA)	-	0.7	2.3	15.5	22.1	14.9	18.4	4.8	9.7	-	-
Reve 3 (IoA)	-	-	1.5	14.4	7.5	13.0	11.7	-	1.7	-	-
Reve 4 (IoA)	-	-	-	38.3	18.2	51.7	42.8	-	1.7	-	-
Milze 2 (IoA)	-	-	-	39.0	16.0	42.0	28.3	-	0.0	-	-
Milze 4 (IoA)	-	-	-	11.0	13.0	17.6	14.7	-	1.3	-	-
Occtve (IoA)	-	-	-	15.8	27.9	21.4	29.9	-	0.9	-	-
Namhe 7	0.9	4.2	3.6	6.8	NT	-	9.5	2.2	1.5	-	-
Namhe 13	3.0	5.9	3.5	10.2	NT	4.6	14.1	3.8	5.1	-	-

NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen UK; IoA: Institute of Aquaculture, Stirling, UK. -: Values equal to 0% and lower. No reaction to positive control: Non-specific. Response is the mean of duplicate wells, expressed as a percentage of the optical density obtained by type strain NCIMB 702928 (OD_{450}=0.89-1.33) in the indirect ELISA for particulate Ags; the mean OD_{450} of negative control (Phosphate Buffered Saline) was 0.07-0.17.