Development of methods to determine prevalence of *Flavobacterium psychrophilum* in farm systems

Thesis submitted for the degree of

Doctor in Philosophy

by

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Declaration

I hereby declare that the work and results presented in this thesis were carried out by me at the Institute of Aquaculture, University of Stirling, Scotland. The work presented in this thesis has not previously been submitted for any other degree or qualification. All information from other sources has been acknowledged.

Farah Manji

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Abstract

Flavobacterium psychrophilum (*Fp*), the aetiological agent of rainbow trout fry syndrome (RTFS) and bacterial cold-water disease (BCWD), is responsible for significant mortalities and economic losses in the salmonid aquaculture industry worldwide. Currently, there is no effective commercial vaccine against RTFS available, and the treatment of the disease depends on the oral administration of a range of anti-microbial compounds, some of which have proven ineffective. With unsuccessful disinfection procedures, possibilities of antibiotic resistance developing and no commercial vaccine available, there is an increased need to rapidly detect *Fp* and reduce mortalities in the industry by improving control measures in the farm system.

The aim of this thesis was to investigate possible sources of *Fp* in a rainbow trout fry farm system and to use this data to develop strategies to reduce the prevalence of the pathogen with this farming system. Novel assays to detect *Fp* (loop-mediated isothermal amplification; LAMP), quantify *Fp* (quantitative PCR; qPCR) and to detect the fishes host response to *Fp* (Luminex™) were developed, and then used alongside bacterial culture and nested PCR to determine the prevalence of *Fp* on a commercial fish farm.

Four batches of eggs from 3 different geographic sources were collected on arrival to the farm and tested for the prevalence of *Fp*. Fry from these batches were monitored as they grew and were moved to different sites at the farm. Kidney, spleen and blood were collected at 3 different life stages from the fry, until they were sold for ongrowing by the farm. Water samples from the inlet, outlet and fry tanks were collected at each sampling point. PCR analysis and bacteriology were the two main methods selected for screening the eggs and fry tissue for *Fp*. All sources of eggs were found to be positive for *Fp* with prevalences ranging from 1.1 % - 1.9 % and there was a significant increase in prevalence over time for all 4 batches of eggs ranging from 19.8 % - 34.6 % by the final life stage sampled. There was also a substantial difference in the numbers of fry samples positive for *Fp* depending on whether nested PCR or bacterial culture were used, as well as the organ (kidney or

spleen) tested. This highlighted the importance of sampling both organs rather than just the one. Nested PCR was more sensitive than culture with 13 % of the fry samples reported as *Fp* positive, by sampling both the kidney and spleen collectively, while only 5 % were *Fp* positive by bacteriology.

The levels of *Fp* in all samples could not be quantified by qPCR due to limits in the sensitivity of the assay. For those samples that were quantified at the levels of *Fp* detected by qPCR ranged from 3.38×10^4 well^{-1} - 2.07×10^6 well^{-1} genome copies in egg samples; from 3.38×10^3 well^{-1} – 3.07×10^7 well^{-1} genome copies well^{-1} in tissue samples (spleen or kidney), and from 7.89×10^3 – 7.22×10^4 genome copies well^{-1} in water samples. The sensitivity of the standard curve was limited to 10^3 copies well^{-1} and following optimisation of the assay the annealing temperature was decreased by 1˚C to 62°C to reduce the cross-reactivity to negligible levels, though this reduced the sensitivity of the assay even further to 10^4 copies well^{-1}. The detection limits by qPCR obtained by spiking samples with known amounts of *Fp* were 192 CFU mg^{-1} from egg samples, 184 CFU mg^{-1} from fry tissue samples, and 220 CFU ml^{-1} from water samples,. The sensitivity of the LAMP assay determined by spiking egg, kidney, spleen and water samples was 18 CFU mg^{-1}, 22 CFU mg^{-1}, 25 CFU mg^{-1} and 16 CFU ml^{-1}, respectively. The latter was similar to, though not as sensitive as nested PCR. Nested PCR limits determined by spiking egg, kidney, spleen and water samples were 14 CFU mg^{-1}, 11 CFU mg^{-1}, 13 CFU mg^{-1} and 11 CFU ml^{-1}. No cross-reactivity was found with any bacteria including other *Flavobacterium* species with nested PCR but cross-reactivity with other *Flavobacterium* species were found with both qPCR (1.51 % with *Flavobacterium aquatile* and 0.30 % with *Flavobacterium johnsoniae*) and LAMP. The LAMP assay showed slight cross-reactivity with *Flavobacterium columnare* and *Flavobacterium branchiophilum*.

A novel Luminex™ assay was also developed and optimised, using microspheres coated with *Fp*, to detect antibodies to *Fp* in the serum of the fry. The Luminex™ allowed small volumes of serum from individual fry to be used to evaluate antibody response as an indirect method to determine exposure to and infection by *Fp*. A large number of fry from all 4 batches (88% - 94%) of eggs were

found to contain anti-*Fp* antibodies though it still remains to be determined whether the antibodies were specific to *Fp*.

From the work carried out in this study, it can be concluded that whether eggs are carrying *Fp* inside and/or on their surface, it should be possible to reduce the prevalence of *Fp* in farm systems by regularly screening the broodstock, eggs and fry. Supply of *Fp*-free eggs and milt is essential to reduce the reservoir of *Fp* on farms. Both the qPCR and LAMP assay require further optimisation but they do offer potential for the future screening of *Fp* at farm sites and in the laboratory.

Future control measures for RFTS should include the screening of broodstock and eggs by sensitive methods so that *Fp*-free seed can be supplied to farms. This, alongside effective disinfection procedures, rigorous husbandry practices and future vaccine development will all be required to manage this very significant fish disease.

List of Abbreviations

ANOVA	: Analysis of variance
APS	: Ammonium per sulphate
ATCC	: American type culture collection
BCA	: Bicinchoninic acid
BCWD	: Bacterial coldwater disease
BGD	: Bacterial gill disease
BIP	: Backward inner primer
BKD	: Bacterial kidney disease
bp	: Base pair
BLAST	: Basic Local Alignment Search Tool
BSA	: Bovine Serum Albumin
CEFAS	: Centre for Environment, Fisheries & Aquaculture Science
CFU	: Colony forming unit
CWSD	: Cold water strawberry disease
d	: Day
dH_2O	: Distilled water
DMSO	: Dimethylsulphoxide
DNA	: Deoxyribonucleic acid
DPBS	: Dulbecco's PBS without Ca and Mg
E	: Efficiency of qPCR reaction
ECP	: Extracellular product
EDTA	: Ethylenediaminetetraacetic acid
ELISA	: Enzyme-linked immunosorbent assay
ERM	: Enteric Redmouth
FACS	: Fluorescence-activated cell sorter
FIP	: Forward inner primer
FLP	: Tryptone yeast salt extracts supplemented with glucose
Fp	: *Flavobacterium psychrophilum*
GLM	: Generalized linear model
GP	: Grouper *Epinephelus* spp.
h	: Hour(s)
H_2O_2	: Hydrogen peroxide
HRP	: Horseradish peroxidase
HSWB	: High salt wash buffer
IFAT	: Indirect fluorescent antibody test
Ig	: Immunoglobulin
IHC	: Immunohistochemistry
IHNV	: infectious hematopoietic necrosis virus
INRA	: L'Institut national de la recherche agronomique
IOA	: Institute of Aquaculture, Stirling
i.p.	: Intraperitoneal

IPNV	: Infectious Pancreatic Necrosis Virus
ISAV	: Infectious Salmon Anaemia Virus
kDa	: Kilodalton
l	: Litre
LAMP	: Loop-mediated isothermal amplification
LB	: Luria-Bertani (broth or agar)
LPS	: Lipopolysaccharide
LSWB	: Low salt wash buffer
M	: Molar
MCA	: Modified *Cytophaga* agar
MCV	: Maintenance, calibration and validation plate
ml	: Millilitre
mM	: Millimolar
min	: Minute(s)
MFI	: Mean Fluorescence Intensity
Mouse IgG-PE	: Goat anti-mouse IgG antibody conjugated with phycoerythrin
mqRT-PCR	: Multiplex real-time quantitative RT-PCR
NASBA	: Nucleic acid-based sequence amplification
NCBI	: National Center for Biotechnology Information
NCIMB	: National Collections of Industrial, Marine and Food Bacteria
OD	: Optical density
OIE	: Office International des Epizooties
PBS	: Phosphate buffered saline
PCR	: Polymerase chain reaction
PE	: Phycoerythrin
qPCR	: Quantitative PCR
Rabbit IgG-PE	: Goat anti-rabbit IgG antibody conjugated with phycoerythrin
RAPD	: Random amplified polymorphic DNA
rDNA	: DNA sequence coding for rRNA
rRNA	: Ribosomal RNA
RMS	: Red mark syndrome
RNA	: Ribonucleic acid
RT	: Room temperature
RTFS	: Rainbow trout fry syndrome
RT-LAMP	: Reverse transcription loop-mediated isothermal amplification
RT-PCR	: Reverse transcription PCR
SDS	: Sodium dodecyl sulphate
SDS-PAGE	: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sec	: Second(s)
SGIV	: Singapore grouper iridovirus
SVCV	: Spring viremia of carp virus
TAE	: Tris acetate EDTA
TBN	: TBS with Tween-20
TBS	: Tris buffered saline

TE	: Tris-EDTA buffer
TEMED	: N,N,N',N'-tetramethyl-ethane-1,2-diamine
TTBS	: Tris buffered saline with Tween-20
TYES	: Tryptone yeast salt extracts
UAC	: Universidal Austal de Chile
µl	: Microlitre
µm	: Micrometer
v/v	: Volume per volume
VHSV	: Viral hemorrhagic septicemia virus
VNC	: Viable but non-culturable cells
VNN	: Viral nervous necrosis
w/v	: Weight per volume

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Chapter 1: General Introduction

Historical background and Taxonomy of Flavobacterium psychrophilum

Flavobacterium psychrophilum (*Fp*) is a bacterial pathogen responsible for substantial economic losses in salmonid aquaculture worldwide (Dumetz *et al*., 2008). The pathogen is ubiquitous in the aquatic environment and is endemic in all fish farms in the UK (Mark James, personal communication, Department for Environment, Food and Rural Affairs, London, UK.). The taxonomy of *Fp* has been revised several times over the years resulting in changes in Genus classification of the bacterium.

The bacterium was first isolated in the USA in 1948 from lesions on juvenile Coho salmon (*Oncorhynchus kisutch*) following high levels of mortalities during a disease episode. The bacterium was later named as *Cytophaga psychrophila* in 1960 by Borg (1960).

The pathology and characteristics of the bacterium (retractile with gliding motility) were found to be similar to *Flavobacterium columnare* (Columnaris disease) which had previously been described by Davis in 1946. In contrast to *F. columnare*, Borg (1960) observed the absence of 'swarming' or production of 'needle-like haystacks'. In 1968, Pacha established that the bacteria did not form microcysts and fruiting bodies, and was therefore re-classified in the genus *Flexibacter*. In the late 1980's the bacterium was isolated in Europe for the first time, from diseased rainbow trout in Germany and France (Weis, 1987; Bernardet *et al*., 1988; Lorenzen *et al*., 1991; Rangdale, 1999). Following isolation of *Fp* in Germany by Weis (1987), the bacterium has since been isolated in other European countries: Denmark, (Lorenzen *et al*., 1991); UK, (Santos *et al*., 1992); Spain, (Toranzo and Barja, 1993); Finland, (Wiklund *et al*., 1994); Switzerland and Northern Ireland, (Lorenzen and Olesen, 1997) as well as in Japan, (Wakabayashi *et al*., 1994); Australia, (Schmidtke and Carson, 1995); Chile, (Bustos *et al*., 1995); Korea, (Lee and Heo, 1998); and Canada, (Ostland *et al*., 1999). A full list of the different fish species affected by *Fp* in different geographical locations is given in

Table 1.1. Though predominantly found in salmonids, the bacterium has been isolated from a variety of other non-salmonid fish.

Bernardet and Grimont (1989) compared the American strains to those isolated in France, and found differences in the polysaccharides produced by the bacterium from different geographical locations. The guanine and cytosine content of deoxyribonucleic acid (DNA) in members of the genera *Cytophagacae, Flexibacter* and *Flavobacterium* was also compared and were found to be highly polyphyletic (Holt *et al*., 1989). Realising that the bacteria in these phyla were closely related, several species were regrouped into the *Flavobacterium* genus including *Cytophaga psychrophilum* giving rise to the current nomenclature *Flavobacterium psychrophilum* (Bernardet and Grimont, 1989). Further investigation using the 16S ribosomal Ribonucleic acid (RNA) of the bacterium suggested that *Fp*, *F. columnare* and *Flexibacter maritimus* were closely related, representing a discrete group known as *Flavobacterium* (Bader and Shotts, 1998).

Table 1.1: Geographic distribution and host species of *Flavobacterium psychrophilum* in order of first reported isolation.

Host Species	Geographic location	Reference
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Sockeye salmon (*Oncorhynchus*	USA	Rucker *et al*., 1953
Chinook salmon (*Oncorhynchus*	USA Canada	Rucker *et al*., 1953 Ostland *et al*., 1999
Brook trout (*Salvelinus fontinalis*)	USA Finland	Bullock, 1972 Madetoja *et al*., 2001
Atlantic salmon (*Salmo salar*)	USA Australia Sweden Canada	Schneider and Nicholson, Schmidtke and Carson, 1995 Ekman *et al*., 1999 Ostland *et al*., 1999
Lake trout (*Salvelinus namaycush*)	USA	Schachte, 1983
Cutthroat trout (*Oncorhynchus clarki*)	USA Finland	Holt, 1987 Crump *et al*., 2001
Chum salmon (*Oncorhynchus keta*)	USA	Holt, 1987
Rainbow trout (*Oncorhynchus*	Germany France Denmark UK Italy Spain Finland Chile Northern Switzerland Canada	Weis, 1987 Bernardet *et al*., 1988 Lorenzen *et al*., 1991 Santos *et al*., 1992 Sarti *et al*., 1992 Toranzo and Barja, 1993 Wiklund *et al*., 1994 Bustos *et al*., 1995 Lorenzen and Olesen, 1997 Lorenzen and Olesen, 1997 Ostland *et al*., 1999
Brown trout (*Salmo trutta*)	Japan Norway Finland	Wakabayashi *et al*., 1991 Lorenzen and Olesen, 1997 Madetoja *et al*., 2001
Carp (*Cyprinnus carpio*) & crucian (*Carassius carassius*)	Germany	Lehmann *et al*., 1991
Eel (*Anguilla anguilla*)	Germany	Lehmann *et al*., 1991
Tench (*Tinca tinca*)	Germany	Lehmann *et al*., 1991
Ayu (*Plecoglossus altivelis*)	Japan Korea	Wakabayashi *et al*., 1994 Lee and Heo, 1998
Amago (*Oncorhynchus rhodurus*)	Japan	Furutsuka-Uozumi *et al*.,
Pale chub (*Zacco platypus*)	Japan	Iida and Mizokami, 1996
Masou salmon (*Oncorhynchus*	Japan	Iida and Mizokami, 1996
Steelhead (*Oncoryhynchus mykiss*)	USA	Brown *et al*., 1997
Forktongue goby (*Chaenogobius*	Japan	Amita *et al*., 2000
Lake goby (*Rhinogobius brunneus*)	Japan	Amita *et al*., 2000
Japanese dace (*Trybolodon*	Japan	Amita *et al*., 2000
Arctic char (*Salvelinus alpinus*)	Finland	Madetoja *et al*., 2001
Grayling (*Thymallus thymallus*)	Estonia	Madetoja *et al*., 2001
Sea trout (*Salmo truta*)	Finland &	Madetoja *et al*., 2001

Perch (*Perca fluviatilis*)	Finland	Madetoja *et al*., 2002
Roach (*Rutilus rutilus*)	Finland	Madetoja *et al*., 2002

Characterisation of Flavobacterium psychrophilum

Flavobacterium psychrophilum is a Gram-negative, rod shaped, aerobic bacterium with weak refractile slender rods with branched or rounded ends which have been found to vary considerably in size (Lorenzen, 1994; Holt *et al*., 1993). Cells tend to be 1.5–7.5 μm in length and 0.75 μm in width with a characteristic cheese-like odour (Bernardet and Grimont, 1989; Lorenzen *et al.*, 1997; Austin, 1999). However, some filamentous (10-40 μm) and pleomorphic forms have also been found to exist in broth cultures older than 48 h. Gliding motility is always reported, although sometimes it is very slow and difficult to observe (Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989). The mechanism behind this gliding motility is unknown, however, there are features within the cell envelope that have been associated with the bacteria's ability to move over a solid surface (Dalsgaard, 1993; Martinez *et al.*, 2004). The gliding motility may be due to the polysaccharide slime layer, as displayed by *Flavobacterium columnaris* (Dalsgaard, 1993).

Flavobacterium psychrophilum is an aerobic mesophilic bacterium with optimal growth observed at 15°C, although growth has been found between 5°C and 25°C. The bacterium does not grow at temperatures above 26°C (Pacha, 1968). The optimal temperature for protease production appears to be slightly lower than the optimal growth temperature at around 13.3 ± 1.9°C (Vatsos *et al.*, 2006). A number of researchers have reported difficulty in sub-culturing this fastidious and slow-growing bacterium. One of the most commonly used agars for the identification of *Fp* is modified *Cytophaga* agar (MCA), developed by Anacker and Ordal (1959). Holt *et al.* (1993) reported that the organism could tolerate up to 1 % NaCl and its growth was greater in cytophaga broth supplemented with tryptone yeast extract salts (TYES). *Flavobacterium psychrophilum* forms yellowish colonies on this agar with a thin-spreading appearance, and usually grow 48-96 h after incubation. Cepeda *et al*. (2004) reported more rapid and greater yields of the bacterium when grown

in a new culture medium, FLP medium (TYES medium supplemented with glucose). Recently, Álvarez and Guijarro (2007) developed a modified medium supplemented with aromatic compounds and activated charcoal which improves the recovery of *Fp*. Despite continuous improvements in the culture media used for pathogen isolation, it is not always possible to recover the bacterium from infected fish (Michel *et al*., 1999). This is reported to be due to the presence of viable but non-culturable cells (VNC). When the bacteria were subjected to suboptimal conditions of survival, this resulted in the formation of VNC cells with inconsistent colony forming units (CFU's) depending on the culture media used (Michel *et al*., 1999). Vatsos *et al*. (2003) investigated this further by observing physical changes in *Fp* over a 19 week starvation period in stream and distilled water. A difference was found between the viability obtained from colony forming units (CFU's) and that observed using a Live/Dead viability kit. The culturability of the organism declined until it was no longer possible to obtain colonies on agar plates, but the bacteria could be resuscitated by placing it into modified *Cytophaga* broth.

Serotyping of Flavobacterium psychrophilum

Various strains of *Fp* share common antigens and therefore it has been possible to distinguish the bacterium from other bacteria belonging to the *Flavobacterium* group (including *Cytophaga* and *Flexibacter*) using serological methods (Pacha, 1968; Holt, 1987; Cipriano *et al*., 1996). However, there can be differences in antigens present within the same species and this is referred as different serotypes. In 1987, Holt identified the presence of two or more serotypes in American isolates of *Fp* using absorbed rabbit antisera. In a subsequent study by Wakabayashi *et al*. (1994) three different serotypes (O-1, O-2, O-3) were identified from isolates obtained from diseased fish in Japan and the USA. Lorenzen and Olesen (1997) also examined the serotyping system of *Fp* and confirmed the presence of three different serotypes (Th, Fd and Fp). The serotyping was later re-classified according to a host-dependant serotype system and they identified 7 host-dependant serotypes (1-salmon, 2-trout, 3-trout, 4-eel, 5-carp, 6-tench and 7-ayu); (Mata *et al*., 2002). A different serotyping

system for *Fp* was proposed Izumi *et al*. (2003a) using antiserum against an *Fp* isolate from amago (*Onchorynchus masou rhodurus*) using absorbed antiserum. From their work they suggested the following serotypes; O-1/2, O-1/2/3, O-1/2/4, O-1/2/3/4, O-2/3/4, O-2/3 with the possibility of others being found in the future.

In 2000, Faruk produced rabbit sera against a virulent and a non-virulent isolate of *Fp* and used them to examine the antigenicity of isolates obtained from different geographical regions. Five different groups of bacteria based on their reactivity in the Enzyme-linked immunosorbent assay (ELISA) were reported, although three of the groups (G1, G2 and G5) were more distinct then the other two (G3 and G4). The most recent serotyping system to be proposed by Morgan *et al*. (2007) recognises four serotypes based on the profiles obtained in western blot analysis using 26 isolates screened with a cocktail of the two rabbit anti-*Fp* polyclonal sera prepared by Faruk (2000).

Disease caused by *Flavobacterium psychrophilum*

The pathogen has been associated with several diseases although the diseases are manifest with different clinical signs i.e. mainly bacterial coldwater disease (BCWD) in the USA and rainbow trout fry syndrome (RTFS) in Europe (Holt *et al*., 1993; Rangdale *et al*., 1999). *Flavobacterium psychrophilum* has recently been associated with red mark syndrome (RMS; Ferguson *et al*., 2006). Bacterial coldwater disease (also known as peduncle disease due to the location of lesions which result) predominantly affects juvenile salmonid fish, particularly Coho salmon (*O. kisutch*) and is most prevalent when the water temperature is less than or around 10˚C (Austin and Austin, 1993). In fish of up to 1 year old, the peduncle lesion is the most typical clinical sign of the disease although dark pigmentation and moribund fish are also seen in the latter stages of the infection. In BCWD epizootics, changes in developing bone and cartilage have been observed; these include the entire head region, such as cephalic osteochondritis and necrotic scleritis, the spinal column, and elements of the pectorals and ribs (Ostland *et al*., 1997).

Kent *et al.* (1989) observed osteitis, meningitis and ganglioneuritis in

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Roach (*Rutilus rutilus*)	Finland	Madetoja *et al*., 2002

Characterisation of Flavobacterium psychrophilum

Flavobacterium psychrophilum is a Gram-negative, rod shaped, aerobic bacterium with weak refractile slender rods with branched or rounded ends which have been found to vary considerably in size (Lorenzen, 1994; Holt *et al*., 1993). Cells tend to be 1.5–7.5 μm in length and 0.75 μm in width with a characteristic cheese-like odour (Bernardet and Grimont, 1989; Lorenzen *et al.*, 1997; Austin, 1999). However, some filamentous (10-40 μm) and pleomorphic forms have also been found to exist in broth cultures older than 48 h. Gliding motility is always reported, although sometimes it is very slow and difficult to observe (Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989). The mechanism behind this gliding motility is unknown, however, there are features within the cell envelope that have been associated with the bacteria's ability to move over a solid surface (Dalsgaard, 1993; Martinez *et al.*, 2004). The gliding motility may be due to the polysaccharide slime layer, as displayed by *Flavobacterium columnaris* (Dalsgaard, 1993).

Flavobacterium psychrophilum is an aerobic mesophilic bacterium with optimal growth observed at 15°C, although growth has been found between 5°C and 25°C. The bacterium does not grow at temperatures above 26°C (Pacha, 1968). The optimal temperature for protease production appears to be slightly lower than the optimal growth temperature at around 13.3 ± 1.9°C (Vatsos *et al.*, 2006). A number of researchers have reported difficulty in sub-culturing this fastidious and slow-growing bacterium. One of the most commonly used agars for the identification of *Fp* is modified *Cytophaga* agar (MCA), developed by Anacker and Ordal (1959). Holt *et al.* (1993) reported that the organism could tolerate up to 1 % NaCl and its growth was greater in cytophaga broth supplemented with tryptone yeast extract salts (TYES). *Flavobacterium psychrophilum* forms yellowish colonies on this agar with a thin-spreading appearance, and usually grow 48-96 h after incubation. Cepeda *et al*. (2004) reported more rapid and greater yields of the bacterium when grown

in a new culture medium, FLP medium (TYES medium supplemented with glucose). Recently, Álvarez and Guijarro (2007) developed a modified medium supplemented with aromatic compounds and activated charcoal which improves the recovery of *Fp*. Despite continuous improvements in the culture media used for pathogen isolation, it is not always possible to recover the bacterium from infected fish (Michel *et al*., 1999). This is reported to be due to the presence of viable but non-culturable cells (VNC). When the bacteria were subjected to suboptimal conditions of survival, this resulted in the formation of VNC cells with inconsistent colony forming units (CFU's) depending on the culture media used (Michel *et al*., 1999). Vatsos *et al*. (2003) investigated this further by observing physical changes in *Fp* over a 19 week starvation period in stream and distilled water. A difference was found between the viability obtained from colony forming units (CFU's) and that observed using a Live/Dead viability kit. The culturability of the organism declined until it was no longer possible to obtain colonies on agar plates, but the bacteria could be resuscitated by placing it into modified *Cytophaga* broth.

Serotyping of Flavobacterium psychrophilum

Various strains of *Fp* share common antigens and therefore it has been possible to distinguish the bacterium from other bacteria belonging to the *Flavobacterium* group (including *Cytophaga* and *Flexibacter*) using serological methods (Pacha, 1968; Holt, 1987; Cipriano *et al*., 1996). However, there can be differences in antigens present within the same species and this is referred as different serotypes. In 1987, Holt identified the presence of two or more serotypes in American isolates of *Fp* using absorbed rabbit antisera. In a subsequent study by Wakabayashi *et al*. (1994) three different serotypes (O-1, O-2, O-3) were identified from isolates obtained from diseased fish in Japan and the USA. Lorenzen and Olesen (1997) also examined the serotyping system of *Fp* and confirmed the presence of three different serotypes (Th, Fd and Fp). The serotyping was later re-classified according to a host-dependant serotype system and they identified 7 host-dependant serotypes (1-salmon, 2-trout, 3-trout, 4-eel, 5-carp, 6-tench and 7-ayu); (Mata *et al*., 2002). A different serotyping

system for *Fp* was proposed Izumi *et al*. (2003a) using antiserum against an *Fp* isolate from amago (*Onchorynchus masou rhodurus*) using absorbed antiserum. From their work they suggested the following serotypes; O-1/2, O-1/2/3, O-1/2/4, O-1/2/3/4, O-2/3/4, O-2/3 with the possibility of others being found in the future.

In 2000, Faruk produced rabbit sera against a virulent and a non-virulent isolate of *Fp* and used them to examine the antigenicity of isolates obtained from different geographical regions. Five different groups of bacteria based on their reactivity in the Enzyme-linked immunosorbent assay (ELISA) were reported, although three of the groups (G1, G2 and G5) were more distinct then the other two (G3 and G4). The most recent serotyping system to be proposed by Morgan *et al*. (2007) recognises four serotypes based on the profiles obtained in western blot analysis using 26 isolates screened with a cocktail of the two rabbit anti-*Fp* polyclonal sera prepared by Faruk (2000).

Disease caused by Flavobacterium psychrophilum

The pathogen has been associated with several diseases although the diseases are manifest with different clinical signs i.e. mainly bacterial coldwater disease (BCWD) in the USA and rainbow trout fry syndrome (RTFS) in Europe (Holt *et al*., 1993; Rangdale *et al*., 1999). *Flavobacterium psychrophilum* has recently been associated with red mark syndrome (RMS; Ferguson *et al*., 2006). Bacterial coldwater disease (also known as peduncle disease due to the location of lesions which result) predominantly affects juvenile salmonid fish, particularly Coho salmon (*O. kisutch*) and is most prevalent when the water temperature is less than or around 10˚C (Austin and Austin, 1993). In fish of up to 1 year old, the peduncle lesion is the most typical clinical sign of the disease although dark pigmentation and moribund fish are also seen in the latter stages of the infection. In BCWD epizootics, changes in developing bone and cartilage have been observed; these include the entire head region, such as cephalic osteochondritis and necrotic scleritis, the spinal column, and elements of the pectorals and ribs (Ostland *et al*., 1997). Kent *et al*. (1989) observed osteitis, meningitis and

ganglioneuritis in juvenile coho salmon, infected with *Fp*. Evensen and Lorenzen (1996) also found bacteria/bacterial products in the vicinity of the vertebrae.

Red mark syndrome was first reported in late 2003 and the association of the *Fp* to RMS and coldwater strawberry disease (CWSD) was reported by Ferguson *et al*. (2006). The DNA of the pathogen was isolated from paraffin wax sections from rainbow trout skin lesions and affected fish typically around 500 g sometimes also have spinal deformities. The association of *Fp* with RMS still remains to be confirmed however.

Rainbow Trout Fry Syndrome

The clinical manifestation of *Fp* infection has been known by a variety of names from the different countries including Fry Anaemia, Fry Mortality Syndrome and Visceral Myxobacteriosis although the commonly accepted name in the UK is now RTFS. In 1984, rainbow trout hatcheries around the UK experienced high mortalities associated with RTFS. Trout hatcheries in the UK lose an average of 10 to 30 % of fry stock to this disease, although losses of up to 80% can occur when the fish is more susceptible to the pathogen when water temperatures are 10°C or below (Bernardet *et al*., 1988; Scott, 1989). Recently, Schering-Plough ranked RTFS to be the highest cause of mortality in trout hatcheries in the UK and fifth in Europe (Chris Gould, personal communication, Intervet/Schering-Plough Animal Health, Milton Keynes, UK.). In more recent years, the disease seems to be affecting larger fish and mortality rates of up to 50 % have been recorded in Scottish hatcheries (Gould, personal communication).

When the temperature rises above 15°C, mortality rates gradually decrease although there are cases of disease occurring above this temperature (Bernardet *et al*., 1988). Young rainbow trout are the most susceptible, especially those weighing between 0.2-10 g, at around 4-7 weeks post-feeding. Increases in mortalities are often associated with spring and early summer due to high stocking densities and water temperatures found at this time (Casey, 1993). Even though rainbow

trout fry have been found to be the most vulnerable species, brown trout (*Salmo trutta* L.) and Atlantic salmon fry also appear to be susceptible (Sarti *et al.*, 1992; Ekman *et al*., 1999).

The gross signs of the disease are the same as found in many other pathological conditions of fish, such as weakness, loss of appetite, melanosis, ascites and exophthalmia (Bernardet *et al*., 1988). In addition, the affected fish sometimes appear lethargic and swim close to the water surface, or to the sides of the tank and the outlet of the troughs (Lorenzen *et al*., 1991). In some hatcheries where RTFS is endemic, juvenile fish exhibit abnormal spiral swimming behaviour due to inflammation of the cranial cavity and anterior vertebrae (Kent *et al.*, 1989). The gills are pale due to anaemia with a severe reduction in the number of erythrocytes. In some cases haemorrhages are also observed in the gills (Sarti *et al.*, 1992). Larger fish show convex lesions associated with the lateral flank (Bruno, 1992) and in severe cases there is muscle damage at one or more sites on the body surface (Santos *et al*., 1992). Some authors have also reported the existence of blisters containing watery blood and occasionally ulcers on the skin (Weis, 1987).

Evensen and Lorenzen (1996) studied the course of the disease in experimentally infected fish and reported that the infected fish showed signs of infection one day after intraperitoneal injection of the pathogen. Peaks in the clinical signs were observed 36 to 48 h post-infection and 94 h post-infection, with survivors showing only a few distinguishable clinical signs or gross pathological changes. Internally, the liver was pale, the head kidney atrophied, the ventricle was often distended, the intestine was empty, fragile and white, except for the caudal part, which was reddish and the spleen greyish, enlarged with soft texture and haemorrhagic petechiae and in later stages completely necrotic (Lorenzen *et al.*, 1991). There was also ascitic fluid in the abdomen. The histopathological signs included focal necrosis in the liver, kidney and heart, hyaline droplet degeneration in the tubules of the secretory part of the kidney and degeneration of the muscles (Lorenzen *et al*., 1991). In addition, weakly-stained Gram negative rods were found in almost all the organs, especially in the vascular system, including head kidney, heart and spleen. Lateral skin lesions are prominent in more chronic infections and are associated with oedema, necrosis, pyknosis

and lymphocyte infiltration of the dermis and underlying muscles (Bruno, 1992). Muscle fibres appeared necrotic without striation and many undergo myophagy by large activated macrophages (Lumsden *et al*., 1996). In many outbreaks, Ostland *et al*. (1997) described intraocular swelling and thickening of the corneal epithelium and in a later stage necrosis and rupture of the epithelium. Evensen and Lorenzen (1996) also detected the pathogen in the eyes and the choroid gland and in addition, they observed inflammatory cells containing bacteria and bacterial products in basal parts of the retina as well as in other organs. Thus, they concluded that RTFS is a severe septicemic infection that heavily involves the monocyte-macrophage system.

Pathogenecity of Flavobacterium psychrophilum

The production of proteases by *Fp* has been suggested to play an important role in virulence of the bacterium (Pacha, 1968; Bertolini *et al*., 1994; Dalsgaard and Madsen, 2000). The bacterium produces enzymes that cause tissue damage as well as enhancing invasion of the bacterium (Nematollahi *et al*., 2003). Other reported virulence factors include adhesins, exotoxins, and endotoxins (Dalsgaard, 1993). Adhesins allow the bacteria to attach to host cells and tissues increasing the invasiveness. Dalsgaard (1993) suggested that the bacterium has a polysaccharide extracellular layer on its surface and which is associated with motility and adhesion to host cells (Dalsgaard, 1993). The organism appears to possess substances that can cause lysis of dead bacterial cells, such as *Escherichia coli* and *Aeromonas hydrophila* (Dalsgaard, 1993). In addition to proteases, endotoxins were found and both of these probably play an important role in the pathogenesis of the disease. In several Gram-negative bacteria including *Fp*, lipopolysacharide (LPS) has been found to be an important protective antigen (Fulop *et al*., 1995; Crump *et al*., 2001; LaFrentz *et al*., 2004, 2007). In 2001, Crump *et al*. identified the link of LPS to pathogenesis suggesting its potential in vaccine development. The first step of bacterial colonisation is the ability of the bacteria to adhere to a surface e.g. aquarium tank, host (Møller *et al*., 2003). This adhesion mechanism is supported by specific and non-specific attachments (Ofek and Doyle, 1994; Vatsos,

2001). Non-specific adherence depends on the hydrophobic interactions between various structures on the surface of the bacterium (Ofek and Doyle, 1994). This hydrophobic mechanism is important as it is believed to help the bacteria interact with other cells as well as helping it to avoid the host defence mechanisms (Santos *et al.*, 1990). Specific adherence requires specific compounds on the surface of the bacterium which allow the organism to bind through rigid bonds to molecules (such as n-hexadecane) on the substrate on which it is attaching. Factors such as salinity and pH are also thought to influence the ability of the bacterium to adhere to fish (Balebona *et al.*, 1995). Even though the bacterium does not produce microcysts, it possibly exists in a vegetative or starved state throughout the year (Pacha and Ordal, 1970; Vatsos *et al*., 2002).

The ability of the bacterium to degrade elastin has been associated with virulence in some isolates and a heat-stable metalloprotease is involved in inducing severe muscle necrosis and necrotic myositis (Madsen and Dalsgaard, 1999; Ostland *et al*. 2000). Lorenzen *et al*. (1997) suggested that the presence or absence of plasmids in *Fp* isolates affects the virulence, although this was disputed by Chakroun *et al*. (1998) and Madsen and Dalsgaard (2000) who found no correlation between the two. A few years later, Wiklund and Dalsgaard (2003) observed the attachment of *Fp* to the surface of rainbow trout kidney phagocytes by an opsonin independent cell receptor adhesin, specifically affected by N-acetylneuraminic acid (sialic acid).

The portal of entry of *Fp* into the fish has not yet been revealed (Rangdale, 1995). To investigate this further, there is a need to reproduce the disease under experimental conditions. Significant mortalities have been obtained when rainbow trout are infected with *Fp* intramuscularly or intraperitoneally (Borg, 1960; Chua, 1991; Bustos *et al*., 1995; Rangdale, 1995). Research has focused on producing an experimental model which imitates natural routes of infection since injection of bacterial cells bypass defence mechanisms such as the mucus, skin and gills (Rombout and Joosten, 1998; Decostere *et al*., 2000). However, bath challenges by several researchers have not been completely successful and fish do not become infected without the addition of an external factor i.e stress or skin or skin mucus abrasion (Mudarris and Austin, 1989; Madetoja *et al*., 2000; Ostland *et*

al., 2000; Ekman, 2003). There have been mixed reports on the infection of the fish by cohabitation experiments. Attempts have been made to set up cohabitation and contact/immersion infection models to mimic the natural route of infection. Several researchers have failed to establish infections by this method (Baudin-Laurencin *et al*., 1989; Mudarris and Austin, 1989; Ostland *et al*., 2000; Decostere *et al*., 2000), however successful infections have been reported by a number of researchers although only when the fish were also stressed (Rangdale, 1995; Madsen and Dalsgaard, 1999; Garcia *et al*., 2000; Madetoja *et al*., 2000; Liu *et al*., 2001). In addition, the presence of the pathogen in a farm system does not always result in a disease outbreak as the disease appears in many forms (Rangdale *et al*., 1997a).

Transmission of Flavobacterium psychrophilum *and RTFS*

The intestinal parasite *Hexamita salmonis* and the ectoparasite *Costia necatrix* have been isolated in many cases of RTFS suggesting the need for further studies to examine possible links between these two parasites damaging the defence mechanisms of the fish skin and RTFS (Lorenzen, 1994). Wild fish or fish escaping from farms could also contribute to the spread of the disease, as they do with many other fish diseases (Wiklund *et al.*, 1994). Besides fish, Brown *et al.* (1997) suggested that amphibians, insects and other animals could possibly act as reservoirs for the bacterium. Stress has been shown to be another factor involved in RTFS as suggested by Rangdale *et al.* (1997a) and Garcia *et al*. (2000). When experimental infections of the pathogen were attempted, a decrease in mortalities was observed in the absence of stress.

Flavobacterium psychrophilum has been isolated from eggs, ovarian fluids and milt of sexually mature females and males respectively (Holt *et al*., 1993; Rangdale *et al*., 1996; Brown *et al*., 1997; Vatsos, 2001; Cipriano, 2005). The source of the contamination was found to be either the broodstock or the water (Brown *et al*., 1997; Vatsos, 2001). Brown *et al*. (1997) showed that the bacterium could be transmitted within salmonid eggs, although it did not cause any detrimental effect to the eggs. They disinfected the surface of infected eggs, homogenised them and placed them on

TYE agar and then identified *Fp* colonies suggesting that the pathogen was localised inside the egg. The contamination of eggs, broodstock and water has had consequences for the whole trout industry as the disease has limited the numbers of grow-on fish. Bustos *et al.* (1995) suggested that imported eggs were responsible for serious epidemics in Chile, and Kumagai and Takahashi (1997) reported that eggs from the USA were responsible for outbreaks of BCWD in Japan. A farm survey by Vatsos (2001), revealed 30 % of the broodstock to be positive, as well as eggs and water samples from the incubators. Small fry were also found to be infected though high levels of mortality were not recorded in the farm. Vatsos (2001) suggested that more farm-based work was required to investigate the transmission of the pathogen further.

Detection of Flavobacterium psychrophilum *and diagnosis of Rainbow Trout Fry Syndrome*

Although scientists have used different techniques (serological and genetic analysis and biochemical profiles) to characterise *Fp* isolates, in the past, detection of the bacterium in infected fish was based on the isolation of the pathogen from the internal organs and skin lesions on *Cytophaga* agar. The morphology of the colonies and the lack of growth on tryptic soya agar and brain heart infusion agar provide some information for the identification of the bacterium. The isolates are then subsequently subjected to morphological, physiological, biochemical and enzymatic tests (e.g. API 50CH and API ZYM systems) (Santos *et al.,* 1992; Madetoja *et al*., 2001). At present, the diagnosis of RTFS is based on the clinical signs of the disease in combination with the successful isolation and identification of *Fp* by culture or nested polymerase chain reaction (PCR). In many cases isolation and identification of *Fp* can be difficult, since there is no antibody-based technique that can identify all isolates (Lorenzen and Karas, 1992; Lorenzen and Olesen, 1997) and culture-based methods can be very problematic (Daskalov *et al.,* 1999; Michel *et al.,* 1999). *Flavobacterium psychrophilum* is considered to be a rather fastidious organism and there are many problems with the culture of the bacterium. Several microbes including *Fp* are known to enter a viable but non-

culturable state. In the study by Vatsos *et al*. (2003), *Fp* was found to survive for more than 4 months when maintained in stream water (starved conditions) and no more than 1 h in distilled water. This characteristic hinders the detection of the pathogen in environmental samples when culture is used as the only means of identifying the bacteria.

Bacterial culture for *Fp* is time consuming and culture of the pathogen is not always successful. An immunofluoresence antibody technique (IFAT) was developed by Lorenzen and Karas (1992) as a rapid diagnostic tool. The authors screened fish tissue and used serial dilutions of anti-*Fp* rabbit serum and found there was a low-level cross reactivity with *Flavobacterium columnare*. The biggest advantage of this method, apart from being quick, was that it could detect viable as well as dead cells. Evensen and Lorenzen (1996) developed an immunhistochemical technique which could detect *Fp* in paraffin-wax-embedded sections. However, the antisera they used cross-reacted with *Aeromonas salmonicida* in the lumen of the intestine and in the pancreas. Nevertheless, in addition to being an important pathogen identification tool, this technique can offer great assistance in a variety of pathogenesis studies i.e. with regard to the distribution of the bacteria in different tissue cells when comparing naturally and experimentally infected fry.

Faruk (2000) produced two anti-*Fp* rabbit polyclonal sera which he used in IFAT to screen the tissue of rainbow trout fry following an intramuscular injection with the bacterium. However, he thought that the antisera he produced may not useful for screening naturally infected fish as the antisera did not react with all *Fp* isolates tested. In 2002, LaFrentz *et al*. developed an ELISA to monitor anti-*Fp* antibody production in trout from experimentally infected rainbow trout. Their study confirmed that rainbow trout produced significant levels of *Fp* specific antibodies.

In 1994, Toyama *et al.* developed primers from the 16S rRNA region and produced a nested PCR to detect the bacterium. Izumi and Wakabayashi (1997) used this technique to screen and detect the pathogen from apparently healthy juvenile ayu and Coho salmon eggs. This method has a great advantage over conventional methods for detecting *Fp*, since it is very specific and it can be performed in one day. Urdaci *et al*. (1998) was able to detect low numbers of *Fp* cells (as low as to

10 bacterial cells) performing a PCR using the primers FP1 and FP2. An adaptation of the nested PCR technique was used by Baliarda *et al*. (2002) to screen ova and spleen from fish infected with RTFS.

A variety of techniques have been used to determine bacterial levels in environmental samples including bacterial isolation by culture, IFAT, nested PCRs and ELISA (Wiklund *et al*., 2000; Vatsos, 2001; del Cerro *et al*., 2002a, 2002b; LaFrentz *et al*., 2002; Madsen *et al*., 2005; Vatsos *et al*., 2002). These techniques have been found to be very useful for epidemiological studies to examine levels of the pathogen before, during, and after a disease outbreak. They have also been used in other studies relating to RTFS such as the efficacy of disinfection or antibiotic treatment. Vatsos (2001) carried out several studies to quantify *Fp* in water samples using nested PCR, IFAT and *in situ* hybridisation but concluded that the *in situ* hybridisation required further optimisation.

In order to minimise the risks of aquatic pathogens in a system, there are a number of universal codes of practice and guidelines available that highlight up to date diagnostic methods. The World Organisation for Animal Health (OIE) *Manual of Diagnostic Tests for Aquatic Animals* 2006 (the *Aquatic Manual*) contains a list of diagnostic methods (traditional, molecular and immunological) for the identification of aquatic pathogens. Although immunological and molecular diagnostics of aquatic animal diseases are always being optimised, prospective novel methods are also frequently under development for a particular pathogen, e.g. micro-array technology, loop mediated isothermal amplification (LAMP) or quantitative PCR (qPCR), which allow either rapid detection and/or quantification of the pathogen (Adams and Thompson, 2008). LAMP is carried out under isothermal conditions allowing it to be performed without the use of a thermocycler (Notomi *et al*., 2000). Products of LAMP amplification are seen by the naked eye through the addition of SYBR Green I to the reaction, which changes from an orange to yellow/green colour when the reaction is positive. Luminex™ xMAP technology offers the potential to develop novel serology and molecular methods for rapid and sensitive detection of antibodies, antigen or DNA of *Fp*. Luminex™

xMAP technology allows multiplexing in the assay and uses a microsphere suspension array in which up to 100 different reactions can be analysed in one single reaction (Dunbar, 2006).

Prevention and control of Flavobacterium psychrophilum

For the time being, the treatment of RTFS depends on the oral administration of a wide range of anti-microbial compounds, some of which have proven ineffective. Soltani *et al.*, (1995) studied the minimum inhibitory concentrations of several anti-microbial agents and recommended amoxcyillin (75-80mg/Kg fish/day), norfloxacin, oxolinic acid and oxytetracycline (300mg/Kg fish/day) for the control of the disease. In a similar study Rangdale *et al*. (1997b) found that the new generation 4-quinolones (e.g. sarafloxacin and enrofloxacin), oxolinic acid and florfenicol when given orally can be very effective against *Fp*. Since 2000, with reduced efficacy of amoxycillin, florfenicol has become the favoured treatment for RTFS in farm outbreaks in the UK and in Europe (Richard Collins, personal communication, University of Stirling, Stirling, UK; Bruun *et al.*, 2003). However some farms still opt for oxytetracycline or amoxicillin at times, and then revert back to florfenicol (Collins, personal communication). In other parts of the world i.e. the USA, oxytetracycline is used for the treatment of outbreaks caused by *Fp* (LaFrentz and Cain, 2004). There is an increasing concern about the excessive use of antibiotics due to build up of resistance. Rangdale (1994) discussed routes of successful treatments of RTFS, and speculated that this problem could be due to either plasmid-mediated or chromosomal resistance.

It is always better to attempt to prevent infection and avoid infection rather than treat with antibiotics and then have to deal with the problems associated with their use. The application of an effective disinfection regime for eggs could decrease the transmission of the disease since the pathogen appears to be vertically transmitted (Holt *et al*., 1993; Rangdale *et al*., 1996; Brown *et al*., 1997). With respect to the disinfection of the eggs, Kumagai and Takahashi (1997) found that 50 ppm of iodine for 15 min failed to prevent outbreaks of the disease in some lots of eggs. Holt *et al.* (1993) also reported the ineffectiveness of such treatments. In 1995, Rangdale carried out a study *in*

vitro to examine the efficacy of disinfectants against *Fp*. The results suggested that gluteraldehyde at 200 ppm for 20 min could potentially disinfect both farm equipment and the surface of eyed ova. On the other hand, Brown *et al.* (1997) reported that 100 ppm povine/iodine for 30 min resulted in a 98% reduction in the number of culturable *Fp* from eggs. However, not all of the bacteria were killed, suggesting that longer exposure may be necessary for effective treatment. Some success has been reported with the use of disinfectants such as formalin, gluteraldehyde and hydrogen peroxide (Branson, 1998). Although disinfection has been found to be ineffective at levels tolerated by the eggs, farm practices still continue with surface disinfection of the eggs. Madsen *et al*. (2005) examined several disinfection procedures (1 and 3 % aqueous iodine solution, 0.1 % Virkon, and 1 % Virkon). Although none of the concentrations or procedures eliminated the bacterial flora on eyed eggs completely, the most successful was 1 % Virkon.

With ineffective disinfection procedures and the fear of antibiotic resistance there is an increased need to develop an effective vaccine for *Fp*. Vaccine strategies are generally aimed at stimulating an adaptive immune response measured by increased antibody production that is capable of providing protection upon pathogen challenge (LaFrentz *et al*., 2003). Holt (1987) reported that vaccination by intraperitoneal injection of formalin-killed cells mixed with adjuvant, or by direct immersion in formalin-killed cells, effectively protected Coho salmon yearlings against experimental challenge with the pathogen. Obach and Baudin-Laurencin (1991) reported that after a vaccination trial of rainbow trout against the visceral form of BCWD, vaccination was only effective in fish greater than 0.5 g (50 days post hatching).

Intraperitoneal injection of *Fp* as the vaccination route can be effective, although in practical terms is not feasible in the case of small fry (the critical size for this disease is 0.5 g). Vaccine development has been complicated due to the fact that mortality rates are highest in fry (1-5g) (Cipriano and Holt, 2005). Immersion, on the other hand, appears to be a more practical way to vaccinate fry. However, as Johnson *et al*. (1982) pointed out, in the case of salmonids the fish should weigh at least 1 g, or 2.5 g, for bath vaccination to be effective. Although there is no effective

commercial RTFS vaccine available, autogenous vaccines formulated with site-specific strains have been reported to be successful in controlling RTFS (Ridgeway Biologicals, Compton, UK.).

The use of epidemiologic methods are essential for understanding the course of infectious diseases in aquaculture systems, which in turn can lead to a better understanding of the causes and management of diseases affecting the aquaculture industry (Georgiadis *et al*., 2001). Determination of prevalence is the most universal epidemiological approach to assess the occurrence of a disease within a farming system. Prevalence can be applied to achieve one of the major objectives of veterinary epidemiology, that is to identify links between environmental and management risk factors and the occurrence of a disease (Thorburn, 1999). A typical epidemiology study requires the selection of a target population (i.e. species, life stages) and to establish a sampling plan which involves the evaluation of the diagnostic tests used and consequently the identification of associated risk factors (Thorburn, 1999). The diagnostic test may be applied to screen apparently health populations of fish for the presence of infected individuals (Thorburn, 1999). Most epidemiological aquaculture case studies have selected a farm, hatchery or fish holding facility as the component for the analysis (Georgiadis *et al*., 2001).

The study by Ryce and Zale (2004) carried out an epidemiology study of *Fp* on cutthroat trout (*Oncorhynchus clarki lewisi*) in hatcheries to identify risk factors and develop control strategies for the reduction of infection. The study was planned by investigating where the pathogen occurred in the hatchery, factors that caused the disease outbreaks and finally to determine avenues of transmission in the hatchery. *Flavobacterium psychrophilum* was detected in the incoming water of the hatchery and some of the broodstock. They also found milt and hatchery water to be possible routes of transmission of the pathogen, stress, experienced by the fish, has also been implicated in disease outbreaks. Control measures highlighted by this study include the need for an effective disinfection regime for eggs, as well as thorough disinfection of incoming pipes, and finally to reduce the levels of stress experienced by the fry through improved farm management.

Aim and Objectives

The aim of this study was to investigate possible sources of *Fp* in a rainbow trout farm system and to use this data to develop strategies to reduce the prevalence of the pathogen with this farming system.

The aim of the study was addressed through the following objectives:

1) To develop novel sensitive molecular-based methods to quantify detect and the level of pathogen in eggs, fry and environmental samples (Chapter 2). Quantitative PCR and LAMP were developed to detect *Fp* in the farm system.

2) To develop a serological-based method to detect fish host response in small fry to *Fp* (Chapter 3). A Luminex™ xMap assay were developed to screen small amounts of serum from individual rainbow trout fry.

3) To use the assays developed in Chapters 2 and 3 (i.e. Luminex™ xMap assay, qPCR and LAMP) together with culture and nested PCR to establish the prevalence of *Fp* in farm systems by sampling eggs upon arrival at the farm, and following the batch of eggs post-hatch through culture until they leave the farm.

juvenile coho salmon, infected with *Fp*. Evensen and Lorenzen (1996) also found bacteria/bacterial products in the vicinity of the vertebrae.

Historical background and Taxonomy of Flavobacterium psychrophilum

Flavobacterium psychrophilum (*Fp*) is a bacterial pathogen responsible for substantial economic losses in salmonid aquaculture worldwide (Dumetz *et al*., 2008). The pathogen is ubiquitous in the aquatic environment and is endemic in all fish farms in the UK (Mark James, personal communication, Department for Environment, Food and Rural Affairs, London, UK.). The taxonomy of *Fp* has been revised several times over the years resulting in changes in Genus classification of the bacterium.

The bacterium was first isolated in the USA in 1948 from lesions on juvenile Coho salmon (*Oncorhynchus kisutch*) following high levels of mortalities during a disease episode. The bacterium was later named as *Cytophaga psychrophila* in 1960 by Borg (1960).

The pathology and characteristics of the bacterium (retractile with gliding motility) were found to be similar to *Flavobacterium columnare* (Columnaris disease) which had previously been described by Davis in 1946. In contrast to *F. columnare*, Borg (1960) observed the absence of 'swarming' or production of 'needle-like haystacks'. In 1968, Pacha established that the bacteria did not form microcysts and fruiting bodies, and was therefore re-classified in the genus *Flexibacter*. In the late 1980's the bacterium was isolated in Europe for the first time, from diseased rainbow trout in Germany and France (Weis, 1987; Bernardet *et al*., 1988; Lorenzen *et al*., 1991; Rangdale, 1999). Following isolation of *Fp* in Germany by Weis (1987), the bacterium has since been isolated in other European countries: Denmark, (Lorenzen *et al*., 1991); UK, (Santos *et al*., 1992); Spain, (Toranzo and Barja, 1993); Finland, (Wiklund *et al*., 1994); Switzerland and Northern Ireland, (Lorenzen and Olesen, 1997) as well as in Japan, (Wakabayashi *et al*., 1994); Australia, (Schmidtke and Carson, 1995); Chile, (Bustos *et al*., 1995); Korea, (Lee and Heo, 1998); and Canada, (Ostland *et al*., 1999).

A full list of the different fish species affected by *Fp* in different geographical locations is given in Table 1.1. Though predominantly found in salmonids, the bacterium has been isolated from a variety of other non-salmonid fish.

Bernardet and Grimont (1989) compared the American strains to those isolated in France, and found differences in the polysaccharides produced by the bacterium from different geographical locations. The guanine and cytosine content of deoxyribonucleic acid (DNA) in members of the genera *Cytophagacae, Flexibacter* and *Flavobacterium* was also compared and were found to be highly polyphyletic (Holt *et al*., 1989). Realising that the bacteria in these phyla were closely related, several species were regrouped into the *Flavobacterium* genus including *Cytophaga psychrophilum* giving rise to the current nomenclature *Flavobacterium psychrophilum* (Bernardet and Grimont, 1989). Further investigation using the 16S ribosomal Ribonucleic acid (RNA) of the bacterium suggested that *Fp*, *F. columnare* and *Flexibacter maritimus* were closely related, representing a discrete group known as *Flavobacterium* (Bader and Shotts, 1998).

Table 1.1: Geographic distribution and host species of *Flavobacterium psychrophilum* in order of first reported isolation.

Host Species	Geographic location	Reference
Coho Salmon (*Oncorhynchus*	USA Japan	Borg, 1960 Wakabayashi *et al*., 1991
Sockeye salmon (*Oncorhynchus*	USA	Rucker *et al*., 1953
Chinook salmon (*Oncorhynchus*	USA Canada	Rucker *et al*., 1953 Ostland *et al*., 1999
Brook trout (*Salvelinus fontinalis*)	USA Finland	Bullock, 1972 Madetoja *et al*., 2001
Atlantic salmon (*Salmo salar*)	USA Australia Sweden Canada	Schneider and Nicholson, Schmidtke and Carson, 1995 Ekman *et al*., 1999 Ostland *et al*., 1999
Lake trout (*Salvelinus namaycush*)	USA	Schachte, 1983
Cutthroat trout (*Oncorhynchus clarki*)	USA Finland	Holt, 1987 Crump *et al*., 2001
Chum salmon (*Oncorhynchus keta*)	USA	Holt, 1987
Rainbow trout (*Oncorhynchus*	Germany France Denmark UK Italy Spain Finland Chile Northern Switzerland Canada	Weis, 1987 Bernardet *et al*., 1988 Lorenzen *et al*., 1991 Santos *et al*., 1992 Sarti *et al*., 1992 Toranzo and Barja, 1993 Wiklund *et al*., 1994 Bustos *et al*., 1995 Lorenzen and Olesen, 1997 Lorenzen and Olesen, 1997 Ostland *et al*., 1999
Brown trout (*Salmo trutta*)	Japan Norway Finland	Wakabayashi *et al*., 1991 Lorenzen and Olesen, 1997 Madetoja *et al*., 2001
Carp (*Cyprinnus carpio*) & crucian (*Carassius carassius*)	Germany	Lehmann *et al*., 1991
Eel (*Anguilla anguilla*)	Germany	Lehmann *et al*., 1991
Tench (*Tinca tinca*)	Germany	Lehmann *et al*., 1991
Ayu (*Plecoglossus altivelis*)	Japan Korea	Wakabayashi *et al*., 1994 Lee and Heo, 1998
Amago (*Oncorhynchus rhodurus*)	Japan	Furutsuka-Uozumi *et al*.,
Pale chub (*Zacco platypus*)	Japan	Iida and Mizokami, 1996
Masou salmon (*Oncorhynchus*	Japan	Iida and Mizokami, 1996
Steelhead (*Oncoryhynchus mykiss*)	USA	Brown *et al*., 1997
Forktongue goby (*Chaenogobius*	Japan	Amita *et al*., 2000
Lake goby (*Rhinogobius brunneus*)	Japan	Amita *et al*., 2000
Japanese dace (*Trybolodon*	Japan	Amita *et al*., 2000
Arctic char (*Salvelinus alpinus*)	Finland	Madetoja *et al*., 2001
Grayling (*Thymallus thymallus*)	Estonia	Madetoja *et al*., 2001

Sea trout (*Salmo truta*)	Finland &	Madetoja *et al*., 2001
Perch (*Perca fluviatilis*)	Finland	Madetoja *et al*., 2002
Roach (*Rutilus rutilus*)	Finland	Madetoja *et al*., 2002

Characterisation of Flavobacterium psychrophilum

Flavobacterium psychrophilum is a Gram-negative, rod shaped, aerobic bacterium with weak refractile slender rods with branched or rounded ends which have been found to vary considerably in size (Lorenzen, 1994; Holt *et al*., 1993). Cells tend to be 1.5–7.5 μm in length and 0.75 μm in width with a characteristic cheese-like odour (Bernardet and Grimont, 1989; Lorenzen *et al.*, 1997; Austin, 1999). However, some filamentous (10-40 μm) and pleomorphic forms have also been found to exist in broth cultures older than 48 h. Gliding motility is always reported, although sometimes it is very slow and difficult to observe (Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989). The mechanism behind this gliding motility is unknown, however, there are features within the cell envelope that have been associated with the bacteria's ability to move over a solid surface (Dalsgaard, 1993; Martinez *et al.*, 2004). The gliding motility may be due to the polysaccharide slime layer, as displayed by *Flavobacterium columnaris* (Dalsgaard, 1993).

Flavobacterium psychrophilum is an aerobic mesophilic bacterium with optimal growth observed at 15°C, although growth has been found between 5°C and 25°C. The bacterium does not grow at temperatures above 26°C (Pacha, 1968). The optimal temperature for protease production appears to be slightly lower than the optimal growth temperature at around 13.3 ± 1.9°C (Vatsos *et al.*, 2006). A number of researchers have reported difficulty in sub-culturing this fastidious and slow-growing bacterium. One of the most commonly used agars for the identification of *Fp* is modified *Cytophaga* agar (MCA), developed by Anacker and Ordal (1959). Holt *et al.* (1993) reported that the organism could tolerate up to 1 % NaCl and its growth was greater in cytophaga broth supplemented with tryptone yeast extract salts (TYES). *Flavobacterium psychrophilum* forms yellowish colonies on this agar with a thin-spreading appearance, and usually grow 48-96 h after

incubation. Cepeda *et al*. (2004) reported more rapid and greater yields of the bacterium when grown in a new culture medium, FLP medium (TYES medium supplemented with glucose). Recently, Álvarez and Guijarro (2007) developed a modified medium supplemented with aromatic compounds and activated charcoal which improves the recovery of *Fp*. Despite continuous improvements in the culture media used for pathogen isolation, it is not always possible to recover the bacterium from infected fish (Michel *et al*., 1999). This is reported to be due to the presence of viable but non-culturable cells (VNC). When the bacteria were subjected to suboptimal conditions of survival, this resulted in the formation of VNC cells with inconsistent colony forming units (CFU's) depending on the culture media used (Michel *et al*., 1999). Vatsos *et al*. (2003) investigated this further by observing physical changes in *Fp* over a 19 week starvation period in stream and distilled water. A difference was found between the viability obtained from colony forming units (CFU's) and that observed using a Live/Dead viability kit. The culturability of the organism declined until it was no longer possible to obtain colonies on agar plates, but the bacteria could be resuscitated by placing it into modified *Cytophaga* broth.

Serotyping of Flavobacterium psychrophilum

Various strains of *Fp* share common antigens and therefore it has been possible to distinguish the bacterium from other bacteria belonging to the *Flavobacterium* group (including *Cytophaga* and *Flexibacter*) using serological methods (Pacha, 1968; Holt, 1987; Cipriano *et al*., 1996). However, there can be differences in antigens present within the same species and this is referred as different serotypes. In 1987, Holt identified the presence of two or more serotypes in American isolates of *Fp* using absorbed rabbit antisera. In a subsequent study by Wakabayashi *et al*. (1994) three different serotypes (O-1, O-2, O-3) were identified from isolates obtained from diseased fish in Japan and the USA. Lorenzen and Olesen (1997) also examined the serotyping system of *Fp* and confirmed the presence of three different serotypes (Th, Fd and Fp). The serotyping was later re-classified according to a host-dependant serotype system and they identified 7 host-dependant serotypes (1-

salmon, 2-trout, 3-trout, 4-eel, 5-carp, 6-tench and 7-ayu); (Mata *et al*., 2002). A different serotyping system for *Fp* was proposed Izumi *et al*. (2003a) using antiserum against an *Fp* isolate from amago (*Onchorynchus masou rhodurus*) using absorbed antiserum. From their work they suggested the following serotypes; O-1/2, O-1/2/3, O-1/2/4, O-1/2/3/4, O-2/3/4, O-2/3 with the possibility of others being found in the future.

In 2000, Faruk produced rabbit sera against a virulent and a non-virulent isolate of *Fp* and used them to examine the antigenicity of isolates obtained from different geographical regions. Five different groups of bacteria based on their reactivity in the Enzyme-linked immunosorbent assay (ELISA) were reported, although three of the groups (G1, G2 and G5) were more distinct then the other two (G3 and G4). The most recent serotyping system to be proposed by Morgan *et al*. (2007) recognises four serotypes based on the profiles obtained in western blot analysis using 26 isolates screened with a cocktail of the two rabbit anti-*Fp* polyclonal sera prepared by Faruk (2000).

Disease caused by Flavobacterium psychrophilum

The pathogen has been associated with several diseases although the diseases are manifest with different clinical signs i.e. mainly bacterial coldwater disease (BCWD) in the USA and rainbow trout fry syndrome (RTFS) in Europe (Holt *et al*., 1993; Rangdale *et al*., 1999). *Flavobacterium psychrophilum* has recently been associated with red mark syndrome (RMS; Ferguson *et al*., 2006). Bacterial coldwater disease (also known as peduncle disease due to the location of lesions which result) predominantly affects juvenile salmonid fish, particularly Coho salmon (*O. kisutch*) and is most prevalent when the water temperature is less than or around 10˚C (Austin and Austin, 1993). In fish of up to 1 year old, the peduncle lesion is the most typical clinical sign of the disease although dark pigmentation and moribund fish are also seen in the latter stages of the infection. In BCWD epizootics, changes in developing bone and cartilage have been observed; these include the entire head region, such as cephalic osteochondritis and necrotic scleritis, the spinal column, and elements of the pectorals and ribs (Ostland *et al*., 1997). Kent *et al*. (1989) observed osteitis, meningitis and

ganglioneuritis in juvenile coho salmon, infected with *Fp*. Evensen and Lorenzen (1996) also found bacteria/bacterial products in the vicinity of the vertebrae.

Red mark syndrome was first reported in late 2003 and the association of the *Fp* to RMS and coldwater strawberry disease (CWSD) was reported by Ferguson *et al*. (2006). The DNA of the pathogen was isolated from paraffin wax sections from rainbow trout skin lesions and affected fish typically around 500 g sometimes also have spinal deformities. The association of *Fp* with RMS still remains to be confirmed however.

Rainbow Trout Fry Syndrome

The clinical manifestation of *Fp* infection has been known by a variety of names from the different countries including Fry Anaemia, Fry Mortality Syndrome and Visceral Myxobacteriosis although the commonly accepted name in the UK is now RTFS. In 1984, rainbow trout hatcheries around the UK experienced high mortalities associated with RTFS. Trout hatcheries in the UK lose an average of 10 to 30 % of fry stock to this disease, although losses of up to 80% can occur when the fish is more susceptible to the pathogen when water temperatures are 10°C or below (Bernardet *et al*., 1988; Scott, 1989). Recently, Schering-Plough ranked RTFS to be the highest cause of mortality in trout hatcheries in the UK and fifth in Europe (Chris Gould, personal communication, Intervet/Schering-Plough Animal Health, Milton Keynes, UK.). In more recent years, the disease seems to be affecting larger fish and mortality rates of up to 50 % have been recorded in Scottish hatcheries (Gould, personal communication).

When the temperature rises above 15°C, mortality rates gradually decrease although there are cases of disease occurring above this temperature (Bernardet *et al*., 1988). Young rainbow trout are the most susceptible, especially those weighing between 0.2-10 g, at around 4-7 weeks post-feeding. Increases in mortalities are often associated with spring and early summer due to high stocking densities and water temperatures found at this time (Casey, 1993). Even though rainbow

trout fry have been found to be the most vulnerable species, brown trout (*Salmo trutta* L.) and Atlantic salmon fry also appear to be susceptible (Sarti *et al.*, 1992; Ekman *et al*., 1999).

The gross signs of the disease are the same as found in many other pathological conditions of fish, such as weakness, loss of appetite, melanosis, ascites and exophthalmia (Bernardet *et al*., 1988). In addition, the affected fish sometimes appear lethargic and swim close to the water surface, or to the sides of the tank and the outlet of the troughs (Lorenzen *et al*., 1991). In some hatcheries where RTFS is endemic, juvenile fish exhibit abnormal spiral swimming behaviour due to inflammation of the cranial cavity and anterior vertebrae (Kent *et al.*, 1989). The gills are pale due to anaemia with a severe reduction in the number of erythrocytes. In some cases haemorrhages are also observed in the gills (Sarti *et al.*, 1992). Larger fish show convex lesions associated with the lateral flank (Bruno, 1992) and in severe cases there is muscle damage at one or more sites on the body surface (Santos *et al*., 1992). Some authors have also reported the existence of blisters containing watery blood and occasionally ulcers on the skin (Weis, 1987).

Evensen and Lorenzen (1996) studied the course of the disease in experimentally infected fish and reported that the infected fish showed signs of infection one day after intraperitoneal injection of the pathogen. Peaks in the clinical signs were observed 36 to 48 h post-infection and 94 h post-infection, with survivors showing only a few distinguishable clinical signs or gross pathological changes. Internally, the liver was pale, the head kidney atrophied, the ventricle was often distended, the intestine was empty, fragile and white, except for the caudal part, which was reddish and the spleen greyish, enlarged with soft texture and haemorrhagic petechiae and in later stages completely necrotic (Lorenzen *et al.*, 1991). There was also ascitic fluid in the abdomen. The histopathological signs included focal necrosis in the liver, kidney and heart, hyaline droplet degeneration in the tubules of the secretory part of the kidney and degeneration of the muscles (Lorenzen *et al*., 1991). In addition, weakly-stained Gram negative rods were found in almost all the organs, especially in the vascular system, including head kidney, heart and spleen. Lateral skin lesions are prominent in more chronic infections and are associated with oedema, necrosis, pyknosis

and lymphocyte infiltration of the dermis and underlying muscles (Bruno, 1992). Muscle fibres appeared necrotic without striation and many undergo myophagy by large activated macrophages (Lumsden *et al*., 1996). In many outbreaks, Ostland *et al*. (1997) described intraocular swelling and thickening of the corneal epithelium and in a later stage necrosis and rupture of the epithelium. Evensen and Lorenzen (1996) also detected the pathogen in the eyes and the choroid gland and in addition, they observed inflammatory cells containing bacteria and bacterial products in basal parts of the retina as well as in other organs. Thus, they concluded that RTFS is a severe septicemic infection that heavily involves the monocyte-macrophage system.

Pathogenecity of Flavobacterium psychrophilum

The production of proteases by *Fp* has been suggested to play an important role in virulence of the bacterium (Pacha, 1968; Bertolini *et al*., 1994; Dalsgaard and Madsen, 2000). The bacterium produces enzymes that cause tissue damage as well as enhancing invasion of the bacterium (Nematollahi *et al*., 2003). Other reported virulence factors include adhesins, exotoxins, and endotoxins (Dalsgaard, 1993). Adhesins allow the bacteria to attach to host cells and tissues increasing the invasiveness. Dalsgaard (1993) suggested that the bacterium has a polysaccharide extracellular layer on its surface and which is associated with motility and adhesion to host cells (Dalsgaard, 1993). The organism appears to possess substances that can cause lysis of dead bacterial cells, such as *Escherichia coli* and *Aeromonas hydrophila* (Dalsgaard, 1993). In addition to proteases, endotoxins were found and both of these probably play an important role in the pathogenesis of the disease. In several Gram-negative bacteria including *Fp*, lipopolysacharide (LPS) has been found to be an important protective antigen (Fulop *et al*., 1995; Crump *et al*., 2001; LaFrentz *et al*., 2004, 2007). In 2001, Crump *et al*. identified the link of LPS to pathogenesis suggesting its potential in vaccine development. The first step of bacterial colonisation is the ability of the bacteria to adhere to a surface e.g. aquarium tank, host (Møller *et al*., 2003). This adhesion mechanism is supported by specific and non-specific attachments (Ofek and Doyle, 1994; Vatsos,

2001). Non-specific adherence depends on the hydrophobic interactions between various structures on the surface of the bacterium (Ofek and Doyle, 1994). This hydrophobic mechanism is important as it is believed to help the bacteria interact with other cells as well as helping it to avoid the host defence mechanisms (Santos *et al.*, 1990). Specific adherence requires specific compounds on the surface of the bacterium which allow the organism to bind through rigid bonds to molecules (such as n-hexadecane) on the substrate on which it is attaching. Factors such as salinity and pH are also thought to influence the ability of the bacterium to adhere to fish (Balebona *et al.*, 1995). Even though the bacterium does not produce microcysts, it possibly exists in a vegetative or starved state throughout the year (Pacha and Ordal, 1970; Vatsos *et al*., 2002).

The ability of the bacterium to degrade elastin has been associated with virulence in some isolates and a heat-stable metalloprotease is involved in inducing severe muscle necrosis and necrotic myositis (Madsen and Dalsgaard, 1999; Ostland *et al*. 2000). Lorenzen *et al*. (1997) suggested that the presence or absence of plasmids in *Fp* isolates affects the virulence, although this was disputed by Chakroun *et al*. (1998) and Madsen and Dalsgaard (2000) who found no correlation between the two. A few years later, Wiklund and Dalsgaard (2003) observed the attachment of *Fp* to the surface of rainbow trout kidney phagocytes by an opsonin independent cell receptor adhesin, specifically affected by N-acetylneuraminic acid (sialic acid).

The portal of entry of *Fp* into the fish has not yet been revealed (Rangdale, 1995). To investigate this further, there is a need to reproduce the disease under experimental conditions. Significant mortalities have been obtained when rainbow trout are infected with *Fp* intramuscularly or intraperitoneally (Borg, 1960; Chua, 1991; Bustos *et al*., 1995; Rangdale, 1995). Research has focused on producing an experimental model which imitates natural routes of infection since injection of bacterial cells bypass defence mechanisms such as the mucus, skin and gills (Rombout and Joosten, 1998; Decostere *et al*., 2000). However, bath challenges by several researchers have not been completely successful and fish do not become infected without the addition of an external factor i.e stress or skin or skin mucus abrasion (Mudarris and Austin, 1989; Madetoja *et al*., 2000; Ostland *et*

al., 2000; Ekman, 2003). There have been mixed reports on the infection of the fish by cohabitation experiments. Attempts have been made to set up cohabitation and contact/immersion infection models to mimic the natural route of infection. Several researchers have failed to establish infections by this method (Baudin-Laurencin *et al*., 1989; Mudarris and Austin, 1989; Ostland *et al*., 2000; Decostere *et al*., 2000), however successful infections have been reported by a number of researchers although only when the fish were also stressed (Rangdale, 1995; Madsen and Dalsgaard, 1999; Garcia *et al*., 2000; Madetoja *et al*., 2000; Liu *et al*., 2001). In addition, the presence of the pathogen in a farm system does not always result in a disease outbreak as the disease appears in many forms (Rangdale *et al*., 1997a).

Transmission of Flavobacterium psychrophilum *and RTFS*

The intestinal parasite *Hexamita salmonis* and the ectoparasite *Costia necatrix* have been isolated in many cases of RTFS suggesting the need for further studies to examine possible links between these two parasites damaging the defence mechanisms of the fish skin and RTFS (Lorenzen, 1994). Wild fish or fish escaping from farms could also contribute to the spread of the disease, as they do with many other fish diseases (Wiklund *et al.*, 1994). Besides fish, Brown *et al.* (1997) suggested that amphibians, insects and other animals could possibly act as reservoirs for the bacterium. Stress has been shown to be another factor involved in RTFS as suggested by Rangdale *et al.* (1997a) and Garcia *et al*. (2000). When experimental infections of the pathogen were attempted, a decrease in mortalities was observed in the absence of stress.

Flavobacterium psychrophilum has been isolated from eggs, ovarian fluids and milt of sexually mature females and males respectively (Holt *et al*., 1993; Rangdale *et al*., 1996; Brown *et al*., 1997; Vatsos, 2001; Cipriano, 2005). The source of the contamination was found to be either the broodstock or the water (Brown *et al*., 1997; Vatsos, 2001). Brown *et al*. (1997) showed that the bacterium could be transmitted within salmonid eggs, although it did not cause any detrimental effect to the eggs. They disinfected the surface of infected eggs, homogenised them and placed them on

TYE agar and then identified *Fp* colonies suggesting that the pathogen was localised inside the egg. The contamination of eggs, broodstock and water has had consequences for the whole trout industry as the disease has limited the numbers of grow-on fish. Bustos *et al.* (1995) suggested that imported eggs were responsible for serious epidemics in Chile, and Kumagai and Takahashi (1997) reported that eggs from the USA were responsible for outbreaks of BCWD in Japan. A farm survey by Vatsos (2001), revealed 30 % of the broodstock to be positive, as well as eggs and water samples from the incubators. Small fry were also found to be infected though high levels of mortality were not recorded in the farm. Vatsos (2001) suggested that more farm-based work was required to investigate the transmission of the pathogen further.

Detection of Flavobacterium psychrophilum *and diagnosis of Rainbow Trout Fry Syndrome*

Although scientists have used different techniques (serological and genetic analysis and biochemical profiles) to characterise *Fp* isolates, in the past, detection of the bacterium in infected fish was based on the isolation of the pathogen from the internal organs and skin lesions on *Cytophaga* agar. The morphology of the colonies and the lack of growth on tryptic soya agar and brain heart infusion agar provide some information for the identification of the bacterium. The isolates are then subsequently subjected to morphological, physiological, biochemical and enzymatic tests (e.g. API 50CH and API ZYM systems) (Santos *et al.,* 1992; Madetoja *et al*., 2001). At present, the diagnosis of RTFS is based on the clinical signs of the disease in combination with the successful isolation and identification of *Fp* by culture or nested polymerase chain reaction (PCR). In many cases isolation and identification of *Fp* can be difficult, since there is no antibody-based technique that can identify all isolates (Lorenzen and Karas, 1992; Lorenzen and Olesen, 1997) and culture-based methods can be very problematic (Daskalov *et al.,* 1999; Michel *et al.,* 1999). *Flavobacterium psychrophilum* is considered to be a rather fastidious organism and there are many problems with the culture of the bacterium. Several microbes including *Fp* are known to enter a viable but non-

culturable state. In the study by Vatsos *et al*. (2003), *Fp* was found to survive for more than 4 months when maintained in stream water (starved conditions) and no more than 1 h in distilled water. This characteristic hinders the detection of the pathogen in environmental samples when culture is used as the only means of identifying the bacteria.

Bacterial culture for *Fp* is time consuming and culture of the pathogen is not always successful. An immunofluoresence antibody technique (IFAT) was developed by Lorenzen and Karas (1992) as a rapid diagnostic tool. The authors screened fish tissue and used serial dilutions of anti-*Fp* rabbit serum and found there was a low-level cross reactivity with *Flavobacterium columnare*. The biggest advantage of this method, apart from being quick, was that it could detect viable as well as dead cells. Evensen and Lorenzen (1996) developed an immunhistochemical technique which could detect *Fp* in paraffin-wax-embedded sections. However, the antisera they used cross-reacted with *Aeromonas salmonicida* in the lumen of the intestine and in the pancreas. Nevertheless, in addition to being an important pathogen identification tool, this technique can offer great assistance in a variety of pathogenesis studies i.e. with regard to the distribution of the bacteria in different tissue cells when comparing naturally and experimentally infected fry.

Faruk (2000) produced two anti-*Fp* rabbit polyclonal sera which he used in IFAT to screen the tissue of rainbow trout fry following an intramuscular injection with the bacterium. However, he thought that the antisera he produced may not useful for screening naturally infected fish as the antisera did not react with all *Fp* isolates tested. In 2002, LaFrentz *et al*. developed an ELISA to monitor anti-*Fp* antibody production in trout from experimentally infected rainbow trout. Their study confirmed that rainbow trout produced significant levels of *Fp* specific antibodies.

In 1994, Toyama *et al.* developed primers from the 16S rRNA region and produced a nested PCR to detect the bacterium. Izumi and Wakabayashi (1997) used this technique to screen and detect the pathogen from apparently healthy juvenile ayu and Coho salmon eggs. This method has a great advantage over conventional methods for detecting *Fp*, since it is very specific and it can be performed in one day. Urdaci *et al*. (1998) was able to detect low numbers of *Fp* cells (as low as to

10 bacterial cells) performing a PCR using the primers FP1 and FP2. An adaptation of the nested PCR technique was used by Baliarda *et al*. (2002) to screen ova and spleen from fish infected with RTFS.

A variety of techniques have been used to determine bacterial levels in environmental samples including bacterial isolation by culture, IFAT, nested PCRs and ELISA (Wiklund *et al*., 2000; Vatsos, 2001; del Cerro *et al*., 2002a, 2002b; LaFrentz *et al*., 2002; Madsen *et al*., 2005; Vatsos *et al*., 2002). These techniques have been found to be very useful for epidemiological studies to examine levels of the pathogen before, during, and after a disease outbreak. They have also been used in other studies relating to RTFS such as the efficacy of disinfection or antibiotic treatment. Vatsos (2001) carried out several studies to quantify *Fp* in water samples using nested PCR, IFAT and *in situ* hybridisation but concluded that the *in situ* hybridisation required further optimisation.

In order to minimise the risks of aquatic pathogens in a system, there are a number of universal codes of practice and guidelines available that highlight up to date diagnostic methods. The World Organisation for Animal Health (OIE) *Manual of Diagnostic Tests for Aquatic Animals* 2006 (the *Aquatic Manual*) contains a list of diagnostic methods (traditional, molecular and immunological) for the identification of aquatic pathogens. Although immunological and molecular diagnostics of aquatic animal diseases are always being optimised, prospective novel methods are also frequently under development for a particular pathogen, e.g. micro-array technology, loop mediated isothermal amplification (LAMP) or quantitative PCR (qPCR), which allow either rapid detection and/or quantification of the pathogen (Adams and Thompson, 2008). LAMP is carried out under isothermal conditions allowing it to be performed without the use of a thermocycler (Notomi *et al*., 2000). Products of LAMP amplification are seen by the naked eye through the addition of SYBR Green I to the reaction, which changes from an orange to yellow/green colour when the reaction is positive. Luminex™ xMAP technology offers the potential to develop novel serology and molecular methods for rapid and sensitive detection of antibodies, antigen or DNA of *Fp*. Luminex™

xMAP technology allows multiplexing in the assay and uses a microsphere suspension array in which up to 100 different reactions can be analysed in one single reaction (Dunbar, 2006).

Prevention and control of Flavobacterium psychrophilum

For the time being, the treatment of RTFS depends on the oral administration of a wide range of anti-microbial compounds, some of which have proven ineffective. Soltani *et al.*, (1995) studied the minimum inhibitory concentrations of several anti-microbial agents and recommended amoxcyillin (75-80mg/Kg fish/day), norfloxacin, oxolinic acid and oxytetracycline (300mg/Kg fish/day) for the control of the disease. In a similar study Rangdale *et al*. (1997b) found that the new generation 4-quinolones (e.g. sarafloxacin and enrofloxacin), oxolinic acid and florfenicol when given orally can be very effective against *Fp*. Since 2000, with reduced efficacy of amoxycillin, florfenicol has become the favoured treatment for RTFS in farm outbreaks in the UK and in Europe (Richard Collins, personal communication, University of Stirling, Stirling, UK; Bruun *et al.*, 2003). However some farms still opt for oxytetracycline or amoxicillin at times, and then revert back to florfenicol (Collins, personal communication). In other parts of the world i.e. the USA, oxytetracycline is used for the treatment of outbreaks caused by *Fp* (LaFrentz and Cain, 2004). There is an increasing concern about the excessive use of antibiotics due to build up of resistance. Rangdale (1994) discussed routes of successful treatments of RTFS, and speculated that this problem could be due to either plasmid-mediated or chromosomal resistance.

It is always better to attempt to prevent infection and avoid infection rather than treat with antibiotics and then have to deal with the problems associated with their use. The application of an effective disinfection regime for eggs could decrease the transmission of the disease since the pathogen appears to be vertically transmitted (Holt *et al*., 1993; Rangdale *et al*., 1996; Brown *et al*., 1997). With respect to the disinfection of the eggs, Kumagai and Takahashi (1997) found that 50 ppm of iodine for 15 min failed to prevent outbreaks of the disease in some lots of eggs. Holt *et al.* (1993) also reported the ineffectiveness of such treatments. In 1995, Rangdale carried out a study *in*

vitro to examine the efficacy of disinfectants against *Fp*. The results suggested that gluteraldehyde at 200 ppm for 20 min could potentially disinfect both farm equipment and the surface of eyed ova. On the other hand, Brown *et al.* (1997) reported that 100 ppm povine/iodine for 30 min resulted in a 98% reduction in the number of culturable *Fp* from eggs. However, not all of the bacteria were killed, suggesting that longer exposure may be necessary for effective treatment. Some success has been reported with the use of disinfectants such as formalin, gluteraldehyde and hydrogen peroxide (Branson, 1998). Although disinfection has been found to be ineffective at levels tolerated by the eggs, farm practices still continue with surface disinfection of the eggs. Madsen *et al*. (2005) examined several disinfection procedures (1 and 3 % aqueous iodine solution, 0.1 % Virkon, and 1 % Virkon). Although none of the concentrations or procedures eliminated the bacterial flora on eyed eggs completely, the most successful was 1 % Virkon.

With ineffective disinfection procedures and the fear of antibiotic resistance there is an increased need to develop an effective vaccine for *Fp*. Vaccine strategies are generally aimed at stimulating an adaptive immune response measured by increased antibody production that is capable of providing protection upon pathogen challenge (LaFrentz *et al*., 2003). Holt (1987) reported that vaccination by intraperitoneal injection of formalin-killed cells mixed with adjuvant, or by direct immersion in formalin-killed cells, effectively protected Coho salmon yearlings against experimental challenge with the pathogen. Obach and Baudin-Laurencin (1991) reported that after a vaccination trial of rainbow trout against the visceral form of BCWD, vaccination was only effective in fish greater than 0.5 g (50 days post hatching).

Intraperitoneal injection of *Fp* as the vaccination route can be effective, although in practical terms is not feasible in the case of small fry (the critical size for this disease is 0.5 g). Vaccine development has been complicated due to the fact that mortality rates are highest in fry (1-5g) (Cipriano and Holt, 2005). Immersion, on the other hand, appears to be a more practical way to vaccinate fry. However, as Johnson *et al*. (1982) pointed out, in the case of salmonids the fish should weigh at least 1 g, or 2.5 g, for bath vaccination to be effective. Although there is no effective

commercial RTFS vaccine available, autogenous vaccines formulated with site-specific strains have been reported to be successful in controlling RTFS (Ridgeway Biologicals, Compton, UK.).

The use of epidemiologic methods are essential for understanding the course of infectious diseases in aquaculture systems, which in turn can lead to a better understanding of the causes and management of diseases affecting the aquaculture industry (Georgiadis *et al*., 2001). Determination of prevalence is the most universal epidemiological approach to assess the occurrence of a disease within a farming system. Prevalence can be applied to achieve one of the major objectives of veterinary epidemiology, that is to identify links between environmental and management risk factors and the occurrence of a disease (Thorburn, 1999). A typical epidemiology study requires the selection of a target population (i.e. species, life stages) and to establish a sampling plan which involves the evaluation of the diagnostic tests used and consequently the identification of associated risk factors (Thorburn, 1999). The diagnostic test may be applied to screen apparently health populations of fish for the presence of infected individuals (Thorburn, 1999). Most epidemiological aquaculture case studies have selected a farm, hatchery or fish holding facility as the component for the analysis (Georgiadis *et al*., 2001).

The study by Ryce and Zale (2004) carried out an epidemiology study of *Fp* on cutthroat trout (*Oncorhynchus clarki lewisi*) in hatcheries to identify risk factors and develop control strategies for the reduction of infection. The study was planned by investigating where the pathogen occurred in the hatchery, factors that caused the disease outbreaks and finally to determine avenues of transmission in the hatchery. *Flavobacterium psychrophilum* was detected in the incoming water of the hatchery and some of the broodstock. They also found milt and hatchery water to be possible routes of transmission of the pathogen, stress, experienced by the fish, has also been implicated in disease outbreaks. Control measures highlighted by this study include the need for an effective disinfection regime for eggs, as well as thorough disinfection of incoming pipes, and finally to reduce the levels of stress experienced by the fry through improved farm management.

Aim and Objectives

The aim of this study was to investigate possible sources of *Fp* in a rainbow trout farm system and to use this data to develop strategies to reduce the prevalence of the pathogen with this farming system.

The aim of the study was addressed through the following objectives:

1) To develop novel sensitive molecular-based methods to quantify detect and the level of pathogen in eggs, fry and environmental samples (Chapter 2). Quantitative PCR and LAMP were developed to detect *Fp* in the farm system.

2) To develop a serological-based method to detect fish host response in small fry to *Fp* (Chapter 3). A Luminex™ xMap assay were developed to screen small amounts of serum from individual rainbow trout fry.

3) To use the assays developed in Chapters 2 and 3 (i.e. Luminex™ xMap assay, qPCR and LAMP) together with culture and nested PCR to establish the prevalence of *Fp* in farm systems by sampling eggs upon arrival at the farm, and following the batch of eggs post-hatch through culture until they leave the farm.

Chapter 1: General Introduction

Historical background and Taxonomy of Flavobacterium psychrophilum

Flavobacterium psychrophilum (*Fp*) is a bacterial pathogen responsible for substantial economic losses in salmonid aquaculture worldwide (Dumetz *et al*., 2008). The pathogen is ubiquitous in the aquatic environment and is endemic in all fish farms in the UK (Mark James, personal communication, Department for Environment, Food and Rural Affairs, London, UK.). The taxonomy of *Fp* has been revised several times over the years resulting in changes in Genus classification of the bacterium.

The bacterium was first isolated in the USA in 1948 from lesions on juvenile Coho salmon (*Oncorhynchus kisutch*) following high levels of mortalities during a disease episode. The bacterium was later named as *Cytophaga psychrophila* in 1960 by Borg (1960).

The pathology and characteristics of the bacterium (retractile with gliding motility) were found to be similar to *Flavobacterium columnare* (Columnaris disease) which had previously been described by Davis in 1946. In contrast to *F. columnare*, Borg (1960) observed the absence of 'swarming' or production of 'needle-like haystacks'. In 1968, Pacha established that the bacteria did not form microcysts and fruiting bodies, and was therefore re-classified in the genus *Flexibacter*. In the late 1980's the bacterium was isolated in Europe for the first time, from diseased rainbow trout in Germany and France (Weis, 1987; Bernardet *et al*., 1988; Lorenzen *et al*., 1991; Rangdale, 1999). Following isolation of *Fp* in Germany by Weis (1987), the bacterium has since been isolated in other European countries: Denmark, (Lorenzen *et al*., 1991); UK, (Santos *et al*., 1992); Spain, (Toranzo and Barja, 1993); Finland, (Wiklund *et al*., 1994); Switzerland and Northern Ireland, (Lorenzen and Olesen, 1997) as well as in Japan, (Wakabayashi *et al*., 1994); Australia, (Schmidtke and Carson, 1995); Chile, (Bustos *et al*., 1995); Korea, (Lee and Heo, 1998); and Canada, (Ostland *et al*., 1999).

A full list of the different fish species affected by *Fp* in different geographical locations is given in Table 1.1. Though predominantly found in salmonids, the bacterium has been isolated from a variety of other non-salmonid fish.

Bernardet and Grimont (1989) compared the American strains to those isolated in France, and found differences in the polysaccharides produced by the bacterium from different geographical locations. The guanine and cytosine content of deoxyribonucleic acid (DNA) in members of the genera *Cytophagacae, Flexibacter* and *Flavobacterium* was also compared and were found to be highly polyphyletic (Holt *et al*., 1989). Realising that the bacteria in these phyla were closely related, several species were regrouped into the *Flavobacterium* genus including *Cytophaga psychrophilum* giving rise to the current nomenclature *Flavobacterium psychrophilum* (Bernardet and Grimont, 1989). Further investigation using the 16S ribosomal Ribonucleic acid (RNA) of the bacterium suggested that *Fp*, *F. columnare* and *Flexibacter maritimus* were closely related, representing a discrete group known as *Flavobacterium* (Bader and Shotts, 1998).

Chapter 2: Development of molecular-based methods for the detection of *Flavobacterium psychrophilum* in eggs, environmental and tissue samples.

2.1 INTRODUCTION

Detection and identification of microorganisms has traditionally relied on isolation of the infectious agent by culture followed by phenotypic and serological characterisation of the organism and histopathology to provide evidence of disease (Mullis, 1990; McKeever and Rege, 1999). Advances in molecular biology over the past 20 years have brought with them a new approach for microbial identification and characterisation (Mullis *et al*., 1987; Erlich *et al*., 1991). There is a large variety of existing molecular techniques available as well as new ones continually being developed for the diagnosis of fish/shrimp diseases, including DNA hybridisation, plasmid profiling, restriction enzyme analysis, nucleic acid-based sequence amplification (NASBA), real-time polymerase chain reaction (PCR), microarray technology, quantitative Luminex™ xMAP assay and PCR (Tang *et al*., 1997; Cunningham, 2002; Adams and Thompson, 2008).

Polymerase chain reaction is a powerful tool for the amplification of trace amounts of nucleic acids, and has rapidly become an essential tool for the detection of many pathogens (Kochanowski and Wolfgang, 1999). This technique provides exceptional sensitivity and is now widely used to detect viruses, parasites and bacterial pathogens in diagnostic samples (Tang *et al*., 1997). The PCR offers reliable detection and identification of pathogens before symptoms of the disease emerge, thereby allowing early treatment of the infected population with appropriate antibiotics. Rapid diagnosis is crucial for the aquaculture industry where symptoms are frequently observed after the disease has progressed to a point where treatment is no longer successful (Pillay and Kutty, 2005). With molecular methods used for routine diagnosis, the answer sought is the presence or absence of

a known gene sequence (i.e. the presence of DNA from the pathogen) in a variety of samples, including environmental and tissue samples (Cunningham, 2002). Although the standard PCR may be able to detect the presence of a pathogen, at times more information is required i.e. differentiation of closely related species, specific identification at a species level, and differentiating between live and dead pathogens (Adams and Thompson, 2006). This has led to the development of variations in the PCR-based methods, such as reverse transcriptase-PCR (RT-PCR) an example of which is the diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout (*Salmo trutta*) (Devold *et al*., 2000); reverse cross blot PCR (rcb-PCR) e.g. for the detection of mycobacteria to a species level (Puttinaowarat *et al*., 2000), random amplified polymorphic DNA (RAPD) e.g. analysis of variability in the pathogenicity of an organism (Thomas-Jinu and Goodwin, 2004), nested PCR e.g. detection of pathogen from a tissue or environmental sample (Wiklund *et al*., 2000) and *in situ* hybridisation e.g. detection of initial infective stages of a pathogen (Carrasco *et al*., 2008).

The requirement of quantifying the amount of a pathogen in a system, for example during disease monitoring and disease outbreaks, has increased over the past decade (Fratamico *et al.*, 2005). The amount of pathogen loading can help prediction of the expected amount of pathogen present during infection. Real-time PCR is the most common molecular method used for quantifying levels of a pathogen in a given sample. For example, Cavender *et al*. (2004) developed a real-time quantitative PCR (RT-qPCR) technique to identify *Myxobolus cerebralis* and to quantify the severity of the infection within rainbow trout, *Oncorhynchus mykiss* fry. A strong correlation was found between the qPCR results and infection levels, and the authors reported that their method was effective for detecting the pathogen in fish tissue and provided an indication of severity of the infection. Quantifying the levels of a microorganism could give an insight into how efficient the antimicrobial therapy has been i.e. the treatment could be said to be ineffective when the pathogen loading did not decrease or in fact continued to increase (Mackay, 2007). Quantification may also help in optimising the treatment strategy by indicating when to begin treatment (Jousson *et al*., 2005).

2.1.2 Real-time quantification PCR (Real-time qPCR)

A result verifying whether a sample is simply positive or negative as obtained with standard PCR amplification, may not be sufficient for when precise quantification of the pathogen is required. For example, Grove *et al*. (2006) developed a real-time assay for the quantification of piscine nodavirus, the causative agent of viral nervous necrosis (VNN). The authors reported that the assay was suitable to study the pathogenesis of the virus, including the establishment of carrier status in healthy fish as well as epidemiological studies by obtaining quantitative data from both fish and environmental samples. Real time PCR is a technique used to monitor the progress of a PCR reaction in 'real time' or during the exponential phase of the reaction following a predictable curve, and therefore combining the amplification and detection in one step without the need to observe the amplification on a gel (Sambrook and Russell., 2001; Patrinos and Ansorge, 2005). Real-time PCR is also known as a quantitative PCR (qPCR) for the obvious reason that it is used to quantify PCR amplicons. Real-time PCR-based fluorescence assays have many advantages over the standard PCR assay e.g.: (i) higher sensitivity; (ii) higher specificity due to the inclusion of a specific primer set and a probe; (iii) allows quantification; (iv) operates in a closed environment, avoiding and reducing possible sources of contamination; and (v) can provide results faster than gel-based PCR assays, allowing rapid intervention of disease (Livak *et al*., 1995; Kochanowski and Wolfgang, 1999; Tucker *et al*., 2001; Tondella *et al*., 2001). The real-time PCR provides a quick and simple alternative to southern or northern-blot analysis for the evaluation of gene copy numbers. The copy numbers of the 16S rRNA gene can be determined by restriction enzyme analysis of the bacterium (Condon *et al*., 1995). Copy numbers of this gene can vary e.g. the copy number of *Rickettsia prowazekii* was determined to be one while *Escherichia coli* has seven as shown by restriction enzyme analysis (Condon *et al*., 1995). Goarant and Merien (2006) developed a real-time PCR quantification assay for the detection of *V. penaeicida* in shrimp and environmental samples to allow accurate surveying for *V. penaeicida* and to investigate seasonal dynamics of the pathogen in relation to the occurrence

of disease outbreaks in shrimp. Balcázar *et al*. (2006) developed a real-time PCR assay for the detection and quantification of *Aeromonas salmonicida* in fish tissue to detect and quantify the amount of pathogens present in different fish tissues e.g. kidney, liver, spleen or intestine. Liu *et al*. (2008) developed a multiplex real-time quantitative RT-PCR (mqRT-PCR) for the detection, identification and quantification of Spring viremia of carp virus (SVCV), infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) in aquaculture species.

Development of the qPCR first starts with the cloning of the target DNA (Figure 2.1) to provide a continuous and reproducible supply of template for use in the standard curve from which the number of 16S copies in a sample are determined (Dorak, 2006). A fluorescent dye or probe of choice is then selected for signal detection. The increase in a fluorescence signal is directly proportional to an increase in the amount of amplified PCR product (Dorak, 2006). A few different methodologies are available using the fluorescent dyes to detect RNA or DNA targets, although most frequently used are, the TaqMan probe and SYBR Green. Real-time PCR was first described using hydrolysis probes which are based on the construction of dual-labelled oligonucleotide probes, also known as TaqMan probes, which emit a fluorescent signal upon cleavage. The probe contains both a fluorochrome and a quencher and when cleaved, the fluorochrome can be detected by the assay. TaqMan assays have a lower background signal and therefore the assay tends to have a higher sensitivity. The simplest system for the qPCR uses the DNA binding dye SYBR Green, which emits fluorescence when it binds to DNA. SYBR Green is highly specific, allowing the detection of the product as it accumulates during the PCR cycle (Simpson *et al*., 2000). Once the method

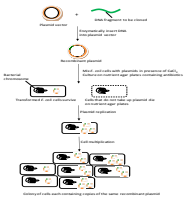


Figure 2.1: Outline of cloning procedure of target DNA to be used in real-time PCR (Figure modified from Griffiths *et al*., 2002).

with the appropriate fluorescent dye and primers has been selected, the assay is ready for optimisation. A melt curve analysis carried out at the end of the assay confirms whether a single product is amplified. As the PCR product melts, SYBR Green is released into the solution, which increases the fluorescent signal observed. A curve of fluorescence intensity over the melting temperature of the PCR product (-dF/dT curve) is produced (Figure 2.2). The efficiency of the PCR reaction (E) is calculated using a formula (Figure 2.2) based on the slope and the efficiency can be affected by variables including presence of inhibitors, controls used for the reaction and primer design (Dorak, 2006). When the efficiency falls below 0.90, it is suggested that the qPCR assay should be optimised further. Care must be taken in the optimisation of each step of the real-time PCR assay, as slight differences in the efficiency of amplification within samples can lead to fairly different amounts of the final product (Nayak and Rose, 2007).

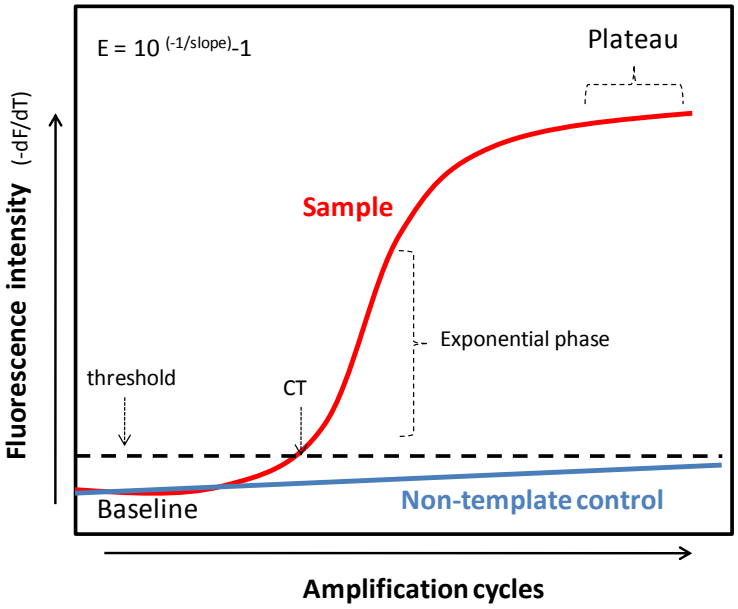


Figure 2.2: Fluorescence signal measurement in a real-time assay (Figure adapted from Dorak, 2006).

2.1.3 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification is used to amplify DNA under isothermal conditions with high specificity, efficiency, and speed. The method has captivated the interest of

researchers because it is able to rapidly detect pathogen DNA without the need for a diagnostic laboratory or special reagents. Strand-displacement by the inclusion of DNA polymerase in the LAMP means there is no need for the high temperatures normally required in template amplification, thus DNA templates can be identified using a constant temperature with extremely high amplification efficiency (Notomi *et al.*, 2000). Due to this, the assay can be carried out using only a water bath, and dismissing the requirement for a thermocycler. This means that the assay can be carried out in the field, possibly by farmers who wish to monitor the pathogen in their farming system themselves (Kuboki *et al.*, 2003; Poon *et al.*, 2005).

The first reported application of LAMP in aquaculture was to detect *Edwardsiella tarda* (Savan *et al.*, 2005), followed by the use of a LAMP-based assay to detect *Edwardsiella ictaluri* in infected catfish (*Ictalurus punctatus*) (Yeh *et al*., 2005). More recently, a LAMP protocol for the detection of spring viraemia of carp virus (SVCV), based on a reverse transcription loop-mediated isothermal amplification (RT-LAMP) has been developed by Shivappa *et al*. (2008) for routine diagnostics in laboratories as well as in farm laboratories. Mao *et al*. (2008) also developed a rapid and sensitive LAMP assay for the detection of Singapore grouper iridovirus (SGIV) in Grouper *Epinephelus* spp. (GP). The authors recommended the use of the assay in GP hatcheries and grow-out ponds for rapid detection of the virus in fish at an early stage in the infection process.

In the LAMP assay, a DNA polymerase with strand displacement and the inclusion of four to six primers (inner and outer) that recognise six different regions of the DNA are used (Notomi *et al.*, 2000; Mori *et al.*, 2001). The Eiken Chemical Co. Ltd website (http://loopamp.eiken.co.jp/e/lamp/principle.html) offers the best explanation of the step-by-step process of how all the primers work in the LAMP assay. Eiken Chemical Co. Ltd recommends that the primers target the F3c, F2c and F1c regions of the target sequence at the 3' side while the B1, B2 and B3 regions target the 5' side (Figure 2.3). The Forward Inner Primer (FIP) consists of the F2 region (3' end) which complements the F2c region, and the F1c region at the 5' end. In contrast, the Backward Inner Primer (BIP) consists of the B2 region (3' end) which complements the B2c region,

and the B1c region at the 5' end. The forward outer primer (B3) with the F3 region complements the F3c region and the backward outer primer (F3) is complementary to the B3c sequence (Eiken Genome Co. Ltd, Tokyo, Japan).

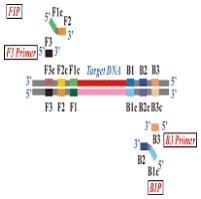


Figure 2.3: Primer target sites for LAMP target sequence (Figure obtained Eiken Genome Co. Ltd, Tokyo, Japan).

Briefly, at 60-65˚C, the FIP primers anneal to the complementary sequence of the double stranded DNA resulting in DNA synthesis which is carried out by using DNA polymerase capable of strand displacement (Figure 2.4 (1)). The strand is displaced and frees a single strand of DNA which is complementary to the template DNA (this starts at the 3' end of the F2 region of the FIP as indicated in Figure 2.4 (2)). The F3 primer anneals to the F3c region on the target DNA, the strand is displaced and a complementary strand linked with FIP is released (Figure 2.4 (3)). The F3 primer and template DNA strand form a double stranded DNA (Figure 2.4 (4)). The complementary strand that is linked to the FIP forms a 'stem-loop' or hairpin structure at the 5' end to the complementary F1c and F1 regions (Figure 2.4 (5)). The stem-loop structure anneals to the BIP primer (3' end) synthesising a complementary DNA strand. At this stage the loop goes back to its linear state. DNA is synthesised and displaced from the BIP and a single strand of DNA is released when the B3 primer anneals to the end of the BIP (Figure 2.4 (6)). During the BIP and B3 step, double stranded DNA is synthesised (Figure 2.4 (7)). The complementary strand (linked to BIP) forms stem-loops at each end which supplies the structure for amplification in the assay (Figure 2.4 (8)). The structure from Figure 2.4 (8) with stem-loops at both ends reverts to a stem-loop and FIP anneals to this single strand which

releases the strand that was synthesised earlier. A stem-loop structure is formed again at the 3' end of the B1c and B1 section of the strand. From the 3' end of the B1 region, DNA synthesis is initiated releasing the complementary strand linked to the FIP (Figure 2.4 (9)). The structure from Figure 2.4 (10) anneals to the single stranded B2c section with the BIP followed by DNA synthesis. Again, the released single stand forms stem-loops at each end with regions that are complementary to the F1-F1c and

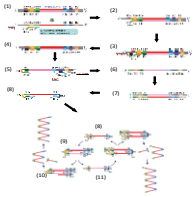


Figure 2.4: Step-by-step process of how LAMP primers work in the assay (Figure obtained Eiken Genome Co. Ltd, Tokyo, Japan)

B1c-B1 section (Figure 2.4 (11)). BIP anneals to the B2c region which releases the B1-primed DNA strand. The final products are stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops. Upto 10^9 copies can be achieved in an hour.

Unlike PCR or real-time PCR, the method does not need the DNA template to be denatured. With the addition of loop primers the reaction can be accelerated even further (Nagamine *et al.*, 2002a,b). These primers will bind to the stem-loops, not including the loop that has been hybridised by the inner primer. LAMP can amplify more DNA than most other methods. The drawback of the technique is the initial design of the primers is complicated. The final product itself is a complex mixture of stem-loop cauliflower-like DNA structures of various sizes but the advanced method was designed to obtain uniform single-stranded DNA (Eiken Genome Co. Ltd, Tokyo, Japan). Specific target sequence is possible due to the 6 different target regions, and theoretically Eiken Chemical Co. Ltd claims that only the target gene can be specifically amplified. However, with the highly conserved 16S sequence of bacteria, it is very important to ensure that the 6 target regions of the sequence from the pathogen of interest does not cross-react with other bacteria. The best way to validate this, like the real-time PCR is to run a cross-reactivity test using DNA template from a variety of bacteria (Ushio *et al*., 2005).

This aim of this chapter was to develop and optimise a real-time PCR assay for the quantification of *Fp* in samples collected from the farm study outlined in Chapter 4. This would allow the pathogen loading in the tank water, fry tissue and eggs to be investigated and to monitor how the loading changes over time. The second aim of this Chapter was to develop and optimise a field-based LAMP assay for the rapid detection of *Fp* in fish tissue and environmental samples from the same study.

2.2 MATERIALS AND METHODS

2.2.1 Real-time quantification (qPCR)

The assay developed here was based on the methodology described by Providenti *et al*., (2006) with modifications. Briefly, the first step in the development of this real-time PCR-based assay to identify *Fp* DNA in infected samples was to extract DNA from the sample and confirm that the sample was positive for *Fp* by nested PCR (Livak *et al*., 1995; Sambrook *et al*., 2001).

2.2.1.1 Nested Polymerase Chain Reaction (PCR)

DNA was extracted from colonies of type strain NCIMB 1947 growing on FLP agar (Appendix: I) using a NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). A nested PCR was carried out using primers synthesised by MWG Biotech (London, UK). For the first round of PCR, universal primers 20F (AGA GTT TGA TCATGG CTC AG) and 1500R (GGT TAC CTT GTT ACG ACT T) which recognise the conserved regions of most eubacterial 16S rRNA were used (Weisburg *et al.,* 1985). Species-specific primers PSY1 (GGT GGC ATC AAC ACA CT) and PSY2 (CGA TCC TAC TTG CGT AG) were used for the second round of PCR (Toyama *et al*., 1994). The nested PCR reactions were amplified using 2x ReddyMix PCR Master Mix (ABgene, Epsom, UK). This mix contained 1.25 units thermoprime plus DNA polymerase; 75 mM Tris-HCL (pH 8.8 at 25˚C); 20 mM $(NH_4)_2SO_4$; 1.5 mM $MgCl_2$; 0.1 % (v/v) Tween®20; 0.2 mM each of dATP, dCTP, dGTP and dTTP; a precipitant and proprietary red dye. The reactions were conducted in 25 µl reactions comprising of 12.5 µl 2x ReddyMix PCR Master Mix together with respective primers and template. For the first round of nested PCR the following were added to the Master Mix: 8 pmol 20F and 1500R, 1 µl of DNA template, and the reaction was made up to 25 µl with dH_2O. The sample was stored at 4˚C overnight. The following day the second round of the nested PCR was performed using the same reagents added to the Master Mix reaction tube exception for substituting 8 pmol of primers PSY1 and PSY2 and 1 µl template from the first round of the nested PCR. The nested PCR

was conducted in a Biometra Thermocycler (Biometra, Göttingen, Germany) and for both rounds of the reaction, samples were denatured at 95˚C for 5 min, followed by 35 cycles of 30 s at 95˚C, 50˚C for 30 s, and 72˚C for 60 s. Following the last cycle, the mixture was incubated for a further 5 min at 72˚C. Amplified products (10 µl) of the second round were detected by electrophoresis (80V, 50 min) using a 1 % agarose gel (ABgene, Epsom, UK.) stained with ethidium bromide (5 µg ml^{-1}) and viewed under ultraviolet (UV) illumination.

2.2.1.2 Purification of nested PCR products

The product from the second round of the nested PCR was purified using a QIAquick PCR purification kit (Qiagen, Crawley UK.), after which the product was run again on a 1 % agarose gel to confirm the product was not lost during purification. The amount of DNA present was determined using a ND-1000 nanodrop spectrophotometer (Labtech Inc., Arizona, USA.). The amount of DNA required for the cloning reaction was determined to be more than 6.85 ng ml^{-1} (Appendix: III), and the concentration obtained and used after purification was 13.7 ng ml^{-1}.

2.2.1.3 Cloning and transformation

The next step in the development of the qPCR was to clone the target DNA as shown in Figure 2.1 for use in a standard curve which would allow the 16S copy numbers from the samples to be determined. This ensures a continuous and reproducible supply of template for the standard curve (Dorak, 2006). The PCR product was ligated into a vector (plasmid) for transformation into a bacterial host. The purified PCR product was cloned using the TOPO TA F' Cloning® kit (Invitrogen, Paisley UK.) with a few modifications to the manufacturer's instructions. One µl of a fresh PCR product was added to 1 µl of salt solution, 3 µl of RNA/DNA-free H_20, and 1 µl of TOP vector in a 0.2 ml thermo PCR tube (Abgene, Epsom UK.) which was gently flicked to mix the contents. The tube was left at room temperature (RT; 20-22°C) for 30 min for the cloning reaction to occur.

The PCR product was inserted into the vector using enzymes that cut open the circular DNA and the PCR product was then inserted into the plasmid (Williams, 2000). A vial of One Shot® Chemically Competent *Escherichia coli* (Invitrogen, Paisley UK.) was used as the host for the transformation. Two µl of the cloning reaction was taken and added to a vial of competent cells. The mixture was left on ice for 25 min. The cells were heat-shocked by placing the vial in a water bath pre-set at 42˚C for exactly 30 s after which it was removed from the water bath and immediately placed back on ice. The vial was shaken prior to adding 250 µl of SOC medium and then vial shaken for 1 h at 37˚C. Thirty µl was taken from the vial and spread onto a pre-warmed Luria–Bertani (LB) agar plate (Appendix: I). The plate was incubated overnight at 37˚C. The following day, a single white colony was picked from the LB plate, added to 4 ml of LB broth (Appendix: I) and left to incubate at 37˚C overnight. The next day the broth was centrifuged at 4500 x *g* for 18 min at 4˚C. The supernatant was discarded and the plasmid was purified using the GenElute™ Plasmid Miniprep Kit (Sigma, Dorset UK.).

2.2.1.4 Sequencing

Once the target DNA has been cloned, the plasmid can be used to create the standard curve for use in the real-time PCR assay. The plasmid was first sequenced to confirm the right target has been cloned and in the correct orientation. The plasmid was sequenced using forward and reverse primers (M13). Sequencing was carried out using CEQ dye terminator cycle sequencing with a quick start kit and protocol (Beckman Coulter, High Wycombe UK.) with modifications. The sequencing reaction was prepared in a 0.2 ml PCR tube containing 2 µl of DNA template (Appendix: II), 1 µl of either forward or reverse primer, and 2 µl of Dye terminator cycle sequencing (DCTS) Quick Start Master Mix. The reaction was mixed thoroughly followed by a rapid pulse at 4000 x *g* to consolidate the mixture. Samples were denatured at 60˚C for 20 s, followed by 30 cycles of 20 s at 50˚C, 60˚C for 4 min, and paused at 4˚C. Stop solution was added (1.25 µl; 3M sodium acetate, 100 mM Na_2EDTA, 20 mg ml^{-1} glycogen) as soon as the tubes were removed from the thermocycler. The

reaction was vortexed before adding 12 µl of cold 95 % (v/v) ethanol/dH_20 stored at -20˚C, vortexed again and immediately centrifuged at 14000 x *g* for 20 min. The supernatant was removed taking care not to disturb the pellet. The pellet was rinsed with 150 µl of 70 % (v/v) ethanol/dH_20 with and immediately centrifuged at 14000 x *g* for 10 min. The supernatant was removed and the pellet allowed to air-dry on the bench at room temperature (RT; 20-22°C) until no alcohol was visible in the tube. The sample was then resuspended in 40 µl of Sample Loading Solution and transferred to the appropriate wells of a CEQ sample plate (Beckman Coulter, High Wycombe UK.), covered with a drop of mineral oil and loaded into the Beckman CEQ 8800 DNA Analysis system (Beckman Coulter) following the manufacturer's instructions. Once the CEQ analysis was complete, the data was analysed using BioEdit® Sequence Alignment Editor (Hall, 1999) for sequence analysis and alignment. Multiple alignment construction and analysis was carried out using Clustal W (Thompson *et al*., 1994) via BioEdit® and the sequences were compared with known *Fp* sequences in GenBank by the BLAST search tool (Altschul *et al*., 1990).

The plasmid containing the insert was also analysed with restriction enzymes to confirm that the gene cloning was in the correct orientation. The restriction enzyme was selected by entering the cloned plasmid sequence into the Lasergene software (DNAstar, Wisconsin USA.) which listed a choice of restriction enzymes that could be used for the *F. psychrophilum* restriction enzyme digestion. Enzyme *Xho I* was selected to cleave the plasmid. Briefly, 1 µl of the plasmid DNA was added to 2 µl of enzyme buffer together with 1 µl of *Xho I*. The reaction was made up to 20 µl and vortexed before incubating for 1 h at 37˚C. Following digestion, 4 µl was taken from the reaction and run on a 1 % agarose gel which was viewed under UV illumination as before.

2.2.1.5 qPCR Primer design

The sequence of the 16S cloned plasmid was aligned individually with the sequences of a variety of *Flavobacterium* and non-*Flavobacterium bacteria* (Table 2.1) using the Clustal W program (Thompson *et al*., 1994).

Table 2.1: List of bacterial strains used for Clustal W in determining sequences specific for *Flavobacterium psychrophilum*.

Species	Isolate	Accession
Flavobacterium johnsoniae	Jm162a	DQ256490
Flavobacterium aquatile	DSM 1132	AM230485
Flexibacter maritimus	NCIMB 2154	D14023
Flavobacterium columnare	EK-28	AB016515
Flavobacterium branchiophilum	IFO 15030	D14017
Flavobacterium flevense	DSM 1076	AM230486
Flavobacterium hydatis	ATCC 29551	M58764
Chryseobacterium indologenes	LMG 8337	AM232813
Aeromonas hydrophila	WAB1875	AM184217
Edwardsiella ictaluri	JCM1680	AB050826
Aeromonas salmonicida	NKCM8904	AB211227
Aeromonas sobria	U75	DQ205458
Aeromonas veronii	B1	AF099024
Bacillus cereus	C10-1	AB244465
Yersinia ruckeri	ATCC 29473	X75275
Lactobacillus plantarum	2.9	AM279758
Micrococcus luteus	B-P 26	AB079788
Pseudomonas anguilliseptica	K29410	DQ298027
Pseudomonas aeruginosa	ESA-5	DQ641680
Serratia sp.	CI20	DQ530081
Bacillus subtilis	A32	DQ631809
Corynebacterium aquaticum	JCM 1368	D45057
Photobacterium damselae subsp. Piscicida	I736	AY147859
Vibrio ordalii	6.30	AF493809
Nocardia asteroides	ATCC 14795	DQ659900
Pseudomonas fluorescens	A1XB1-4	AY512614
Vibrio vulnificus	MP-4	AY911393
Renibacterium salmoninarum	ATCC33209	AF180950
Enterococcus faecalis	RO90	AF515223

2.2.1.6 Selection of Fp *specific primers at its optimum annealing temperature*

The first step for choosing the primers for the qPCR was to determine the optimum annealing temperature for each set of primers. This was done by running a 20˚C temperature gradient (45˚C - 65˚C) in a PCR thermocycler with the standard PCR protocol as described in Section 2.2.1.1, substituting the first round of PCR primers with each of these two sets. The PCR was run on a 1 % agarose gel, viewed by UV illumination and the best temperature for each primer set selected by eye based on the temperature which gave the clearest band. The two selected primer sets (Table 2.2) were then used in a standard PCR reaction, testing DNA template from the bacteria listed in Table 2.3 for primer specificity. The primer set that reacted with bacteria other than *Fp* were disregarded and the primer set that reacted only with *Fp* was used to optimise a qPCR reaction.

Table 2.2: Primer sets selected from Clustal W alignment of *Flavobacterium psychrophilum* sequences to determine sites specific for *Flavobacterium psychrophilum*.

Pair	Primer	Sequence
1	spec1R	GCCCATCGCTCAACGATGG
1	spec1F	ACTACCTCGTGAGGTAGC
2	spec2R	GCTACCTCACGAGGTAGT
2	spec2F	CAGCGAGTCATGTCGGGA

Table 2.3: List of bacterial strains used for testing specificity of *Flavobacterium psychrophilum* primers for the qPCR and LAMP assay.

Species	Isolate
Escherichia coli	NCIMB 86[a]
Edwardsiella ictaluri	NCIMB 13272
Aeromonas hydrophila	NCIMB 9240
Aeromonas salmonicida	NCIMB 1102

Aeromonas sobria	NCIMB 12065
Bacillus subtilis	NCIMB 3610
Corynebacterium spp.	NCIMB 2025
Bacillus cereus	ATCC 11778[b]
Yersinia ruckeri	NCIMB 1316
Vibrio ordalii	NCIMB 2167
Lactobacillus plantanum	NCIMB 1406
Listonella anguillarum	NCIMB 6
Micrococcus luteus	NCIMB 8553
Nocardia asteroides	NCIMB 1290
Pseudomonas anguilliseptica	NCIMB 2185
Pseudomonas fluorescens	NCIMB 1953
Pseudomonas aeruginosa	ATCC 27853
Renibacterium salmoninarum	NCIMB 1112
Edwardsiella tarda	NCIMB 2034
Photobacterium damselae subsp. piscicida	ATCC 11778
Vibrio vulnificus	ATCC 27562
Serratia sp.	B96234[c]
Flavobacterium branchiophilum	NCIMB 2537
Flavobacterium columnare	NCIMB 2248
Flavobacterium johnsoniae	ATCC 29585
Flavobacterium aquatile	NCIMB 2215
Flavobacterium flevense	NCIMB 12056
Flavobacterium hydatis	NCIMB 2215
Flexibacter maritimus	NCIMB 2153
Flavobacterium indologenes	NCIMB 2533

[a] NCIMB: The National Collection of Industrial, Marine and Food Bacteria. NCIMB Ltd. Aberdeen, UK

[b] ATCC: American Type Culture Collection. Middlesex, UK.

[c] Landcatch Ltd. Argyll, UK.

2.2.1.7 Optimisation and selection of qPCR assay parameters

Optimum primer concentration and annealing temperature were performed using DNA extracted from the *Fp* type strain NCIMB 1947 as described in Section 2.2.1.1. A serial dilution was

performed on this DNA and tested in both PCR and qPCR to compare the sensitivities of the two. The signal in this study was generated by the fluorophore SybrGreen™ (Abgene, Epsom UK.) to the DNA. The SYBR green master mix recommends the primers be used at a concentration of 7 pmol μl^{-1} (a gradient of 6 pmol μl^{-1} – 8 pmol μl^{-1} was tested and 7 pmol μl^{-1} was found to be optimum).

2.2.1.8 Standard curve and qPCR assay

The assay was based on the method by Providenti *et al*., (2006) with modifications. To evaluate the performance of the chosen primer set, a serial dilution (e.g. 10-fold dilution of plasmid DNA for 5 to 7 log) of the target DNA was analysed using these primers. The standard curve was run following the method recommended by Invitrogen using the plasmid DNA after confirming successful inclusion of the *Fp* gene. After spectrophotometric determination of plasmid DNA concentration, the copy number of standard DNA molecules can be calculated using the following formula:

(X g/µl DNA / [plasmid length in base pairs x 660]) x 6.022 x 1023 = Y molecules/µl

The highest detection limit of the assay was 10^8 copies $well^{-1}$ determined from the standards used and the lowest detection limit was 10^3 copies $well^{-1}$. Using Absolute SYBR green master mix (Abgene, Epsom UK.) the amplification procedure followed a three-step PCR with 20 s denaturation (94°C), 20 s annealing and elongation at 61°C (60˚C - 63˚C was tested with the temperature selected from the PCR gradient above) for 120 s. Baseline and threshold values were automatically calculated by the Quantica software. Validation of the qPCR was performed using triplicate samples of DNA, ranging from 30 ng μl^{-1} from water and tissue samples (all confirmed as *Fp* positive by nested PCR in Chapter 4) and added to the assay. Melt curve data of the products from the assay was obtained by the software and analysed to test for cross-reactivity from the samples in Table 2.4.

Table 2.4: List of *Flavobacterium psychrophilum* isolates tested with the selected primers (second set) by nested PCR, qPCR and LAMP.

Isolate	Fish species	Tissue	Origin	Source
NCIMB	Coho	Kidney	USA	NCIMB
UP193/97	Rainbow	Spleen	UK	CEFAS
B97026	Rainbow	Lesion	UK	IOA
59/95	-	-	Chile	UAC
32/97	Rainbow	Kidney	Chile	UAC
904/10	Rainbow	Spleen	UK	CEFAS
JIP 02/86	Rainbow	Kidney	France	INRA
DI01	Rainbow	Spleen	UK	IOA
DI-EGG	Rainbow	Egg	UK	IOA
DII01	Rainbow	Spleen	UK	IOA
DII-	Rainbow	Egg	UK	IOA
IM01	Rainbow	Spleen	UK	IOA
IM-	Rainbow	Egg	UK	IOA
TL01	Rainbow	Spleen	UK	IOA
TL-	Rainbow	Egg	UK	IOA
MOF17K	Rainbow	Kidney	UK	IOA
MOF25	Rainbow	Spleen	UK	IOA
ARF05	Rainbow	Lesion	UK	IOA
ARF06	Rainbow	Spleen	UK	IOA

NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, UK; CEFAS: Centre for Environment, Fisheries & Aquaculture Science, Dr. Rachel E. Rangdale, UK; IOA: Institute of Aquaculture, University of Stirling, Scotland, UK; UAC: Universidal Austal de Chile, Dr. Carlos Farais, Chile; INRA: L'Institut national de la recherche agronomique, Dr. J. F. Bernardet, France; Coho salmo (*Oncorhynchus kisutch*) and Rainbow trout (*Oncorhynchus mykiss*).

The sensitivity of the assay was compared to that of the nested PCR using the plate count method described by Vatsos (2001). Briefly, a bacterial suspension was diluted 10-fold ten times with fresh FLP media. Eggs, kidney and spleen samples were spiked with these dilutions, the DNA extracted (Section 2.2.1.1) before analysing the samples by qPCR and nested PCR. Six replicate drops (20 µl $drop^{-1}$) from each dilution were placed onto a FLP agar plate previously divided into six sections. The plates were allowed to dry before incubating at 15˚C for at least 96 h before colonies

were counted. The average number of colonies per drop was counted and the CFU ml^{-1} was determined for the bacterial suspension using the following equation:

CFU ml^{-1} = colony numbers x dilution factor x 50

2.2.2 LAMP

2.2.2.1 Primer design

The same sequence of DNA as used in the qPCR were used here for the LAMP assay primer design. The sequence was run through PrimerExplorer Version 3 program using the pre-set settings suggested by the manufacturer for optimum primer design and included the following criteria:

1) The distance between 5' end of F2 and B2 should be 120-180bp.
2) The distance between F2 and F3 and B2 and B3 is 0-20bp.
3) The distance for loop forming regions (5' of F2 to 3' of F1, 5' of B2 to 3' of B1) is 40-60bp.
4) The melting temperature for the primer regions is around 60-65°C.
5) The GC content should be around 50-60 %.
6) Primers should be designed so secondary structures do not easily form. The 3' end sequence should not be AT rich or complementary to other primers.

Over 50 primer sets were generated by PrimerExplorer, and each primer sequence was run through NCBI Blast, with 2 sets of primers found to contain specific target sites for *Fp* using Clustal W work as carried out for the qPCR. Two sets of six primers were selected and synthesised by MWG Biotech (London, UK.) (Table 2.5).

Table 2.5: Primer sequences generated by PrimerExplorer v.3 for using in the LAMP assay.

Set	Primer	Sequence
1	FIP	AGGGCCGTGATGATTTGACGTCACTCTAGCAAGACTGCCAGT
1	BIP	GTACAGAGAGCAGCCACTACGCGCAGACTCCGATCCGAACT
1	F3	TGCCAGCGAGTTATGTTGG
1	B3	AGCTTCACGGAGTCGAGTT
1	LF	ATCCCCACCTTCCTCACAGTTTAC
1	LB	TCTAGCAAGACTGCCAGTGTA
2	FIP	AGGGCCGTGATGATTTGACGTCACTCTAGCAAGACTGCCAGT
2	BIP	GTACAGAGAGCAGCCACTACGCGCAGACTCCGATCCGAACT
2	F3	TGCCAGCGAGTTATGTTGG
2	B3	CCAGCTCACGGAGTCGA[1]
2	LF	ATCCCCACCTTCCTCACAGTTTAC
2	LB	TCTAGCAAGACTGCCAGTGTA

[1] Primer B3 was the only difference between the two sets

2.2.2.2 LAMP assay

The assay was based on the method published by Savan *et al.* (2005) for the rapid detection of *E. tarda*. The same DNA template as used in the nested PCR carried out to generate plasmid in the cloning reaction of the qPCR assay for the LAMP optimisation. A 25 µl reaction was set up in a PCR tube (Abgene, Epsom UK.) containing 40 pmol of FIP and BIP, 5 pmol of F3 and B3, 5 pmol of LF and LB, 12.5 µl 40 mM Tris-HCl (pH 8.8) (Sigma, Dorset UK.), 20 mM KCl (Sigma, Dorset UK.), 18 mM $MgSO_4$ (Sigma, Dorset UK.), 20 mM $(NH)_4SO_4$ (Sigma, Dorset UK.), 0.1 % Tween-20 (Sigma, Dorset UK.), 1.6 M betaine (Sigma, Dorset UK.), 1 µl of 2.8 mM deoxynucleotide triphosphates (dNTPs) (Promega, Southampton UK.), *Bst* DNA polymerase (New England BioLabs, Hertforshire UK.), and finally 1 µl of template DNA. The reaction tube was mixed by gently flicking the tube and incubated at 65°C for 45 min followed by 2 min at 80°C to stop the reaction. SYBR

Green dye (0.5 µl; 1:10 dilution at a concentration of 10,000x; Sigma, Dorset UK.) was added to each reaction tube. Based on the results of this assay, a range of different parameters were tested to optimise the assay including incubation temperature, $MgS0_4$ concentration, Betaine concentration, DNA template and dNTP concentration (Table 2.6).

Once the optimum assay condition was determined, serial dilutions of DNA were tested by LAMP to determine sensitivity of the assay. Isolates from Table 2.3 were also analysed to examine the specificity of the assay. The sensitivity of the assay was compared to that of the nested PCR by spiking samples and using the plate count method described in Section 2.2.1.8. A change in colour from orange to yellow indicated that the reaction was positive, while no change in colour meant the reaction was negative (Figure 2.5).

Table 2.6: Parameters tested for the optimisation of a LAMP assay to detect *Flavobacterium psychrophilum*.

Sample	dNTP (µl)	Bst (µl)	rx buffer (µl)	Betaine (µl)	Oligo's (µl)	MgS (µl
1	0.5	1	2.5	8	6	1
2	0.8	1	2.5	8	6	1
3	1	1	2.5	8	6	1
4	0.5	1	2.5	8	6	0
5	0.8	1	2.5	8	6	0
6	1	1	2.5	8	6	0
7	0.5	1	2.5	8	6	1
8	0.8	1	2.5	8	6	1
9	1	1	2.5	8	6	1
10	0.5	1	2.5	8	6	0
11	0.8	1	2.5	8	6	0
12	1	1	2.5	8	6	0
13	0.5	1	2.5	8	6	1
14	0.8	1	2.5	8	6	1
15	1	1	2.5	8	6	1
16	0.5	1	2.5	8	6	0
17	0.8	1	2.5	8	6	0
18	1	1	2.5	8	6	0
19	0.5	1	2.5	8	6	1
20	0.8	1	2.5	8	6	1
21	1	1	2.5	8	6	1
22	0.5	1	2.5	8	6	0
23	0.8	1	2.5	8	6	0
24	1	1	2.5	8	6	0

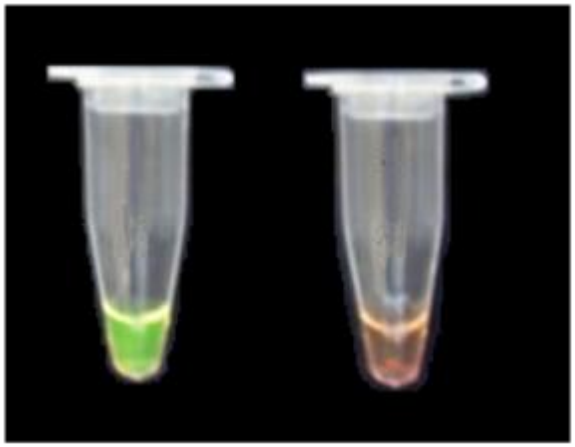


Figure 2.5: Colour change of LAMP assay; yellow colour indicates positive reaction whereas orange colour indicates a negative reaction.

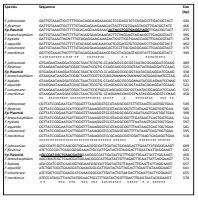
2.3 RESULTS

2.3.1 qPCR

2.3.1.1 Selection of primer set for qPCR assay

A selection of *Flavobacterium* isolates were aligned against the *Fp* plasmid sequence to determine sites specific for *Fp* which could be used as potential primer sets (Table 2.7).

Table 2.7: Primer selected for qPCR using CLUSTAL W alignment of *Flavobacterium* sequences.



The final primer set selected for the qPCR underlined in Table 2.7 above, is primer Set 2 from Table 2.5. The cross-reactivity of the first set of primers detected all bacteria and the second set only detected *Fp* (Figure 2.6). The highest temperature at which the primers detected *Fp* was 60.9˚C and therefore 61˚C was selected as the optimum annealing temperature for the assay (Figure 2.7).

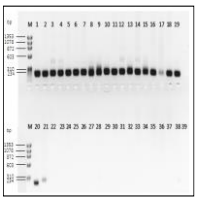


Figure 2.6: Electrophoresis gel of PCR products to test the specificity of two sets of primers designed for the *Flavobacterium psychrophilum* qPCR assay. M = IX Roche molecular ladder. Lanes 1 to 20 are using the first set of primers (181 bp) to test DNA samples from *F. psychrophilum, E. coli, E.ictaluri, P. fluorescens, R. salmoninarum, F. branchiophilum, F. columnare, F. johnsoniae, F. aquatile, F. flevense, F. hydatis, F. maritimus, F. indologenes.* The isolates were tested with the second set of primers in Lane 21 to 39 (222 bp) are from *F. psychrophilum, E. coli, P. fluorescens, R.*

salmoninarum, F. branchiophilum, F. columnare, F. johnsoniae, F. aquatile, F. flevense, F. hydatis, F. maritimus, F. indologenes.

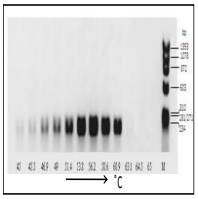


Figure 2.7: Electrophoresis gel of *Flavobacterium psychrophilum* PCR amplicons produced using different temperatures in the qPCR, as indicated as a band at 222 bp on a 1 % agarose gel. M = IX Roche molecular ladder.

2.3.1.2 Sensitivity of qPCR assay

The standard curve had a high level of efficiency (E = 1.96; Figure 2.8 (a)) when the plasmid standards run were between 10^{-8} to 10^{-3} copies well^{-1}, although this dropped when lower dilution standards were used (E = 1.61; Figure 2.8 (b)). The sensitivity or detection limit of the assay

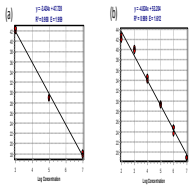


Figure 2.8: Standard curve for *Flavobacterium psychrophilum* plasmid dilution selection: (a) 10^7, 10^5 and 10^3 plasmid copies well^{-1} (b) 10^7, 10^5, 10^4, 10^3 and 10^2 copies well^{-1}.

was found to be 10^3 copies well^{-1} as determined from the standard curve shown in Figure 2.9. Lower dilutions of the standard curve were not always detected or reproducible.

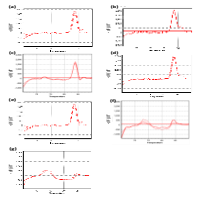


Figure 2.9: Plasmid standard curve to establish detection limit for qPCR assay (a) 10^8, (b) 10^7, (c) 10^6, (d) 10^5, (e) 10^3, (f) 10^2 and (g) 10 copies well^{-1}.

As only 3 standards are required to run an assay, the 10^7, 10^5 and 10^3 copy well^{-1} dilutions were selected to maintain a high efficiency for the assay. When the temperature of the qPCR was lowered by 1˚C to 62˚C, the repeatability of readings for the detection of plasmid copies increased. The sensitivity of the qPCR for eggs, kidney and spleen spiked with known quantities of *Fp* was determined to be 237 CFU mg^{-1}, 184 CFU mg^{-1} and 192 CFU mg^{-1}, respectively, while for nested PCR it was 14 CFU mg^{-1}, 11 CFU mg^{-1} and 13 CFU mg^{-1}. For bacterial cultures the obtained detection limit of *Fp* was 172 CFU ml^{-1} by qPCR and 9 CFU ml^{-1} by nested PCR while for water samples spiked with known quantities of *Fp* the sensitivity was determined to be 220 CFU ml^{-1} by qPCR and 11 CFU ml^{-1} by nested PCR.

The melting curve obtained for the optimised assay is shown in Figure 2.10, with the blue lines indicating background primer-dimer between 70˚C and 75˚C, although this can be disregarded as it is above the –dF/dT threshold i.e. before the melting cure analysis which took place between 80˚C and 85˚C. The red lines (plasmid standard curve) peaked at the same point as the green lines, indicating that the samples were positive for *Fp*.

2.3.1.3 Specificity of qPCR primers for the detection of Fp

DNA from the non-*Flavobacterium* samples was not detected in the qPCR assay. However bacteria from two other *Flavobacterium* species were detected at low levels (Table 2.8; Figure 2.11 i.e. *F. aquatile* and *F. johnsoniae*). The average copy numbers well^{-1} detected were 1.37E+06 for the positive control (*Fp* DNA sample), 1.82E+02 for the negative control (water), while values of 2.07E+04 were detected for *F. aquatile* and 4.08E+03 for *F. johnsoniae*. A slight peak was detected above the background threshold values for *F. aquatile* and *F. johnsoniae* as shown in Figure 2.11 (a)

and 2.11 (g) respectively. The readings for all the other *Flavobacterium* species that were not *psychrophilum* are shown in Table 2.8; none of these were detected by the assay.

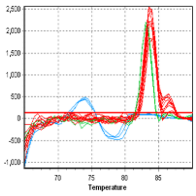


Figure 2.10: Melting curve analysis of qPCR for the detection of *Flavobacterium psychrophilum.* Red lines indicate plasmid standards, blue lines indicate non-template control (water) and green lines indicate sample tested.

Table 2.8: Average copy numbers detected by testing primer specificity on *Flavobacteria* species for the qPCR assay.

Sample	Average copies of DNA well^{-1}	% cross-reactivity to ***F. psychrophilum*** (+ve
Standard 10^7 (plasmid µl^{-1})	1.09E+07	-
Standard 10^5 (plasmid µl^{-1})	9.53E+04	-
Standard 10^3 (plasmid µl^{-1})	1.09E+03	-
F. aquatile (30 ng µl^{-1})	2.07E+04	1.51 %
F. branchiophilum (30 ng µl^{-1})	8.77E+02	0.06 %
F. columnare (30 ng µl^{-1})	3.24E+03	0.24 %
F. flevense (30 ng µl^{-1})	8.04E+02	0.06 %
F. hydatis (30 ng µl^{-1})	7.58E+02	0.06 %
F. johnsoniae (30 ng µl^{-1})	4.08E+03	0.30 %
F. indologenes (30 ng µl^{-1})	7.24E+01	0.01 %
F. psychrophilum (+ve control; 30 ng µl^{-1})	1.37E+06	100 %
Non template control (-ve control; 30 ng µl^{-1})	1.82E+02	0.01 %

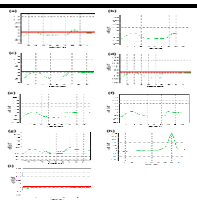


Figure 2.11: Testing primer specificity in the qPCR assay using different *Flavobacteria* species: (a) *Flavobacterium aquatile* (b) *Flavobacterium branchiophilum* (c) *Flavobacterium columnare* (d)

Flavobacterium flevense (e) *Flavobacterium hydatis* (f) *Flavobacterium indologenes* (g) *Flavobacterium johnsoniae* (h) *Flavobacterium psychrophilum* (i) negative control (water).

When the annealing temperature was lowered by a degree the detection limit decreased from 10^3 to 10^4 copies well^{-1} (Figure 2.12 (a) and 2.12 (b)). By lowering the temperature, the two *Flavobacterium* species previously detected by the assay were no longer detected (Figure 2.12 (c) and 2.12(d)).

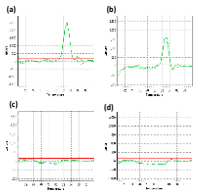


Figure 2.12: Effect of lowering annealing temperature of qPCR assay by 1˚C to 62˚C: (a) results for *Flavobacterium psychrophilum* prior to lowering the temperature by 1˚C from 63˚C; (b) results for *Flavobacterium psychrophilum* after lowering the *t*emperature by 1˚C to 62˚C; (c) results for *Flavobacterium aquatile* after lowering the temperature by 1˚C to 62˚C; (d) results for *Flavobacterium johnsoniae* after lowering the temperature by 1˚C to 62˚C.

2.3.2 LAMP assay

2.3.2.1 Optimisation of LAMP assay for the detection of Fp

The results from testing different assay parameters for the LAMP assay are shown in Table 2.9. The different shades of colour represent the colour observed by eye after SYBR Green was added to each tube (i.e. yellow being positive, orange being negative). Assay parameters 3 and 21 were found to give the strongest positive reaction and therefore were used in the assay protocol (i.e. 1 µl of dNTP, 1 µl Bst, 2.5 µl rx buffer, 8 µl betaine, 6 µl of Oligo's, 1 µl of $MgSO_4$, 2 µl of DNA template, 3.5 µl of water and the reaction was run at 63˚C (reaction 3) and 65˚C (reaction 21). A

decrease in reaction was noticed when less than 0.8 µl of dNTP was used whereas reactions improved with the addition of 1 µl of $MgSO_4$.

2.3.2.2 Specificity of LAMP primers to other bacterial species

Reactions 3 and 21 were selected for further optimisation. A variety of *Flavobacterium* sp. and non-*Flavobacterium* indicated in Table 2.3 was tested using the parameters shown for Reaction 3 and 21 (Table 2.10). The conditions used for Reaction 3 reacted with quite a few bacterial species other than *Fp*, whereas there was only a slight cross-reaction with *F. columnare* and *F. branchiophilum* when the protocol for Reaction 21 was used (Table 2.10). The tubes changed colour from orange to a dark yellow in the presence of these two bacteria, however, the yellow was not as bright as the yellow colour observed when the tube contained *Fp*. *Flavobacterium columnare* and *F. branchiophilum* were still detected when the temperature of the LAMP assay was increased or lowered.

Table 2.9: Reaction of the LAMP using different assay parameters assessed by eye.

Tube [1]	dNTP (µl)	Bst (µl)	rx buffer (µl)	Betaine (µl)	Oligo's (µl)	$MgS0_4$ (µl)	
1	0.5	1	2.5	8	6	1	
2	0.8	1	2.5	8	6	1	
3	1	1	2.5	8	6	1	
4	0.5	1	2.5	8	6	0	
5	0.8	1	2.5	8	6	0	
6	1	1	2.5	8	6	0	
7	0.5	1	2.5	8	6	1	
8	0.8	1	2.5	8	6	1	
9	1	1	2.5	8	6	1	
10	0.5	1	2.5	8	6	0	
11	0.8	1	2.5	8	6	0	
12	1	1	2.5	8	6	0	
13	0.5	1	2.5	8	6	1	

14	0.8	1	2.5	8	6	1	
15	1	1	2.5	8	6	1	
16	0.5	1	2.5	8	6	0	
17	0.8	1	2.5	8	6	0	
18	1	1	2.5	8	6	0	
19	0.5	1	2.5	8	6	1	
20	0.8	1	2.5	8	6	1	
21	1	1	2.5	8	6	1	
22	0.5	1	2.5	8	6	0	
23	0.8	1	2.5	8	6	0	
24	1	1	2.5	8	6	0	

[1] Orange shading indicates negative result, yellow shading indicates positive result. Degrees of variation in colour between orange and yellow observed are also highlighted.

Table 2.10: List of bacterial strains used for testing specificity of *Flavobacterium psychrophilum* primers using Reaction 3 and 21 of the LAMP assay.

Species	Positive/negative by LAMP (Reaction 3)	Reaction 3 colour [1]	Positive/negative by LAMP (Reaction 21)	Reaction 21 colour
Escherichia coli	-		-	
Edwardsiella	-		-	
Aeromonas	-		-	
Aeromonas	-		-	
Aeromonas	-		-	
Bacillus subtilis	-		-	
Corynebacterium	-		-	
Bacillus cereus	-		-	
Yersinia ruckeri	-		-	
Vibrio ordalii	-		-	
Lactobacillus	-		-	
Listonella	-		-	
Micrococcus	-		-	
Nocardia	-		-	
Pseudomonas	-		-	
Pseudomonas	-		-	
Pseudomonas	-		-	
Renibacterium	-		-	
Edwardsiella	-		-	
Photobacterium	-		-	
Vibrio vulnificus	-		-	
Serratia sp.	-		-	
Flavobacterium	+		-/+	
Flavobacterium	+		-/+	
Flavobacterium	-/+		-	
Flavobacterium	-		-	
Flavobacterium	+		-	
Flavobacterium	-		-	
Flexibacter	-		-	
Flavobacterium	+		-	

[1] Orange shading indicates negative result, yellow shading indicates positive result. Degrees of variation in colour between orange and yellow observed are also highlighted.

2.3.2.3 Sensitivity for LAMP assay to detect Fp

The sensitivity of the LAMP assay was determined using the parameters for Reaction 21 as this protocol resulted in the lowest cross-reactivity with non-*Fp* bacteria. Egg, kidney, spleen and water samples were spiked with known quantities of *Fp* and the detection limit was determined to be 18 CFU mg^{-1}, 22 CFU mg^{-1}, 25 CFU mg^{-1} and CFU 16 ml^{-1},respectively, while for bacterial cultures it was 14 CFU ml^{-1}.

2.4 DISCUSSION

The development of molecular tools to detect fish pathogens has advanced greatly over recent years, which in turn has helped in the rapid diagnosis of fish disease. This is essential to allow speedy treatment of disease outbreaks to limit mortality in farming systems and also to help prevent the spread of the pathogen (Cunningham, 2002). The tools and equipment required for molecular diagnosis can be expensive, with the application of techniques requiring trained staff and facilities of a high standard (Cunningham, 2002). The development of methods that are suitable for on-site diagnosis at fish farms has been sought by the aquaculture industry for many years (Anderson, 1998).

Primer design is a crucial step in the development of nucleic acid amplification assays be it traditional PCR, qPCR, LAMP or any other DNA-based assay, especially since specificity is essential for these assays (Casali and Preston, 2003; Pham *et al*., 2005). The primer-design for *Fp* was very difficult. Selection of optimal primers for the qPCR and LAMP assay was restricted due to the limited parts of the 16S sequence that are specific to only *Fp*. The best primers to use for both assays were of course primers that did not detect bacteria other than *Fp*. Primers for both assays were selected on the basis of specificity, which in turn reduced the quality and choice of primers available and in turn the sensitivity of the assays. The qPCR assay had limitations with regards to both sensitivity and specificities while the LAMP's sensitivity was only slightly lower than the nested PCR (12 CFU ml^{-1} by LAMP for bacterial culture and 9 CFU ml^{-1} by nested PCR), but it also showed cross-reactivity with the two other *Flavobacterium s*pecies.

The primers selected for the qPCR assay affected the outcome of the assay in many ways. Firstly, the sensitivity of the standard curve was limited to 10^3 copies $well^{-1}$. The obtained detection limit of *Fp* obtained by qPCR was 237 CFU mg^{-1}, 184 CFU mg^{-1} and 192 CFU mg^{-1} for eggs, kidney and spleen tissue respectively while for bacterial culture it was 172 CFU ml^{-1} by qPCR. In contrast, the detection limit by nested PCR was 14 CFU mg^{-1}, 11 CFU mg^{-1}, 13 CFU mg^{-1} and 9 CFU ml^{-1} for eggs, kidney and spleen tissue and bacterial culture respectively. Water samples spiked with a known

quantity of quantity of *Fp* were also detected at a lower level by qPCR (220 CFU ml^{-1}) in comparison to 11 CFU ml^{-1} by nested PCR.

Optimising the annealing temperatures by decreasing the temperature by 1˚C to 62°C helped reduce the cross-reactivity to negligible levels, though this reduced the sensitivity of the assay even further to 10^4 copies $well^{-1}$. Rondini *et al*. (2003) developed a qPCR Assay for the quantification of *Mycobacterium ulcerans* DNA and reported the detection limit to be 0.2 genome copies ml^{-1} (4 copies $well^{-1}$) making it 10 times more sensitive than the conventional PCR. Another assay which quantified the amount of *A. salmonicida* in fish tissue found similar sensitivity levels to that of the PCR with sensitivity of around 16 copies ml^{-1} (Balcázar *et al*., 2006).

It has been reported by several authors that the annealing temperature is the most important step in determining the specificity of the assay. At lower temperatures, the primers could anneal to similar though irrelevant sequences elsewhere in the genome (Martin and Timmers, 1997). Though the genomic material of some bacterial species such as *E. coli* has been completely sequenced, the sequencing of *Fp* was completed after this study was performed (Duchaud *et al*., 2007). When a sequence from an organism is incomplete, the primer sequences may be present in another species despite being absent in the sequence database (Martin and Timmers, 1997). This could possibly explain why the qPCR primers appeared to be specific when selected from the NCBI Blast database, and they were specific in traditional nested PCR but not specific in the qPCR assay.

The specificity of the assay can also be increased by reducing the concentration of primers, although in this case this was difficult as the assay already had sensitivity problems, and decreasing the primer concentration would have reduced the sensitivity of the assay even further. When the annealing temperature of the qPCR assay was increased by 1˚C, the detection limit of the assay, and therefore the sensitivity, dropped by ten-fold. To obtain optimum results, each step of the procedure from DNA isolation through to cloning and assay optimisation is important. There were only two other possible options available after having optimised the assay i.e. to design more primers from the cloned sequence or start over and use a Taqman based probe rather than SYBR green. Minor

differences in the efficiency of amplification among samples can give rise to markedly different amounts of the final product, due to the exponential nature of amplification (Bustin and Nolan, 2004).

As the primers used were selected from the few *Fp*-specific regions of the 16S sequence, it was very difficult to find another set suitable for qPCR as the remaining primer sequences detect other *Flavobacterium* species as indicated from the NCBI Blast search. The SYBR Green probe used was also found to have some disadvantages. It was the cheapest probe available commercially, however the method can lack discrimination between amplicon-specific and unspecific DNA in the reaction tube (Winn *et al*., 2006). Melt curve analysis is used to verify the specificity of the resulting product using SYBR Green which can potentially separate unspecific sites from the specific product and can therefore increase the sensitivity of the assay (Winn *et al*., 2006).

Another method to increase the specificity of the qPCR is the use of additional hybridisation oligonucleotides with the two PCR primers (McClatchey, 2002), the advantage of this being that internally hybridising fluorescence-labelled probes can increase target sequence specificity and amplification efficiency of the assay. With well-designed primers, SYBR Green is known to work extremely well, with non-specific background only showing in the exponential phase of the amplification (Nitsche, 2007). The reason that SYBR Green was selected here, apart from being the simplest method for qPCR, was if it had worked there would have been no need for further assay optimisation or having to switch to a more time-consuming and/or expensive method. Although melting curve analysis with SYBR Green is sometimes considered less specific compared to the use of fluorescent probes, the need for more expensive probes is not always necessary when conditions of amplification have been correctly optimized (especially primer sequences and concentrations) (Nitsche, 2007). In aquaculture, the introduction of real-time PCR amplification methods using SYBR Green to detect pathogens has made detection of bacterial pathogens such as *Vibrio parahaemolyticus* and *Vibrio vulnificus* in oyster samples rapid and cost-effective (Blackstone *et al*., 2003; Panicker *et al*., 2004). Goarant and Merien (2006) also developed a SYBR Green based qPCR

assay for the quantification of *Vibrio penaeicida*, the etiological agent of Syndrome 93 in New Caledonian shrimp.

The quantification of bacteria can also be performed using Taqman probes. A quantitative TaqMan PCR assay was developed to assess the presence of *Ovipleistophora ovariae* in the tissues of the cyprinid fish *Notemigonus crysoleucas* (golden shiner; Phelps and Goodwin., 2007). Overturf and LaPatra (2006) designed specific primers and probes for the detection of two IHNV sequences from samples of contaminated water and infected fish. Though the assay was developed successfully, the drawback was that the sensitivity of the assay was limited to one thousand copies in water samples. A sensitive TaqMan assay was also developed for the detection and differentiation of three subtypes of salmonid alphavirus (salmon pancreas disease virus, sleeping disease virus and Norwegian salmonid alphavirus; Hodneland and Endresen, 2006).

A major disadvantage of real-time PCR is the need for a special thermocycler and expensive reagents. LAMP, on the other hand, does not require special equipment and allows rapid, specific, and sensitive detection of the pathogen. It also offers the possibility of a field test. It was difficult to select primers for the LAMP assay for two reasons. Firstly, due to the high numbers of primers used in the assay even though at times only one of the six primers was actually different to other sets, and secondly the sites specific to *Fp* in the sequence could not be selected by the software for the primers to be designed on. There is now an updated version of Primer Explorer (version 4) which allows this. The 16S sequence of *Fp* that was cloned to produce the standard curve for the qPCR was run in Primer Explorer Version 3 to produce 100 different possible primer sets, and finally the primer set selected included sequences that only matched with *Fp* sequences indicated in NCBI Blast search before carrying out the LAMP assay and testing for specificity in the actual LAMP assay.

This is similar to how primer sets were selected for other LAMP assays reported in the literature (Kamachi *et al*., 2006; Mekata *et al*., 2006). However since LAMP is a fairly new technique, publications highlighting problems with assay development are very limited. For the

LAMP assay, testing more parameters could increase the sensitivity and specificity of the assay. Like the qPCR assay, the sensitivity and specificity of LAMP depends on the primer selection.

The assay depends primarily on primer design and optimisation of primers; finding a balance between the two is not always straightforward. The high sensitivity of the LAMP system makes it susceptible to false positives because of carry-over or cross-contamination (Savan *et al*., 2005). Savan *et al*. (2005) suggested that the assay should be carefully validated to guarantee that the primers only amplify the target sequence of the specific pathogen. With the cross-reaction of the *F. columnare* and *F. branchiophilum* albeit at a low level the only option seemed to be to design other primers for the assay. Yeh *et al*. (2006) developed the LAMP assay for the rapid detection of *F. columnare* and reported the assay to be specific. However, it seems that *F. aquatile* and *F. johnsoniae* were the only bacteria belonging to the *Flavobacterium* species that were actually tested in their study. Regarding sensitivity of the assay, the LAMP assay had sensitivity levels similar to those obtained in the nested PCR for *Fp*. Previous studies have reported that LAMP can detect target DNA at levels as low as 6 copies (Nagamine *et al*., 2001). Reports on the detection levels of fish pathogens have varied from 10 to 10^3 CFU ml^{-1} for *E. tarda* to 20 CFU ml^{-1} for the detection of *E. ictaluri* (Yeh *et al*., 2005). They used the same amount of DNA in the traditional PCR and the LAMP assay using the F3 and B3 primers to compare the two methods. Ikadai *et al*. (2004) assessed the diagnostic potential of a LAMP assay to detect *Babesia gibsoni* parasites in Japan and found the sensitivity of the LAMP assay also comparable to that of the PCR. Seki *et al*. (2005) also found a similar level of detection to PCR with their LAMP assay for *Streptococcus pneumonia* and Song *et al*. (2005) for *Shigella* and enteroinvasive *E. coli*. The detection of koi herpesvirus (KHV) in common carp, *Cyprinus car*pio L., by loop-mediated isothermal amplification was found to be at similar levels to that of PCR (10^{-6} dilution of the initial concentration though the initial concentration was not reported; Gunimaladevi *et al*., 2004). The sensitivity of the LAMP assay was determined as 12 CFU ml^{-1} for bacterial cultures and 18-25 CFU mg^{-1} for tissue samples spiked with known quantities of *Fp*

(eggs, kidney and spleen) in this study. The detection limit of water spiked with the same quantity of *Fp* obtained was CFU 16 ml^{-1}.

Despite the limitation in the sensitivity and specificity of the two assays developed in this chapter, the novel qPCR and LAMP assays were used in Chapter 4 to quantify and detect *Fp* in selected field samples. The use of both assays has potential, as explained earlier, although more work is required for these to be used routinely at sensitive and specific levels. The qPCR needs to be more sensitive than 10^3 copies $well^{-1}$ and cross-reactivity with *F. aquatile* and *F. johnsoniae* needs to be addressed. *Flavobacterium aquatile* has been associated with diseased fish and *F. johnsoniae*-like organisms have been frequently isolated from lesions of freshwater fish (Rintamäki-Kinnunen *et al*., 1997; Crump *et al*., 2001).

In addition, restriction enzyme mapping to determine how many copies of 16S are in the genome of *Fp* is required to complete assay development. The sensitivity of the LAMP was higher than qPCR and comparable to the nested PCR, but the cross-reactivity to *F. columnare* and *F. branchiophilum* needs to be overcome. Both organisms have been associated with diseased fish (Rintamäki-Kinnunen *et al*., 1997; Crump *et al*., 2001). The recent sequencing of the whole genome of *Fp* will enable the design of new primers for the qPCR assay on a different part of the sequence or gene available to target regions specific to *Fp*. New primers for the LAMP assay can now also be designed with the newer version of the appropriate software becoming available.

Chapter 3: Development of a Luminex™ assay to detect anti-*Fp* antibodies in rainbow trout fry serum.

3.1 INTRODUCTION

The application of control strategies to minimise the severity of a disease is one of the most important measures for firstly reducing transmission of the infectious agent and secondly reducing the impact of the disease on the farming system (Cunningham, 2002). The strategies used to control *Fp* in farming systems have not been very successful to date (LaFrentz *et al*., 2002; Nematollahi *et al*., 2003). For example, the failure of various disinfection procedures, have shown how difficult it is to successfully eliminate the bacterium from the surface of eggs since it is still possible to recover the pathogen from eggs after disinfection (Brown *et al*., 1997; Madsen *et al*., 2005). Also, no commercial vaccine is available against *Fp*, and thus the use of antibiotics such as florfenicol and/or oxytetracycline are frequently used to control *Fp* outbreaks (Madsen *et al*., 2001; Merle *et al.*, 2003). Apart from the cost of these treatments, the presence of antibiotic resistant strains of bacteria and the accumulation of antibiotics in the environment have been a worry for the aquaculture industry (Bruun *et al*., 2000; Merle *et al*., 2003; Aarestrup, 2006).

These concerns have lead to an increased demand for the development of an efficacious vaccine to protect fish against *Fp*. Progress in formulating such a vaccine has been hindered by a lack of understanding in the pathogenic mechanisms used by *Fp*, the fastidious nature of the organism in culture and the early age at which fish are affected by the disease (Crump *et al*., 2001; LaFrentz *et al*., 2003). The development of a vaccine against *Fp* also requires a better understanding of the immune system of fish against the pathogen and the interaction between pathogen and host (Vatsos, 2001; Wiklund and Dalsgaard, 2002). Despite being a very important pathogen to the industry, the serotypes, antigenicity and pathogenicity of *Fp* are not very well understood (Thompson and Adams, 2004). Pathogens (or antigens) trigger the production of specific antibodies by the immune system

and these recognise and bind specifically to the pathogen and help eradicate it from the fish (Williams, 2000; Zapata *et al*., 2006). The humoral component of the adaptive response mediated by antibodies helps to clear bacteria from the fish and prevent re-infection through memory B-cells (Roitt, 1997; Campo, 2006). When the B-cells re-encounter the pathogen, they differentiate into plasma cells and secrete antibodies (Roitt, 1997). The pathogen is eliminated in a number of ways; the antibody can bind to the pathogen, neutralising it and therefore blocking its ability to invade host cells. It can also act as an opsonin coating the pathogen and facilitating phagocytes in phagocytosis.

The binding of antibodies to antigen to form immune complexes can activate the complement system (Roitt, 1997; Boyle, 2006). The complement system enhances phagocytosis through opsonisation and chemotactic attraction of phagocytic cells to the site of infection. Complement can also directly kill some microorganisms (Roitt, 1997; Campo, 2006; Schaechter *et al*., 2006). The level of antibodies in the blood are often used as a measure of the immune response. Vaccines mimic the exposure of a host to a pathogen so that memory cells are produced when they re-encounter the pathogen (Dorson, 1981; Ellis, 1989; Roberts, 2001; LaFrentz *et al*., 2002). Lipopolysaccharide (LPS) has been found to be an important molecular antigen on the surface of the bacterium and is believed to be an important protective antigen in *Fp* (Fulop *et al*., 1995; Crump *et al*., 2001; LaFrentz *et al*., 2004). The link of LPS to pathogenesis was identified by Crump *et al*. (2001) who suggested its potential as a vaccine candidate. The presence of specific antibodies in the serum of fish can also be useful for indicating whether they have been previously exposed to a pathogen, and this is why the Luminex™ assay was developed in this Chapter. Serological screening for the detection of anti-KHV antibodies from the serum of populations of common carp (*Cyprinus carpio carpio*) using an ELISA was developed by St-Hilaire *et al*. (2007). The authors suggested that the test would be useful in identifying exposure status of the fish at a population level. The use of an indirect ELISA for the detection of specific antibodies to nodavirus was developed to screen spawners in a sea bass, (*Dicentrarchus labrax*, L.) hatchery (Breuil and Romestand, 1999). This could then be used to test for and therefore reduce the vertical transmission of the virus in sea bass hatcheries.

Immunological methods such as ELISA (LaFrentz *et al*., 2002), serum agglutination (Rahman *et al*., 2002) and western blot analysis (Crump *et al*., 2001; LaFrentz *et al*., 2002) are all established methods for screening the antibody response of fish to *Fp*. Each of these established techniques has advantages and disadvantages depending on the requirement of the assay (Table 3.1).

A major constraint for using a standard 96-well plate ELISA to screen fish for antibodies responses in fish against *Fp* has been to obtain sufficient quantities of serum to measure the antibody response in small fry infected with *Fp*. Most studies using sera from small fry employing ELISAs, have been carried out using pooled sera samples rather than individual samples or by using considerably larger fish (LaFrentz *et al*., 2002). These constraints

Table 3.1: Advantages and disadvantages of different immunoassays used for screening fish serum for the presence of *Flavobacterium psychrophilum* antibodies.

Immunoassay	Advantages	Disadvantages
Western blot	• Able to associate specific bands with reactivity to a particular antibody • Provides information on molecular weight and subunit structure	• Not quantitative • High consumption of serum in test • Some proteins are denatured during the process • Limitation on samples number • Time-consuming
ELISA	• Rapid • Sensitive • Large number of samples can be screened	• Organism has to be cultured before performing assay • Can be strain specific

have resulted in the requirement for an alternative method for measuring antibodies in small quantities of serum.

In 1977, Horan and Wheeless Jr. were the first to describe the use of flow cytometry combined with microspheres to determine analytes in serum and other fluids. In 1975, Knapp *et al.* introduced antigen-coated microspheres with fluorescent labels for the capture and quantification of antigen-antibody reactions using a fluorescence-activated cell sorter (FACS). The Luminex™ 100 analyser also known as xMAP technology, was designed by the Luminex™ Corporation© (Texas, USA) specifically for fluorosphere-based flow cytometry (Nolan and Mandy, 2001). Flow cytometry based methods such as FACS and Luminex™ allow measurements of optical signals from a single particle such as cells or beads (Edwards *et al*., 2004). Bead-based immunoassays can reduce the assay time measuring antibodies compared to traditional plate-based assays as they rely on the rapid association of antibodies and antigens in a solution, as well as the use of a much smaller surface area. As a result, smaller quantities of reactants are required compared to traditional immunoassays (Kellar *et al*., 2001).

The technology is based on a 5.6 µm microsphere (Figure 3.1 (a)) although other sizes can be used. When excited by a 635-nm laser, the fluorophores emit light at different wavelengths, 658 and 712 nm (Figure 3.1 (b)).

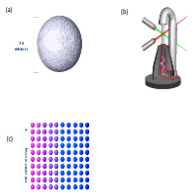


Figure 3.1: Components of the Luminex™ assay: (a) 5.6 µm microsphere; (b) Dual lasers identifying the beads; (c) panel of 100 microspheres available for coupling (Diagram obtained from Bio-plex applications pack, Bio-Rad, Hertfodshire, UK)

With the use of precision fluidics, digital signal processors and advanced optics the Luminex™ 100 analyser can classify each individual microsphere with a high precision according to its predefined fluorescent emission ratio (Shapiro, 2003). Multiple microspheres coupled to different analytes can be combined within a single sample thereby providing a multiplex assay. A third

fluorophore (i.e. phycoerythrin) coupled to a reporter molecule enables quantification of the interaction that is occurring on the surface of the fluorescent microsphere. With slight variations in the amount of each of these fluorochromes, an array can be created consisting of up to 100 different microsphere sets (Figure 3.1 (c)). Because of the ability to bind different substances to the beads, they can be used to quantify either antibody, DNA or protein depending on what is coupled to the beads.

For antibody detection, covalent coupling of the antigen to the beads is recommended (McHugh and Stites, 1991) through carboxyl, amine groups (Kellar and Iannone, 2002). Individual interrogation of the microsphere occurs in a rapidly flowing fluid stream as they pass by two separate lasers in the Luminex™ 100 analyser. Rapid digital signal processing classifies each microsphere based on its spectral address and quantifies the reaction. In a matter of a few seconds per sample, thousands of microspheres can be interrogated with up to 100 different reactions being reported. The assay for measuring antibodies in the serum of fish is based on the protocol shown in Figure 3.2.

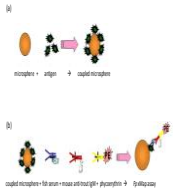


Figure 3.2: Sequence of steps performed in the Luminex™ assay to detect antibodies in the serum of fish. (a) Coupling of capture reagent to microsphere (b) Luminex™ assay using a mouse anti-trout IgM monoclonal antibody followed by anti-mouse IgM conjugated to phycoerythrin.

The capture reagent can be either an antigen or an antibody. The analyte is then added to the microsphere. A fluorescent reporter molecule added to the reaction binds to the analyte and finally the reaction is quantified by the amount of fluorescence present on the surface of the beads. The results are analysed by the software which detects individual bead sets and determines the average amount of binding of analyte to each bead (Vignali, 2000).

The Luminex™-100 used for this study was bought from Bio-Rad (Hertfordshire, UK) who renamed the machine as Bio-plex™. This suspension array system consists of an array reader, an XY platform, an HTF system and a computer (Figure 3.3).

Figure 3.3: Bio-plex™ suspension array system: (a) HTF system, (b) array reader and (c) XY platform. (Diagram obtained from Bio-plex applications pack, Bio-Rad, Hertfodshire, UK).

The array reader is a compact flow analysis unit integrating a dual laser detection system, optics, fluidics and advanced digital processing. The XY platform allows automated processing of samples from a 96 well microplate. The HTF system is designed to automate the introduction of sheath fluid into the Bio-plex™ array reader from a non-pressurised bulk container to constantly maintain a reservoir of pressurised sheath fluid. The Bio-plex™ system also comes with a maintenance, calibration and validation (MCV) plate (Figure 3.4) that is designed for the following uses: needle adjustments, calibration and validation of the system, start-up of the system, washing the system in between assays, and shutting-down of the system after use.

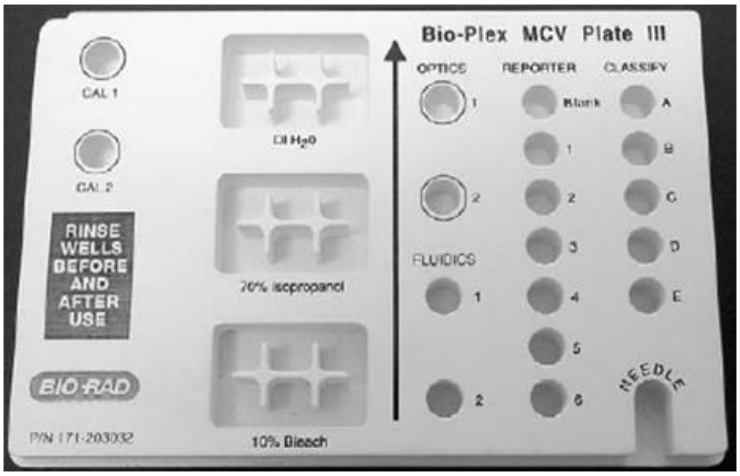


Figure 3.4: Luminex™ maintenance, calibration and validation (MCV) plate. (Diagram obtained from Bio-plex applications pack, Bio-Rad, Hertfodshire, UK).

Bio-plex Manger™ software version 4.0 was the software used to analyse the data obtained from the Bio-plex. The software records fluorescent signals simultaneously for each bead translating the signal into data that can be exported to Microsoft Excel. To date, Luminex™ assays have predominantly been used in a variety of immunological based studies, such as the quantification of cytokines (Lash *et al*., 2006; Dehqanzada *et al*., 2007), quantification of antibodies (Dasso *et al*., 2002; Opalka *et al*., 2003), detection of antibodies in autoimmune studies (Mahler *et al*., 2004), antibody characterisation (Muro *et al*., 2005; Zachary *et al*., 2005) and to detect pre-transplant donor-specific antibodies and anti-human leukocyte antigen antibodies (Gibney *et al*., 2006; Zou *et al*., 2006).

The aim of this Chapter was to develop a Luminex™ xMAP assay for sensitive, rapid and reproducible measurements of anti-*Fp* antibodies in individual sera from small fry, thus eliminating the need to use pooled sera. This would allow the antibody response of the fry to be investigated to determine if they had been previously exposed to *Fp*, and the use of the Luminex™ in serological analysis is discussed in more detail in Chapter 4.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of bacteria and Extracellular products

The *Fp* isolates were cultured and various extractions prepared for coupling to the Luminex™ microspheres including whole cell, lysed *Fp* and extracellular proteins (ECPs). The *Fp* isolates [type strain 1947 obtained from the National Collection of Industrial Bacteria (NCIMB) (Aberdeen, UK) and field isolate B97026P isolated from infected rainbow trout fry in Scotland in 1997] were cultivated in 25 ml of tryptone–yeast extract–salts broth supplemented with glucose (FLP media; Cepeda *et al*., 2004; Appendix: I) for 4 days at 15°C. The bacteria were pelleted and washed twice at 3500g for 15 min with Dulbecco's phosphate-buffered saline pH 7.4 (DPBS) (Gibco, Paisley UK) and used to make the bacterial preparations shown in Section 3.2.5.1.

The cellophane plate technique described by Liu (1957) was used to obtain extracellular products (ECP) from the *Fp* isolates, with slight modifications. Briefly, isolates were grown for 3 days on FLP broth as described above. A 0.5 ml aliquot of bacterial culture was spread over the FLP plates which were overlaid with a sterile cellophane sheet using a sterile scraper. Plates were then incubated for 48 h at 15 °C. The ECPs were harvested by washing the cellophane sheet with 25ml of PBS followed by centrifugation at 2000 x *g* for 30 min at 4°C. The supernatant was collected and concentrated using an Amicon Ultra-15 concentrating tube (PLTK Ultracel-PL Membrane, 30 kDa; Millipore, Watford UK) by centrifuging at 3000 x *g*.

3.2.2 Production of anti-*Fp* rainbow trout serum

Forty rainbow trout fry (average of 15 g) were randomly selected from the stock obtained from Glenwyllin Fish Farm, Isle of Man, UK. Before performing the challenge (i.e. experimentally infecting the fish with *Fp*), fish were screened to establish that they did not have any anti-*Fp* antibodies present in their serum as an indicator of pre-exposure to infection. Blood was collected from the fish by caudal puncture using a 22-gauge needle and this was allowed to clot overnight at

4°C. Serum was collected following centrifugation of the blood at 15,000 x *g* for 7 min. The serum samples were stored at −20°C until they were screened by ELISA to confirm that the fish had produced anti-*Fp* antibodies (Section 3.2.3). Spleen and kidney samples were also taken and tested by polymerase chain reaction (PCR) (Wiklund *et al.*, 2000) for the presence of *Fp* as described in Section 4.3. A virulent strain of *Fp* (B97026P) was used for the pathogen challenge. The isolate was cultured in FLP broth at 15°C for 72 h. Thirty fish were infected with a sub-lethal dose (2.2×10^5 ml^{-1} cells) of live *Fp* by intra-muscular (IM) injection. Five fish were also injected with PBS for use as a negative control. Blood was collected by caudal puncture on Day 0, 2, 8, 24, 35, 45 and 64 with 5 fish per time point. The titre of the fish sera was measured over the course of time to obtain the peak (positive) titre which was found to be at Day 45.

3.2.3 ELISA

Protein and LPS bacterial preparations were made in Section 3.2.5 and tested by ELISA and western-blot analysis to confirm that the antigenic components of the preparations were retained. A similar ELISA protocol was used to measure the antibody response of fish to *Fp*. The ELISA protocol described here indicates where appropriate modifications for each assay.

ELISA was performed using the method described by Faruk *et al*. (1999). ELISA plates (Immulon® 4 HBS flat bottom, Thermo Labsystems) were coated with 50 µl $well^{-1}$ of 0.01 % (v/v) poly-L-lysine (Sigma, Dorset UK) for 60 min at room temperature (RT; 20-22°C). The plates were washed 3 times with low salt washing buffer (LSWB; Appendix: I) before adding 100 µl $well^{-1}$ of the different bacterial extraction preparations prepared in Section 3.2.5 and the plates were incubated overnight at 4°C. The following morning, 50 µl $well^{-1}$ of 0.05 % (v/v) gluteraldehyde was added and incubated for 20 min at RT (20-22°C) to fix the bacteria. Plates were washed 3 times with LSWB, and 250 µl $well^{-1}$ of blocking buffer (Appendix: I) was added for 2 h at RT (20-22°C). The washing procedure above was repeated before adding rabbit anti-*Fp* polyclonal serum produced using isolate

B97926 and 32/97 (Faruk, 2000) (100 µl well^{-1}) at a 1:5,000 dilution in antibody buffer (Appendix: I) and the ELISA plate incubated for 180 min at RT (20-22°C). Antibody buffer alone was used as a negative control. With serum obtained from fish, 100 µl well^{-1} serum titred from 1:16 to 1:2,048 in antibody buffer, was added to the plate and incubated overnight at 4°C. Antibody buffer and trout serum from fish injected with PBS (Section 3.2.2) were used as negative controls. Following incubation, the wells were washed 5 times with high salt washing buffer (HSWB; Appendix: I) and soaked for 5 min on the last wash. When assaying the fish serum an extra step was added at this point where 100 µl well^{-1} of anti-trout IgM (Aquatic Diagnostics Ltd., Stirling UK) produced in rabbit was prepared according to the manufacturer's instructions was added to wells that had previously been incubated with fish serum, and incubated for 1 h at RT (20-22°C). Either 100 µl well^{-1} of anti-mouse IgG conjugated with horseradish peroxidise (HRP; Sigma, Dorset UK) in the case of the fish serum or anti-rabbit IgG conjugated with HRP (Sigma, Dorset UK) in the case of the rabbit anti-*Fp* polyclonal serum was added to the plates diluted at 1:1000 (v/v) in conjugated buffer (Appendix: I) and incubated for 1 h at RT (20-22°C). The washing procedure with HSWB was repeated. Substrate (Appendix: I) was added to each well and incubated for 10 min at RT (20-22°C). After this time, 50 µl well^{-1} of stop solution (Appendix: I) was added. The results were read at 450 nm using an ELISA Reader (Dynex Technologies MRXII). The average of the negative control was calculated and samples were considered positive when their values were 3 times higher than the negative control average.

3.2.4 Western Blot Analysis

SDS-PAGE and western blot analysis was performed using the method as described by Faruk *et al*. (1999). A dual gel caster system (Might™ Small SE245, Hoefer Pharmacia Biotech Inc.) was assembled according to the manufacturer's instructions. A 12 % polyacrylamide gel (Appendix: I) was prepared and degassed for 30 min before adding 15 µl of N,N,N',N'-tetramethyl-ethane-1,2-

diamine (TEMED; Sigma, Dorset UK) and 70 µl of 10 % (w/v) ammonium persulphate (APS) to the solution. The solution was mixed briefly, poured into the gel caster, covered with a layer of butan-2-ol (Fisher Scientific, Leicester UK) and allowed to polymerise for 60 min at RT (20-22°C). Following polymerisation, the butan-2-ol was rinsed off the gel and a comb was inserted into the top of the gel caster and covered with a 4 % stacking gel (Appendix: I) which was allowed to polymerise for 60 min at RT (20-22°C). The plates containing the gels were assembled into the electrophoresis chamber according to the manufacturer's instructions and the chamber was filled with reservoir buffer (Appendix: I). Whole bacteria and other bacterial preparations (OD 1 at 520 nm) were added to sample buffer (1:5 dilution; Appendix: I) and boiled for 5 min in an Eppendorf tube, then centrifuged for 5 min at 15,000 *x* g. Ten µl of molecular weight rainbow markers (BioRad, Hemel Hempstead, UK) was loaded into the first well of the gel, and 10 µl of each sample into the remaining wells, and then electrophoresis was performed for 90 min at 120 V. The gel was stained with Coomassie brilliant blue (Sigma) for 4 h and destained with multiple washes of destain (Appendix: I) to examine the protein profile on the gel.

An unstained duplicate gel was used for the Western blot analysis. Six pre-cut filter papers (Whatman No. 1), and a pre-cut nitrocellulose membrane (Hybond™-EC, Amersham) cut to the size of the gels were soaked together in transblot buffer (Appendix: I) for 20 min after which they were assembled into the transblot system (Fisher Scientific, Leicester UK) according to the manufacturer's instructions. The membrane was blotted for 60 min at 60 V, then non-specific binding sites on the membrane were blocked by incubating the membrane overnight at 4°C in 1 % (w/v) bovine serum albumin (BSA; w/v) in Tris buffered saline (TBS; Appendix: I). The following morning the membrane was washed 3 times with Tris buffered saline with Tween-20 (TTBS; Appendix: I) with 5 min incubation between washes. One membrane was incubated with rabbit anti-*Fp* polyclonal serum (1:5,000 dilution) and the other with fish serum (1:10 dilution) for 3 h at RT (20-22°C). The membrane containing fish serum was washed as before with TTBS and incubated for 1 h with anti-

trout monoclonal antibody (1:10 diluted with antibody buffer) at RT (20-22°C). The membrane was washed 3 times with TTBS with 5 min incubations each time and the membrane was incubated for 1 h at RT (20-22°C) with anti-mouse IgG conjugated to HRP (1:100 dilution with PBS) (Sigma) for the fish serum or anti-rabbit conjugated to HRP (1:100 dilution with PBS) for the rabbit anti-*Fp* polyclonal serum blot. The TTBS wash was repeated followed by a 1 min TBS wash. The membrane was developed with chromagen/substrate solution (4CN Membrane Peroxidase Substrate System, KPL). The reaction was stopped after 10 min with distilled water.

3.2.5 Preparation of beads for Luminex™

The preparations which gave the highest response in the ELISA were then selected for coupling to the Luminex™ beads.

3.2.5.1 Preparation of bacterial antigens

The washed bacterial pellets from Section 3.2.1 were re-suspended in 10 ml of DPBS and split into two 5 ml samples before centrifuging them at 3500 x *g* for 15 min. The supernatant was discarded from both samples, and the first sample was re-suspended in 2 ml DBPS without protease inhibitors and the second in 2 ml lysis buffer (Appendix: I). The lysis buffer was added to lyse the bacteria with the addition of an EDTA-free protease inhibitor cocktail (Roche Applied Science, West Sussex UK) (one tablet per 50 ml of lysis buffer) and 10 µl ml^{-1} of 100 mM phenylmethylsulfonyl fluoride (Pierce, Northumberland, UK) to protect the proteins from degradation.

3.2.5.1 (a) Protein extraction

A variety of different methods were used to extract protein from *Fp* for coupling to the Luminex™ beads. These consisted of a selection of mechanical or chemical methods. For mechanical extraction, bacteria were sonicated (Branson Sonifier 150, USA) on ice at 5 sec intervals at speed '3' with 5 sec and rested over 15 min. Briefly 150-212 µm glass beads (Sigma) in a 1:2 proportion were added to the bacteria in lysis byffer and vortexed for 1 min. The preparation was

centrifuged at 3000 x *g* for 10 min and the supernatant was collected. For the chemical extraction, Triton X-100 and sodium dodecyl sulphate (SDS) (Sigma) were added to the samples at a 0.5 % (v/v) or a 1 % (v/v) concentration respectively then sonicated as described above using glass beads. The total protein contents of all bacterial preparations described were quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce, USA) and the protein concentration adjusted to 150, 25, 5 and 1 µg ml^{-1}.

3.2.5.1 (b) LPS extraction

LPS was extracted following the method of Gratacap (personal communication, University of Stirling) which is based on a modification of the method described by Morrison and Lieve (1975). Cellophane sheeting (Medicell International Ltd, London, UK) was cut into 15 cm diameter circles and placed between 15 cm sheets of filter paper (Whatman No. 1, London, UK). The cellophane was pre-wet prior to autoclaving at 121˚C for 20 min. A circle of the sterile cellophane was then placed over FLP agar (Cepeda *et al*., 2004) in a 13 cm Petri dish. Five ml of *Fp* isolate B97026P (OD 1 at 520 nm) was cultured in FLP broth for 4 days as described in Section 3.2.1, then placed over the cellophane insert on FLP agar plates and incubated at 15°C for 4 days. Eight plates were prepared for each isolate.

Bacteria were harvested from the plates using the blunt end of a flamed microtome blade, collected into pre-weighed sterile glass bottles and re-suspended in 20 ml PBS. The bottles were placed in boiling water for 10 min after which 20 ml of butan-1-ol (BDH, VWR, Leicester, UK) was added and the bottles vortexed for 30 sec. The contents of the bottles were transferred into two sterile polypropylene copolymer conical centrifuge tubes (Nalgene, Hereford, UK) vortexed briefly for 30 sec and centrifuged at 35,000 x *g* for 20 min at 4˚C in a Sorvall RC-5B superspeed centrifuge (Thermo scientific, Basingstoke, UK). Using a 5 ml sterile syringe the bottom layer was collected and re-centrifuged for 20 min at 4˚C. The bottom layer was again collected and concentrated through a 0.20 µm vivaspin tube by centrifugation at 3000 x *g* for 30 min at 4˚C. The concentrate was collected and

passed through a 300 kDa vivaspin tube followed by a 10 kDa by continuous centrifugation at 3000 x *g* until a final volume of 1 ml was obtained. This was collected and washed three times with 2 ml PBS before incubating overnight in a water bath at 60˚C with the addition of 2.5 mg ml^{-1} proteinase-K (Sigma). The following day 8000 units ml^{-1} DNAse and 120 units ml^{-1} RNAse (Amersham Biosciences, Buckinghamshire, UK) were added at a 1:100 dilution and incubated at RT (20-22°C) for a further 60 min. The solution was then placed in boiling water for 10 min and re-suspended with 10 ml of distilled water. Ten ml of butan-1-ol was added to the sample which was vortexed and centrifuged as above. The bottom layer was collected with a 5 ml syringe and passed through a 10 kDa vivaspin concentrator at 3000 x *g* for 30 min and washed twice with 5 ml of distilled water by centrifugation at 3000 x *g*. It was then aliquoted into freeze-drying glass ampoules and stored at -70˚C overnight, before freeze drying the sample overnight. The next day the lyophilised LPS samples were collected into sterile Eppendorfs and stored at -20˚C.

3.2.5.2 Conjugation of antigens to beads

3.2.5.2 (a) Protein conjugation

Carboxy-coated beads (COOH; Bio-Rad, Hertfodshire, UK) were selected for the coupling reaction. Three different coupling methods were performed: (1) Bio-plex amine coupling kit (Bio-Rad) as per manufacturer's instructions, (2) LiquiChip (Qiagen, West Sussex, UK.) coupling protocol according to the manufacturer's instructions, and (3) the two-step carbodimide coupling of protein to carboxylated microspheres (Luminex™ Corporation©, Austin, USA) with several modifications as described. Briefly, 100 µl of stock uncoupled Bio-plex COOH microspheres (Bio-Rad) were vortexed for 30 sec before adding to a protein LoBind Eppendorf tube (Eppendorf, Cambridge, UK) and centrifugation at 11,000 x *g* for 3 min. The supernatant was carefully removed and the pellet was resuspended in 100 µl of distilled water. The microspheres were sonicated in a Bransonic Ultrasonic cleaner (Bransonic Ultrasonic Corporation, Connecticut, USA) for 30 sec. The supernatant was removed prior to resuspending the washed microsphere in 80 µl of 100 mM

monobasic sodium phosphate, pH 6.2 (BDH, VWR, Leicester, UK), vortexing for 30 sec and sonicating for another 30 sec. Ten µl of 50 mg ml^{-1} of *N*-hydroxysulfosuccinimide (Sulfo-NHS; Pierce, Nothumberland, UK) was added to the microspheres and mixed gently by vortexing for 20 sec. Ten µl of 50 mg ml^{-1} 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC; Pierce) was added to the microspheres and mixed gently by vortexing for a further 20 sec. The microspheres were incubated for 20 min at RT (20-22°C) in the dark with 30 sec vortexing at 10 min intervals. The activated microspheres were pelleted by centrifugation at 11,000 x *g* for 2 min and the supernatant discarded. The microspheres were resuspended in 250 µl of PBS, vortexed for 30 sec, sonicated for 30 sec and centrifuged at 11,000 x *g* for 2 min. This last wash step was repeated twice. The supernatant was carefully removed, discarded and the microspheres were resuspended in 100 µl of PBS, followed by vortexing for 30 sec and sonicating for 30 sec. At this point, either 125, 25, 5 or 1 µg protein was added to the resuspended microspheres and the total volume of the suspension was made up to 500 µl with PBS. The coupling reaction was vortexed for 30 sec and incubated for 2 h on a rotator in the dark at RT (20-22°C). The microspheres were centrifuged at 11,000 x *g* for 2 min before removing the supernatant and resuspending in 500 µl of PBS-Tween-20 (PBS-TBN; Appendix I), vortexing and sonicating for 30 sec. The microspheres were incubated for 30 min with continuous mixing on a rotator at RT (20-22°C) followed by centrifugation at 11,000 x *g* for 2 min. The supernatant was removed and resuspended in 1 ml of PBS-TBN vortexing for 30 sec, then sonicated and centrifuged at 11,000 x *g* for 3 min. This PBS-TBN wash step was repeated twice with a final resuspension of the microspheres in 250 µl of PBS-TBN.

Following all coupling reactions, bead concentrations were determined using a cell-counting haemocytometer and stored at 4°C in the dark.

3.2.5.1 (b) LPS conjugation

The LPS was conjugated to 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM; Sigma) modified polysaccharides, which were then conjugated to carboxyl

microspheres (COOH–DMTMM) using the method of Schlottmann *et al*. (2006) with a few modifications. Briefly, 2.5 ml of the LPS preparation from Section 3.2.5.1 was added to a 200 µl solution of 200 mg ml^{-1} of DMTMM. The LPS/DMTMM mixture was incubated for 1 h on a rotator mixer at RT (20-22°C). A buffer exchange was carried out with Sephadex G-25M PD10 columns (Amersham Biosciences, Piscataway, USA) and eluted with 3.5 ml of PBS to separate the DMTMM modified LPS from free DMTMM. Five hundred µl of the modified LPS was added to 100 µl COOH microspheres, vortexed and sonicated for 30 sec before incubating overnight at RT (20-22°C). The LPS-DMTMM microspheres were washed twice with 1 ml of PBS-TBN by centrifuging at 11,000 for 3 min and they were then stored in 1 ml PBS-TBN at 4 °C in the dark.

3.2.6 Luminex™

3.2.6.1 Luminex™ validation assay

The Luminex™ 100™ (renamed by BioRad as Bio-plex™) was purchased from BioRad (Hertforshire UK). The assay was initially validated based on the protocol suggested with the Bio-plex amine coupling kit to confirm successful coupling of the antigen to the beads. A range of incubation times and quantities of reagents were tested during the optimisation process (Figure 3.5).

Briefly, wells of a 96 well MultiScreen HTF™ (High Throughput Fuidics) plate (Millipore, Watford, UK) were blocked with 150 µl of blocking buffer (DPBS with 1 % BSA (w/v) and 0.05 % sodium azide (w/v) (Sigma) for 30 min. The plate was placed on a vacuum manifold (Bio-Rad) connected to a vacuum pump with an average pressure of around 8-10 mm Hg. Fifty µl containing 3,000 beads was added to each well. The plate was applied to the vacuum manifold again, followed by 3 washes with 150 µl of assay buffer (DPBS with 1 % BSA (w/v) and 0.05 % sodium azide (w/v)). One hundred and fifty µl of rabbit anti-*Fp* polyclonal antibody, diluted 1:10,000 (dilution determined from the titre obtained by the ELISA in Section 3.2.4) was added to the wells of the plate and incubated for 3 h at RT (20-22°C). The plate was washed three times as described above and

incubated for 30 min with 50 µl well^{-1} of anti-rabbit IgG conjugated to phycoerythrin (PE; Vector Labs, Peterborough, UK) diluted in assay buffer. The plate was washed a further 3 times, and the beads were re- suspended in 125 µl well^{-1} of assay buffer, before reading the plate on the Bio-Plex™ (Bio-Rad).

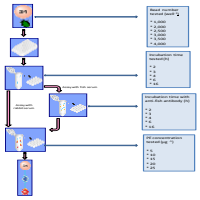


Figure 3.5: Parameters used for the optimisation of the Luminex™ assay to measure anti-*Flavobacterium psychrophilum* antibodies in rabbit and rainbow trout sera.

Figure 3.5: Parameters used for the optimisation of the Luminex™ assay to measure anti-*Flavobacterium psychrophilum* antibodies in rabbit and rainbow trout sera.

3.2.6.2 Luminex™ serum assay

Once the assay had been validated, it was then optimised to detect anti-*Fp* antibodies in the serum of fish artificially infected with the bacterium (Section 3.2.2.). The LPS preparation along with three protein extracts were chosen for conjugation to the beads based on their activity in the ELISA i.e. sonicated bacteria, ECP and 0.5 % Triton extracted bacteria. Briefly, wells were blocked for 30 min with 150 µl of blocking buffer on 96 well MultiScreen HTF™ plates. The plate was applied to a vacuum manifold and 50µl of beads, conjugated with the antigen, were added to each well. The plate was applied to the vacuum manifold again followed by 3 washes of 150 µl of DPBS with 1 % BSA (w/v) and 0.05 % sodium azide (w/v). Rainbow trout serum was titrated with DPBS in doubling dilutions starting from a 1 in 2 dilution to a final dilution of 1 in 2048. The plate was incubated overnight at 4°C. The plate was washed three times as described previously and incubated with anti-trout IgM (20 µg ml^{-1}). The plate was washed again and incubated for 30 min with 50 µl of phycoerythrin anti-mouse IgG (PE) per well (20 µg ml^{-1} diluted with assay buffer). The plate was washed a further 3 times and 125 µl $well^{-1}$ of buffer was added to each well before reading the assay on the Bio-Plex™.

3.2.7 Data Analysis

Data was captured by the Bio-plex™ software. Readings of wells containing DPBS instead of fish serum were subtracted from the 'raw sample' data (mean fluorescent intensity). All readings less than 50 median fluorescence intensity (MFI) are normally classed as background levels (Carter, 1994; Luminex™ Corp©, USA), however, for studying the immune response of fish this was adjusted to 15 MFI due to lower backgrounds obtained by fish in comparison to the human background levels (Tina Rakos, personal communication, Luminex™ Corporation©, Austin, Texas, USA). Readings of 3 times the background wells were considered to be positive. Samples of positive fish sera and rabbit anti-*Fp* polyclonal antibody were tested simultaneously in the Luminex™ assay and by ELISA. Antibody titres were determined for both methods and subjected to linear regression analysis and the

results of these then compared. Finally, all validation data and a selection of the data obtained during the development of the assay was sent to Luminex™ Corp© technical/support services for verification and validity of data.

3.3 RESULTS

3.3.1 Evaluating antigen preparations for coating Luminex™ beads by ELISA

The antigenicity of the *Fp* preparations after chemical and mechanical extraction was first assessed by ELISA. The reaction of the preparations with the rabbit anti-*Fp* polyclonal antibody is shown in Table 3.2.

All preparations gave a high reaction with the rabbit polyclonal antibody in the ELISA (89.5 % - 104.9 %), except for the sonicated bacteria prepared without lysis buffer, which had a 35 % and 39.2 % reactivity compared to whole bacteria with a 1:1000 and 1:5000 dilution of the anti-*Fp* sera, respectively. There was a slight variation in the % level of reactivity compared to whole bacteria depending on the dilution of rabbit anti-*Fp* polyclonal antibody used. The three highest reactions obtained compared with the whole bacteria, when using a 1:1,000 dilution of the serum, were sonicated bacteria (104.9 %), 1 % SDS + bacteria (102.2 %) and 1 % Triton-100 + bacteria (98.4 %). The other preparations were of a similar level of reactivity between glass bead + Triton-100 (89.5 %) and 0.5 % Triton-100 + bacteria (98.1 %) used to extract the bacteria. The three highest reactions obtained when the serum was used at a 1:5,000 dilution were LPS (130.7 % reactivity), glass bead + bacteria (110.9 % reactivity) and glass bead & Triton-100 (108.6 % reactivity).

The *Fp* preparations were then screened in ELISA using trout serum known to contain antibodies against *Fp*. The response of these preparations with the anti-trout antibody is shown in Table 3.3 with median absorbance values (A_{450}) and % reactivity to whole bacteria reported for each of the samples.

Table 3.2: A comparison of the antigenicity of the *Flavobacterium psychrophilum* preparations by ELISA using rabbit anti-*Fp* polyclonal antibody.

	Antigen preparation[a]	**OD at 410 nm[b]**		**% reactivity to whole bacteria**	
		1:1,000[c]	**1:5,000[c]**	**1:1,000[c]**	**1:5,000[c]**
	Whole bacteria	1.150	0.914	100.0 %	100.0 %
	Sonicated bacteria without lysis buffer	0.423	0.384	35.0 %	39.2 %
	Sonicated bacteria	1.205	0.983	104.9 %	107.9 %
	Extra-cellular protein	1.071	0.968	92.9 %	106.2 %
	Glass bead + bacteria	1.117	1.009	97.1 %	110.9 %
	Glass bead & Triton-100 + bacteria	1.033	0.989	89.5 %	108.6 %
	0.5% Triton-100 + bacteria	1.129	1.027	98.1 %	113.0 %
	1% Triton-100 + bacteria	1.132	0.956	98.4 %	104.8 %
	0.5% SDS + bacteria	1.065	0.832	92.4 %	90.6 %
0	1% SDS + bacteria	1.175	0.869	102.2 %	94.8 %
1	LPS	1.046	1.182	90.7 %	130.7 %

2	Negative control[d]	0.03 1	0.042	2.8 %	4.60 %

[a] All samples were prepared using lysis buffer containing protease inhibitor cocktails except for sample (1) and (2); samples were run in duplicates and mean values were calculated

[b] All samples were run in duplicates

[c] dilution of anti-rabbit polyclonal *Flavobacterium psychrophilum* antibody used

[d] Negative control serum was screened against whole bacteria and obtained from rabbit prior to being injected with the bacterium.

Table 3.3: A comparison of the antigenicity of the *Flavobacterium psychrophilum* preparations and whole bacteria preparations by ELISA using trout anti-*Fp* serum.

	Sample[a]	OD at 410 nm[b]	% reactivity to whole bacteria
	Whole bacteria	0.914	100.0 %
	Sonicated bacteria without lysis buffer	0.315	30.8 %
	Sonicated bacteria with lysis buffer	0.836	91.0 %
	Extra-cellular protein	0.596	63.3 %
	Glass bead + bacteria	0.515	53.9 %
	Glass bead & Triton-100 + bacteria	0.044	-0.5 %
	0.5% Triton-100 + bacteria	0.939	102.9 %
	1% Triton-100 + bacteria	0.240	22.2 %
	0.5% SDS + bacteria	0.252	23.6 %
0	1% SDS + bacteria	0.435	44.7 %
1	LPS	0.331	32.7 %
2	Negative control[c]	0.048	3.08 %

[a] All samples were prepared using lysis buffer containing protease inhibitor cocktails except for sample (1) and (2); samples were run in duplicates and mean values were calculated

[b] All samples were run in duplicates

[b] fish serum was added at 1/64 dilution in phosphate buffered saline

[c] Negative control serum was screened against whole bacteria and obtained from fish injected with phosphate buffered saline

at 4˚C. Therefore the shortest incubation time of 3 h was used for all subsequent optimisation steps in the experiments using the anti-rabbit *Fp* polyclonal antibody.

Once the incubation time with the anti-rabbit *Fp* polyclonal antibody was optimised, the concentration and incubation time of the anti-rabbit IgG (PE) was optimised at 20 µg ml^{-1} for 30 min. Finally, the optimal number of beads per well was determined. The sensitivity of the assay dropped when less than 2,000 beads $µl^{-1}$ were added, however the MFI readings did not appear to increase when more than 3,000 beads $µl^{-1}$ were added, and therefore 3,000 beads $µl^{-1}$ was chosen for subsequent assays.

3.3.3 (b) Optimisation of the assay to detect Fp *antibodies in trout serum*

The first step in the optimisation of the assay to detect the fish antibody was to establish the optimal incubation time with the fish serum (Figure 3.7).

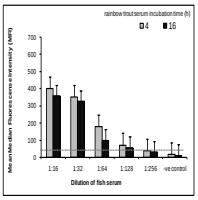


Figure 3.7: Assessment of incubation times with rainbow trout serum against Fp in the Luminex™ assay (values represent mean median fluorescence intensity (MFI) values for triplicate samples). Dotted line indicates 3 times negative control threshold. Negative control used was rainbow trout serum at 1:32 dilution.

Incubation with the serum overnight (16 h) gave slightly higher readings for most dilutions than using a 4 h incubation at RT (20-22°C), but either seemed suitable for the assay and 16 h was chosen for convenience so the assay could be incubated overnight. Once all other parameters were optimised, the incubation time was re-evaluated but the results obtained for 16 h were still similar to those obtained with a 4 h incubation (Figure 3.7).

The concentrations of anti-trout IgM MAb tested in the assay were 10, 15, 20, and 25 µg ml^{-1}. The first three concentrations all gave the same level of MFI values (Figure 3.8). The 20 and 25 µg ml^{-1} concentrations gave the highest MFI readings (380 and 311 at 1 in 32 dilution), although 20

µg ml^{-1} gave slightly better MFI values than the 25 µg ml^{-1} (219 and 133 respectively at 1 in 128 dilution) and was therefore selected as the optimum concentration of anti-trout antibody for the assay.

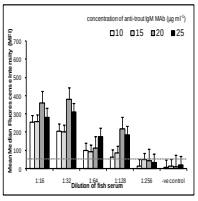


Figure 3.8: Optimisation of the concentration of anti-fish IgM MAb for Luminex™ assay (values represent mean median fluorescent intensity (MFI) values for triplicate samples). Dotted line indicates 3 times negative control threshold. Negative control used was rainbow trout serum at 1:32 dilution.

Lower concentrations of anti-mouse IgG labelled with phycoerythrin (5, 10 and 15 µg ml^{-1}) gave consistently lower MFI values compared to 20 and 25 µg ml^{-1} when tested in the Luminex™ assay (Figure 3.9). Overall 20 µg ml^{-1} of anti-mouse IgG PE gave the highest MFI values and was therefore selected for subsequent assays.

Fish	Mean ELISA titre ± SE[1]	Mean Sonicated *Fp* Luminex™ titre ± SE[2]	Mean *Fp* LPS Luminex™ titre ± SE[3]
Positive control	7.2 ± 0.14	8.3 ± 0.14	8.1 ± 0.23
Negative control[4]	4.3±0.33	4.7±0.33	3.7±0.33

[1] Plate coated with *Flavobacterium psychrophilum* (whole bacteria)

[2] Beads coated with sonicated *Flavobacterium psychrophilum*

[3] Beads coated with *Flavobacterium psychrophilum* LPS

[4] Negative control serum was obtained from challenge fish injected with phosphate-buffered saline

3.4 DISCUSSION

A Luminex™ assay was developed to detect anti-*Fp* antibodies in the serum of small rainbow trout fry to be used in the epidemiological study carried out in Chapter 4. This would determine if the fry had an antibody response to *Fp* indicating previous exposure to the pathogen. It was difficult to find appropriate information relating to the development of this assay as there are no reports in the literature using the Luminex™ technology to detect antibodies in the serum of fish. In fact there are very few Luminex™ assays reported for non-clinical work, with the majority of publications focusing on human-based research mainly in the field of cytokines (Probst *et al*., 2003).

The first step in developing this assay was to determine the best antigen preparation for coating the microspheres, after verifying by ELISA and western blot that the proteins were still antigenic after processing. Different preparations of the antigen were recommended rather than using the whole bacterial cells due to the large size of the bacteria in comparison to the beads (Rakos, personal communication). A variety of mechanical and chemical methods were used to break up the bacterial cells to allow exposure of antigenic epitopes present on the bacteria. It was crucial to determine which method (mechanical, chemical or a combination of both) was the best for coupling to the beads without having a detrimental effect on the antigen i.e. denaturing of proteins. Aggressive extraction methods were used, including sonication of the bacteria, protein extraction by the addition of a detergent or breaking up of the cells with glass beads. Non-ionic detergents (chemical) such as Triton X-100 are recommended as they are less denaturing than ionic detergents (Ahmed, 2004). However at lower concentrations, detergents such as SDS can be used to partition and unfold the membrane without a detrimental effect to the protein structure (Ahmed, 2004). This is not always the case however, and it is therefore recommended that a selection of detergents of different concentrations be used to determine the best detergent and ideal conditions both the rabbit and the fish sera in the ELISA and the western blot, sonicated bacteria gave consistent high MFI readings in all tests and were therefore selected as the antigen preparation for

The reproducibility of the Luminex™ assay depends on the instrument being used as results can vary between instruments and therefore correct calibration is very important. The Bio-plex used here was routinely calibrated and care was taken during optimisation steps and subsequent experiments to maintain the calibration of the instrument.

Once optimised, the assay was used to measure the antibody titres with LPS-coupled beads. It has been suggested that the LPS of *Fp* is strongly immunogenic (Crump *et al*., 2001) and may be involved in eliciting a protective immune response in rainbow trout (LaFrentz *et al*., 2004; Rahman *et al*., 2002). LPS is often cited as a virulence factor of *Fp* and a possible target for vaccine development (Lerouge *et al*., 2001) and immunisation with LPS-based vaccines have been shown to provide protection in a variety of diseases (Aoki *et al*., 1984; Soltani and Kalbassi, 2001). Although the LPS preparation did not give the highest titres in the Luminex™ assay, its use may generate some useful results. Crump *et al*. (2001) found that LPS has an O chain that may be unique to *Fp* and therefore be a specific target for use in diagnostics. The methods used for coating LPS to ELISA plates has been reported to give large variations in results between researchers (Poxton, 1995), and therefore the coating of LPS to the microspheres was tested in parallel to the other bacterial preparations described. However, it is important to note that there are several serotypes for *Fp* and that LPS changes between serotypes which might affect the results depending on which serotype the antibody was raised against (Brade, 1999). Also, Wiklund and Dalsgaard (2002) were able to detect LPS from the surface of *Fp* though the role of LPS in serum resistance requires further analysis. It has not been possible to compare the results of this Luminex™ assay with similar Luminex™-based assays for fish since this appears to be the first report of it used to measure antibodies in the serum of fish.

Further improvements to the assay presented in this Chapter would be the use of a mouse anti-trout IgM monoclonal antibody conjugated directly with PE, allowing the assay time to be

Chapter 4: Prevalence of *Flavobacterium psychrophilum* in four populations from a commercial rainbow trout hatchery.

4.1 INTRODUCTION

With most bacterial diseases, prevention is a key element in the control of disease outbreaks (Wagner *et al.*, 2002). Therefore, it is essential for both aquaculture producers and researchers to identify and understand the sources of infection and the other risk factors associated with outbreaks of disease (Martin *et al*., 1987). The sources of infection and risk factors for outbreaks are questions that must be addressed at a population level through the application of epidemiological principles.

It has been suggested that transmission of *Flavobacterium psychrophilum* (*Fp*) occurs both horizontally and vertically (Brown *et al*., 1997; Ekman *et al*., 1999). Vertical transmission was suggested as a possible route of infection by Holt (1987), who found *Fp* in the ovarian fluid of fish. These findings were further supported by Vatsos (2001) and Madetoja *et al*. (2002). As well as culturing the bacterium from the ovarian fluid of infected fish, it has been possible to culture it from the surface of rainbow trout (*Oncorhynchus mykiss*) eggs (Brown *et al*., 1997; Rangdale *et al*., 1999; Cipriano, 2005). There are, however, few reports in the literature relating to the occurrence and persistence of *Fp* in the natural aquatic environment (Wiklund *et al*., 2000). Several potential sources of infection of *Fp* in farms have been reported, including infected fish, dead fish, fish farm rearing water, algae and other fish species and broodstock (Lehmann *et al*., 1991; Amita *et al*., 2000; Vatsos, 2001; Madetoja *et al*., 2002). The presence of *Fp* in the mucus and ovarian fluid of fish suggested that transport of fish between farms might be a possible source of infection. The bacterium has also been detected in the tank water of fish farm sites (Rangdale, 1995; Bruun *et al*., 2000; Wiklund *et al*., 2000; Vatsos, 2001). The detection and identification of *Fp* in fish samples based on bacterial isolation is known to be difficult due to the bacterium's fastidious nature and associated with bacterial gill disease (BGD) in young, hatchery-reared rainbow trout, O*ncorhynchus mykiss*. The results of

their questionnaire-based study highlighted the presence of wild fish in the hatchery water as a risk factor for BGD. They also determined that disinfection of water neither killed the pathogen nor prevented outbreaks of BGD occurring.

PCR is a useful tool in epidemiology studies relating to RTFS (Rainbow trout fry syndrome), for example studying the distribution and transmission of the disease (Baliarda *et al*., 2002; Wiklund *et al.*, 2000). Baliarda *et al*. (2002) showed that their nested PCR assay could be applied to a number of different biological samples including spleen, kidney, eggs and ovarian fluid.

Another method that can be applied in epidemiology studies is the detection of specific antibodies to *Fp* in the serum of rainbow trout as an indicator of previous exposure to the pathogen (Lorenzen *et al*., 1997; Faruk *et al*., 2002).

The aim of the present Chapter was to monitor changes in prevalence of *Fp* over time in a commercial farm system and to identify possible sources of infection. A hatchery with a history of RTFS outbreaks, which was regularly supplied with sources of eggs from different geographical regions was selected as the study site. Bacteriological isolation of the pathogen and PCR (both nested and the quantitative PCR (qPCR) developed in Chapter 2) were selected as the methods used to detect the pathogen in the various samples collected (i.e. eggs, fish tissue, and water). An alternative molecular method, loop-mediated isothermal amplification (LAMP; developed in Chapter 2) was also used to test selected samples. This method is rapid and requires no specialized equipment. Batches of eggs were randomly selected from four different sources, and screened from their arrival on the farm to the point where they were sold for on-growing at 8 g. Along with the nested PCR and bacteriological sampling, a sample of blood was also collected from each fry of suitable size for the detection of antibodies to *Fp* using the Luminex™ protocol developed in Chapter 3. Due to the large size of the population under investigation, it was necessary to use sufficiently sensitive tests and large sample sizes to be able to determine the prevalence of the bacterium in the fish population and in the aquatic environment.

4.2 MATERIALS AND METHODS

4.2.1 Sampling Site

The commercial hatchery was split into two separate sites, the layouts of which are shown in Figure 4.1. Both sites had separate water inlet and outlet points. Site I (Figure 4.1a) was supplied with spring water, while Site II (Figure 4.1b) was fed with stream water. Eggs were bought from various UK and international sources and after disinfection with 0.1 % Virkon® (Antec Int. Ltd, Suffolk, UK) were placed in hatchery troughs at Site 1, following normal practice. Eggs were hatched at Site I and maintained at this site as fry for up to 4 months (~ 1g) post-hatch before being transferred to Site II.

4.2.2 Sampling Regime

Samples were collected from 4 different batches of eggs brought to the farm from 3 different geographical sources (source 1 – Denmark I; source 2 – Denmark II; source 3 – UK; source 4 – USA). The batches of eggs were followed from hatch to fingerling stage and were sampled 4 times over the course of the study. Eggs were sampled on arrival at the farm and prior to disinfection before being put into the troughs to make sure there was no contact with hatchery water prior to sampling. Once the eggs had hatched, every batch was sampled again prior to transferring the fry to Site II (~ 1g, life stage I), then again within 1-2 weeks (life stage II) post-transfer, and finally sampled once more prior to leaving Site II when they were around 6-8 months old (~5-8 g, life stage III). At the same time as the fish were sampled, 3L of inlet and outlet water was also sampled from the respective site. The sampling regime and dates of sampling are summarised in Table 4.1.

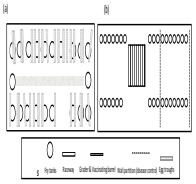


Figure 4.1: Layout of the farm surveyed in this study (a) Site I (hatchery) (b) Site II (on-growing 1-10 g)

4.2.2.1 Eggs

Three hundred eggs were randomly sampled from the four batches of eggs shown in Table 4.1. This was carried out by generating a list of random numbers which were allocated to individual compartments within the box of eggs. Eggs were then pooled into 50 samples containing six eggs per sample, and were immediately homogenised in 200 µl of sterile phosphate-buffered saline (Appendix I). A sterile loopful of the homogenised eggs was inoculated onto tryptone yeast salts (TYES) agar (Appendix I; Holt *et al.*, 1993) in 14 cm Petri dishes and incubated at 15˚C for 4 days. Samples were then examined for the presence of *Fp* using the biochemical tests described in Section 4.2.3.

Table 4.1: Details of sampling schedule and samples collected.

Country of origin of eggs	Sampling date	Degree days	Stage tested
Denmark I	15-Mar-04	0	Eggs I
	04-Jun-04	585	I
	07-Jul-04	979	I I
	26-Aug-04+	2031	I II
Denmark II	18-Mar-04	0	Eggs I
	17-Jun-04	668	

	7-Jul-04	89	8 I	I
	16-Sep-04+	827	1 II	I
UK	11-Mar-04		0 ggs	E
	04-Jun-04	30	6	I
	06-Jul-04	78	8 I	I
	06-Sep-04+	205	1 II	I
USA	29-Apr-04		0 ggs	E
	30-Jul-04	98	6	I
	01-Aug-04	86	8 I	I
	29-Oct-04+	827	1 II	I

The remainder of the homogenised egg samples were stored in 95 % ethanol at -20˚C for nested PCR analysis. A further three hundred eggs from the batches of eggs obtained from the UK were collected on arrival at the farm and transferred to the Aquatic Research Facility at the Institute of Aquaculture aquarium to hatch in troughs and water pre-screened by nested PCR to confirm that no *Fp* was detected before the eggs were laid out. The eggs were raised in parallel to the equivalent batch of eggs hatched at the farm. The eggs and the first sampling of fry was carried out on samples from the aquarium at the same time as the samples were taken from the farm. A water sample was also collected from the inlet and tank water at each sampling point.

4.2.2.2 Fry

A random sample of 50 fry was collected at 3 different sampling time-points as indicated in Table 4.1. The sampling consisted of 4 scoops with a net, taken from 4 different points around the tank, to ensure a representative sample of the population. The fish were sacrificed by an overdose of 2-phenoxyethanol (0.2 ml L^{-1}; Sigma-Aldrich, UK). The fish were placed on ice and taken back to the laboratory (25 minute car journey) for examination. A sample of blood was also collected from

the fry at the last sampling point. The fish were too small to collect blood at the first and second fry life stages. The fry were aseptically dissected, and the kidney and spleen inoculated on to TYES agar plates using a sterile loop and incubated at 15˚C for 4 days. Individual samples of kidney and spleen were frozen at -20˚C in pre-weighed sterile Eppendorf tubes containing 95 % ethanol for nested PCR analysis.

4.2.2.3 Water

Water samples were taken on all the dates when biological samples were collected (see Table 4.1) from the water inlet and outlet points, as well as from the egg trough or fry tanks. Each 3L water sample was collected, split into aliquots and centrifuged (5500 *x g*, 30 min, 4°C). The centrifuged aliquots were pooled and the final water sample was collected by drawing up 1 ml of the centrifuged water from the bottom of the tube with a sterile pipette. A sterile loop was inserted into the sample and streaked on to TYES agar plates. Water samples were frozen at -20°C for DNA extraction for nested PCR analysis.

carried out in a Biometra Thermocycler using the kit's recommended cycling programme with a few modifications: 35 cycles of denaturation at 96˚C for 30 s, annealing at 56˚C for 25 s and extension at 60˚C for 4 min. To adjust the reaction to the recommended total of 20 µl, 15 µl of ultra-pure water was added to each reaction and stored at 4˚C. The sequencing product was purified by using the ethanol precipitation protocol recommended by Beckman (Beckman, Coulter®, UK), with some modifications (described below). A combination of 2 µl sodium acetate (3M; Sigma-Aldrich, Dorset, UK), 2 µl of 0.5 mM EDTA (Sigma-Aldrich, Dorset, UK and 1 µl of glycogen (Roche, UK) was added to the reaction tube. Sixty µl of chilled (-20˚C) 95 % molecular grade ethanol (Sigma-Aldrich, Dorset, UK) was added to the reaction tube before vortexing briefly and centrifuging at 15000 g for 5 min. The supernatant was carefully removed with a pipette after which 200 µl of chilled 70 % (-20˚C) molecular grade ethanol was added. The sample was centrifuged for a further 2 min at 15000 x *g*, after which the supernatant was carefully removed and the pellet left to dry at

23˚C. The pellet was re-suspended in 30 µl of sample loading solution (SLS; Quick Start Kit) for 10 min before transferring to a 96 well sample plate (CEQ, Beckman, Coulter®) and loaded on to an automated capillary sequencer, CEQ^{TM}8800 (Beckman, Coulter®).

Each set of forward and reverse sequences were assembled by the BioEdit® Sequence Alignment Editor (Hall, 1999). The sequences were edited using BioEdit® where required. The sequence was then compared (by BLAST, Altschul *et al*., 1990) to the *Fp* nucleotide sequences on the DNA bank (http://www.ncbi.nlm.nih.gov/BLAST) for percentage similarity.

4.2.3.4 Testing field samples by qPCR to determine the level of Fp

The qPCR assay was first developed and optimised with the *Fp* type strain (Chapter 2). Then a batch of samples obtained from the farm sampling and positive by the nested PCR were tested in the qPCR assay using the protocol outlined in Chapter 2. These samples included a selection of DNA samples from eggs, fry tissue (spleen and kidney) and water collected at various 2.0 software (De Blas *et al*., 2000). Survey Toolbox 1.0 (Cameron, 2002) and the AusVet EpiTools pooled prevalence calculator (http://www.ausvet.com.au; Sergeant, 2004) were used to determine true and estimated prevalence from pooled samples. AusVet software (http://www.ausvet.com.au) was used to obtain estimates of individual prevalence determined by PCR and bacteriology. Estimates of prevalence with confidence intervals were produced by applying a predicted distribution, for example if one pool out of 50 is positive, it is probable that only a small number of the samples within that pool will be positive, whereas if 49 pools are positive out of 50 then it is probable that there will be more than one positive sample per pool (see Cowling *et al*. 1999 for detailed description). True prevalence (an estimate of prevalence taking into account test sensitivity and specificity) was calculated for the individual fry samples (life stage II-III). All *true prevalence* data is referred to as **prevalence** and *estimated individual egg prevalence* as **estimated prevalence** from this point, unless further details are required for clarification.

AusVet software was also used to compare the prevalence of *Fp* for the Denmark I and USA sources (the two sources for which a full set of bacteriology and nested PCR data had been collected). Epi Info™ (v3.4.3, Centres for Disease Control and Prevention, USA; Thrusfield (1995), was used for contingency tables to determine if being PCR positive increased the risk of having an antibody response (positive by serology).

A generalized linear model (GLZ) (Statistica© 6.0, Statsoft 1984-2001) was produced to investigate the relationship between the dependant variable (prevalence) and the two independent variables, "life stage" (expressed as degree day) and "source". Degree day information was obtained from farm records for all 4 batches. The degree day reflects the life stage of the fish as shown in Table 4.1. but is a continuous variable and therefore more suitable for the GLZ.

Mortality rates in the tanks from which fish were sampled, were collected from farm records. Unfortunately these records were incomplete due to some mortalities not being recorded by the farm. These data were used to look at the effect of mortality rates on levels of prevalence of the pathogen in the tanks in relation to the population at risk.

4.3 RESULTS

The results are comprised mainly of a comparison of two of the techniques used for the detection of *Fp* (bacteriology and nested PCR), followed by the determination of prevalence of *Fp* from eggs to on-growing fish from four different sources (Table 4.2). Two new molecular techniques developed in Chapter 2 (LAMP and qPCR) were also used on selected samples (eggs, fry tissue and water) to detect and quantify the levels of Fp, respectively. The detection of *Fp* antibodies in the fry from the second site in relation to the nested PCR results was also investigated.

Table 4.2: Results of nested PCR and bacteriology analysis.

C ountry of origin	tage tested	Samples positive by bacteriology Numbers of sample per life stage =50	Samples positive by nested PCR Numbers of sample per life stage =50

o f eggs		0 batches of 6	idney nly	pleen nly	S c	K idney and spleen	otal	5 0 batches o f 6	idney nly	pleen nly	S c idney nd spleen	otal
D enmark I	ggs				4	4		3			3	0
	I				3	1					2	
	II				4	6	0				3	0 4
D enmark I	ggs				n	n		5			4	
I			/a[1]	/a		/a	/a					
	I				3	3					2	
	II				4	7	1				3	0 3
U K	ggs				n	n		6			0	
			/a	/a		/a	/a					
	I		/a	/a	n	/a	/a				0	
	II				2	0					1	0 2
U SA	ggs				1	1		3			1	
					2	0					0	
	I											
	II				2	1					0	

[1] – indicates samples with overgrowth by other bacteria species.

4.3.1 Comparison of nested PCR and bacteriology as a method for assessing prevalence

A full set of bacteriology data was only available for two of the four sources of eggs over the 4 life stages due to bacterial overgrowth (i.e. USA and Denmark I) and this was used for comparing the increase of prevalence over time when both nested PCR and bacteriology were being compared. The detection of the pathogen from the eggs, spleen, and kidney was assessed using both bacteriology

and nested PCR, and the sensitivity of the two techniques compared. Representative nested PCR samples that were reported positive for *Fp* were confirmed by sequencing to validate that the identification of the pathogen by nested PCR was correct.

The ability to detect *Fp* in infected fish varied significantly depending on the methods used (i.e. nested PCR and bacteriology) as well as the organ sampled. The total prevalence from all samples was 18 % by nested PCR. *Fp* was detected in both the kidney and spleen from 13 % of the samples by nested PCR (Figure 4.2), while *Fp* was detected only in spleens of 3 % and only in the kidney of 2 %. The total prevalence from all samples was 12 % by bacteriology, excluding samples where overgrowth precluded interpretation. *Fp* was detected in both the kidney and spleen from 5 % of the samples by bacteriology (Figure 4.2), while *Fp* was detected only in spleens of 6 % and only in the kidney of 2 %.

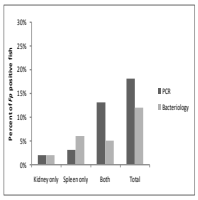


Figure 4.2: Percent of all fish sampled in which *F. psychrophilum* was detected in the kidney only, spleen only or in both organs by PCR and bacteriology (PCR n = 600; Bacteriology n=450).

4.3.2 Differences in the prevalence of *Fp* between different sources of eggs and in relation to fry mortalities

The prevalence levels of the bacterium established by PCR for all sources (adjusted for life stag prev (1.1 % - 1.9 % by nested PCR) were shown to grow into fry with much higher prevalences (19.8 % - 34.6 % by nested PCR in life stage III). A comparison of the prevalence of *Fp* between the two sources of eggs that were tested by both PCR and bacteriology was carried out (see Fig. 4.3). The

prevalence rate of *Fp* from the USA batch ranged from 1.14 % to 19.8 % of *Fp* detected by PCR by any organ whereas by bacteriology it ranged from 1.14 % to 11.1 %. The total prevalence of *Fp* in all stages from both batches was found to be 13 % by bacteriology, whereas by PCR it was 21 %. No *Fp* was detected in the inlet water from all sampling points although it was detected in all the outlet and tank water samples.

The prevalence of *Fp* in the batch of eggs taken to the Institute of Aquaculture (i.e. source I (UK)) increased from 2 % in the eggs to 10 % in the fry at life stage I. No *Fp* was detected in the inlet water of the tank or in the tank prior to stocking with the eggs.

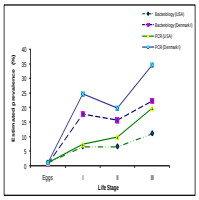


Figure 4.3: A comparison between estimated and prevalence levels from two different sources (USA and Denmark I) demonstrated by bacteriology and nested PCR (samples considered positive when detected by spleen and/or kidney).

The prevalence of Fp over time in the fry hatched from all 4 sources of eggs, showed a significant increase over the 8 month period of screening (p=0.001). The true and estimated prevalence rates, estimated by the AusVet EpiTools pooled prevalence calculator, were plotted against mortality figures obtained from the farm for each relevant batch of eggs and fry to observe any trends between mortalities and changes in prevalence (Figure 4.4).

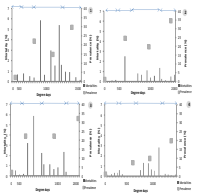


Figure 4.4: Estimated and true prevalence of *F. psychrophilum* determined by nested PCR against mortality figures. The arrows separates mortality rates data over the 4 life stages. (1) Source 1 (UK); (2) Source 2 (Denmark I); (3) Source 3 (Denmark II); (4) Source 4 (USA). (samples were considered positive when detected in spleen and/or kidney).

4.3.3 Sequencing of samples that tested positive for *Fp*.

The nested PCR products from a selection of samples were randomly selected for sequencing following nested PCR. The isolates were confirmed as *Fp* by running the sequenced nested PCR products through BLAST analysis. All sequences tested positive for *Fp* by the BLAST analysis with a minimum of 99 % homology to the sequences in GenBank.

4.3.4 Analysis of field samples by qPCR to determine the level of *Fp* present

DNA extracted from water, eggs and fry tissue (kidney and spleen) sampled and tested by nested PCR was also analysed in the qPCR assay developed in Chapter 2. Out of the ten water samples tested, only 4 samples were amplified by the qPCR assay along with the positive control (higher peak) (Figure 4.5). Out of the fifty-one tissue (egg, spleen and kidney) samples and water samples, only 20 samples were amplified by the qPCR assay (Figure 4.5 (b)). Table 4.3 lists the values obtained in the qPCR with readings varying from 3.48E+03 (for one of the water samples) to 3.07E+07 (from a spleen sample) copies well^{-1}.

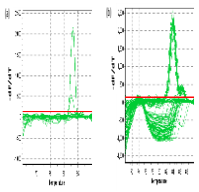


Figure 4.5: qPCR assay testing of (a) farm water samples, and (b) egg, spleen and kidney samples.

Only seven out of seventeen egg samples, three out of ten water samples, four out of twelve spleens and six out of twelve kidney samples were detected by the qPCR assay. The negative control was negative for both assays.

4.3.5 Analysis of field samples by LAMP to determine the presence of *Fp*

A number of field samples collected that tested positive by nested PCR were tested in the LAMP assay (Table 4.4). Negative control samples were negative in both assays. All egg samples tested were positive with both Reaction 3 and 21, LAMP protocols showing full agreement with the nested PCR results tested was negative in the LAMP assay though it was positive by nested PCR. All spleen samples were positive with both assays though with the kidney samples, two of samples that were positive by nested PCR were negative in the LAMP assay.

Table 4.3: A representative selection of qPCR and nested PCR analysis for water and rainbow trout eggs, spleen and kidney sampled during the farm study.

Sample	Average copies well^{-1}	Positive/negative by qPCR	Positive/negative by nested PCR
Non Template Control	6.11E+02	-	-
Standard 10^7	1.02E+07	+	+
Standard 10^5	9.39E+04	+	+
Standard 10^3	1.03E+03	+	+
Egg 1	-[1]	-	+
Egg 2	2.24E+05	+	+
Egg 3	-	-	+
Egg 4	3.38E+04	+	+
Egg 5	2.07E+06	+	+
Egg 6	4.17E+03	+	+
Egg 7	-	-	+
Egg 8	-	-	+
Egg 9	5.08E+05	+	+
Egg 10	-	-	+
Egg 11	-	-	+
Egg 12	-	-	+
Egg 13	7.64E+04	+	+
Egg 14	-	-	+
Egg 15	6.39E+05	+	+
Egg 16	-	-	+
Egg 17	-	-	+
Water 1	6.75E+03	+	+
Water 2	7.22E+04	+	+
Water 3	-	-	+
Water 4	-	-	+
Water 5	-	-	+
Water 6	-	-	+
Water 7	3.48E+03	+	+
Water 8	-	-	+
Water 9	-	-	+
Water 10	-	-	+

[1] No value obtained for negative readings below detection level.

Table 4.3 (cont'd): A representative selection of qPCR and nested PCR analysis for water and rainbow trout eggs, spleen and kidney sampled during the farm study.

Sample	Average copies well^{-1}	Positive/negative by qPCR	Positive/negative by nested PCR
Non Template Control	4.81E+02	-	-
Standard 10^7	1.11E+07	+	+
Standard 10^5	1.04E+05	+	+
Standard 10^3	1.02E+03	+	+
Spleen 1	-[1]	-	+
Spleen 2	-	-	+
Spleen 3	-	-	+
Spleen 4	-	-	+
Spleen 5	5.47E+05	+	+
Spleen 6	-	-	+
Spleen 7	7.89E+03	+	+
Spleen 8	-	-	+
Spleen 9	3.07E+07	+	+
Spleen 10	1.87E+05	+	+
Spleen 11	-	-	+
Spleen 12	-	-	+
Kidney 1	2.07E+04	+	+
Kidney 2	-	-	+
Kidney 3	-	-	+
Kidney 4	3.44E+06	+	+
Kidney 5	4.06E+04	+	+
Kidney 6	8.24E+03	+	+
Kidney 7	-	-	+
Kidney 8	2.73E+05	+	+
Kidney 9	-	-	+
Kidney 10	-	-	+
Spleen 11	-	-	+
Spleen 12	8.69E+05	+	+

[1] No value obtained for negative readings below detection level.

Table 4.4: A comparison of the field samples collected from the farm sampling using LAMP assay with traditional nested PCR.

Sample	Positive/negative by LAMP	Positive/negative by nested PCR
Negative control (water)	-	-
Positive control (*Fp* NCIMB 1947)	+	+
Egg 1	+	+
Egg 2	+	+
Egg 3	+	+
Egg 4	+	+
Egg 5	+	+
Egg 6	+	+
Egg 7	+	+
Egg 8	+	+
Egg 9	+	+
Egg 10	+	+
Egg 11	+	+
Egg 12	+	+
Egg 13	+	+
Egg 14	+	+
Egg 15	+	+
Egg 16	+	+
Egg 17	+	+
Water 1	+	+
Water 2	+	+
Water 3	+	+
Water 4	+	+
Water 5	+	+
Water 6	+	+
Water 7	+	+
Water 8	+	+
Water 9	-	+
Water 10	+	+

Table 4.4 (Cont'd): comparison of the field samples collected from the farm sampling using LAMP assay with traditional nested PCR.

Sample	Positive/negative by LAMP	Positive/negative by nested PCR
Negative control (water)	-	-
Positive control (*Fp* NCIMB 1947)	+	+
Spleen 1	+	+
Spleen 2	+	+
Spleen 3	+	+
Spleen 4	+	+
Spleen 5	+	+
Spleen 6	+	+
Spleen 7	+	+
Spleen 8	+	+
Spleen 9	+	+
Spleen 10	+	+
Spleen 11	+	+
Spleen 12	+	+
Kidney 1	+	+
Kidney 2	-	+
Kidney 3	+	+
Kidney 4	+	+
Kidney 5	+	+
Kidney 6	+	+
Kidney 7	+	+
Kidney 8	+	+
Kidney 9	-	+
Kidney 10	+	+
Spleen 11	+	+
Spleen 12	+	+

4.3.6 Serology

Serology raw data was obtained from the Luminex™ assay for all batches from Life Stage III (Appendix: IV). In the Denmark I fish, 88 % of the fish had antibodies to *Fp*, 92 % of UK and the Denmark II batch and 94 % of the USA fish. Mean titres of the anti-*Fp* antibodies from life stage III fish ranged from 8.2 (Denmark I), 8.5 (UK), 8.9 Denmark II), and 9.0 (USA) (Table 4.5).

Table 4.5: Comparison of life stage III fry by serology (mean fluorescence intensity titers (MFI)) and positive by nested PCR.

Source	Samples positive by nested PCR	Samples positive by Serology	Serology (mean titre) (-Log2+1)
Denmark	28	88[o]	8.2 ± 1.5
UK	24	92[Δ]	8.5 ± 1
Denmark	26	92[•]	8.9 ± 1.5
USA	16	94[+]	9.0 ± 1.4

[o] Negative mean fluorescence intensity (MFI) values = 21.5; Positive mean MFI values = 143

[Δ] Negative mean MFI values = 16.7; Positive mean MFI values = 168

[•]Negative mean MFI values = 19.6; Positive mean MFI values = 184

[+] Negative mean MFI values = 32; Positive mean MFI values = 234

A relative risk analysis was also carried out comparing the results from the two techniques, serology and nested. The percentage of positive results obtained from both techniques in Table 4.5 suggests that the decrease in samples positive by nested PCR is correlated to the increase in the samples positive by serology. However, the association of the results from both techniques using the contingency tables by Epi Info™ showed there was no significant difference (Chi square - Yates corrected; $p = 0.06$).

4.4 DISCUSSION

It has been shown in past studies that it is possible to detect *Fp* in several different organs and in the reproductive fluids of infected fish (Holt, 1987; Brown *et al.*, 1997; Vatsos, 2001). Holt (1987) reported finding the bacterium in the ovarian fluid, milt, kidney, spleen and skin mucus. Generally kidney and spleen are sampled for the presence of *Fp* in infected fish (Madsen *et al.*, 2005; Madetoja *et al*., 2002) although there are a few studies in which only the spleen has been sampled (del Cerro *et al*., 2002b; Cepeda and Santos, 2000). Izumi and Wakabayashi (1997) used a nested PCR to detect *Fp* in kidney tissue and on the surface of eggs from ayu *Plecoglossus* ativelis, whereas Baliarda *et al*. (2002) used a nested PCR to detect *Fp* in the ovarian fluid of rainbow trout. In this study, it was observed that the number of fish in which *Fp* could be detected varied depending on the organ (spleen or kidney) sampled. The total percentage of *Fp* detected by sampling only the spleen by nested PCR was 3 % and 6 % by bacteriology while by sampling only the kidney by nested PCR and bacteriology was 2 %. When both organs were sampled the total percentage of *Fp* detected by nested PCR was 13 % and 5 % by bacteriology. It is therefore important to sample both organs when attempting to detect *Fp*.

There was also a substantial difference in *Fp* detected depending on the detection method used. The use of nested PCR (using primers of Toyama *et al*., 1994) as a tool for detecting *Fp* in farm water samples, eggs and fish tissues was examined and validated in the study by Vatsos (2001) and found to detect 10 bacterial cells ml^{-1}. *Flavobacterium psychrophilum* detected in samples (eggs, kidney and spleen) was consistently higher with nested PCR than by culture of the samples on agar. Similar findings were reported in a study by Wiklund *et al*. (2000) in which they seeded *Fp* into the brain tissue of the fish. More *Fp* was detected in the brain by nested PCR (76 %) compared with the level detected by culture (52 %). Detection by culture has always been considered problematic due to the slow and fastidious growth of the bacterium as well as the problem of contamination by other rapidly multiplying bacteria (Wiklund *et al*., 2000; Lorenzen, 1994). Although contaminants hampered the isolation of pure cultures of *Fp*, the presence of these bacteria does not affect the amplification of *Fp* in nested PCR (Wiklund *et al*., 2000). PCR

also has the advantage of being a faster detection method (24 h) unlike culture of the bacteria which can take up to a week (Cipriano and Holt, 2005) and it also has higher sensitivity compared with culture (Wiklund *et al*., 2000). The disadvantage of using a method such as PCR is that it does not differentiate between live and dead cultures and it is not quantitative.

The qPCR assay developed in Chapter 2 was used to test a number of samples collected from the farm sampling in an attempt to quantify the levels of *Fp*. A number of samples positive by nested PCR were negative in the qPCR because the amount of *Fp* in the sample was below the detection limit of the assay i.e. it was not sensitive enough to detect *Fp* in these samples and so the samples were recorded as negative for the presence of the pathogen. However, all field samples except for one water sample, tested positive in the LAMP assay showing that this assay, although not quantitative, was more sensitive than the qPCR developed in Chapter 2. Egg, kidney, and spleen samples were spiked with known quantities of *Fp* and the detection limit was determined to be 18 CFU mg^{-1}, 22 CFU mg^{-1}, and 25 CFU mg^{-1},respectively, while for water and bacterial cultures it was 16 CFU ml^{-1} and 14 CFU ml^{-1}. In comparison the detection limit for egg, kidney and spleen samples spiked with known quantities of *Fp* by nested PCR was 14 CFU mg^{-1}, 11 CFU mg^{-1} and 13 CFU mg^{-1}. For bacterial cultures the obtained detection limit of *Fp* in water and bacteria cultures was 11 CFU ml^{-1} and 9 CFU ml^{-1} by nested PCR.

The LAMP assay did, however, show slight cross-reactivity to *F. columnare* and *F. branchiophilum*. Since the cross-reactivity was found to be near the threshold levels (detection limit) of the assay it may still have a role. Both the LAMP and qPCR require further optimisation before they can be used as a reliable tool for the detection and quantification of *Fp*. The presence of *Fp* on rainbow trout eggs was reported by Holt *et al.* (1993) and is considered to be the most important source of *Fp* in many hatcheries worldwide (Brown *et al*., 1997; Vatsos *et al*., 2001). Prevalence of *Fp* in all sources of eggs imported into the farm was found.

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APPENDICES

Appendix I: Buffers

FLP Media

tryptone–yeast extract–salts agar and broth supplemented with glucose

	1 000 ml
Tryptone	4 g
Yeast extract	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
CaCl	0.2 g
Glucose	0.5 g

For FLP agar, supplement with Agar 14 g l^{-1}.

Dissolve in distilled water and adjust pH to 7.2. Autoclave at 121˚C for 15 min.

TYES Media

	1 000 ml
Tryptone	4 g
Yeast extract	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
CaCl	0.2 g

For TYES agar, supplement with Agar 14 g l^{-1}.

Dissolve in distilled water and adjust pH to 7.2. Autoclave at 121˚C for 15 min.

ELISA Buffers

Low Salt Wash Buffer (LSWB) *10x concentrated solution*

0.02 M Tris; 0.38 M NaCl; 0.05 % Tween-20; pH 7.3

	1000 ml
Trisma base	2 4.2 g
NaCl	22.2 g
Tween-20	5 ml

Dissolve in distilled water and adjust pH to 7.3 with HCl.

Blocking buffer

Dissolve 1 g of Bovine Serum Albumin (BSA; Sigma, Dorset U.K.) in 100 ml of distilled water.

Make up fresh and use the same day.

Antibody Buffer

Dissolve 1 g of BSA in 100 ml phosphate buffered saline (PBS).

Make up fresh and use the same day.

High Salt Wash Buffer (HSWB) *10x concentrated solution*

0.02M Tris; 0.5 M NaCl; 0.1% Tween 20

	1000 ml
Trisma base	2 4.2 g
NaCl	92.2 g
Tween 20	1 0 ml

Dissolve in distilled water and adjust pH to 7.7 with HCl.

Phosphate Buffered Saline (PBS)

0.02 M phosphate; 0.15 M NaCl

	1000 ml
NaCl	8.77 g
$NaH_2PO_4.2H_2O$	0.876 g
$Na_2HPO_4.2H_2O$	2.56 g

Dissolve in distilled water and adjust pH to 7.2 with HCl.

Conjugate Buffer

Dissolve 1 g of BSA in 100 ml LSWB.

Make up fresh and use the same day.

Substrate

To 15 ml of substrate buffer add 150 µl of substrate solution and 5 µl of hydrogen peroxidase.

Substrate buffer

0.1 M Citric acid; 0.1 M Sodium acetate

	1000 ml
Citric acid	21 g
Sodium acetate	8.2 g

Dissolve in distilled water and adjust pH to 5.4 with NaOH.

Stop Solution

(H_2SO_4 2 M)

Add 55.5 ml of sulphuric acid (98%) to 444.5 ml of distilled water.

Gel Electrophoresis and Western Blot Buffers

Polyacrylamide Gel (Separating Gel) Buffer

(1.5 M Tris; 0.4 % (w/v) Sodium Dodecyl Sulphate (SDS))

	1000 ml
Trisma base	182 g
SDS	4 g

Dissolve in distilled water and adjust pH to 8.7 with HCl.

Make up fresh and use the same day.

Stacking Gel Buffer

(0.5M Tris; 0.4% (w/v) Sodium Dodecyl Sulphate (SDS))

	1000 ml
Trisma base	60.5 g
SDS	4 g

Dissolve in distilled water and adjust pH to 6.8 with HCl.

Make up fresh and use the same day.

Ammonium Persulphate Solution

(10 % v/v ammonium persulphate)

Dissolve 0.1 g ammonium persulphate solution in 1 ml distilled water.

Make up fresh and use the same day.

Reservoir Buffer *5x concentrated solution.*

	1000 ml
Tris	15 g
Glycine	43.2 g
SDS	5 g

Dissolved in distilled water and adjust pH to 8.3 with HCl.

Sample Buffer *5x concentrated solution.*

(100 mM Tris; 4% SDS; 2mM DTT; 0.02 % Bromophenol blue)

Tris HCl 0.5 M (pH 6.8)	2.5 ml
Glycerol	2 ml
SDS (10% (w/v))	4 ml
DTT	0.31 ml
Bromophenol blue	2 mg
Distilled water	0.9 ml

Transblot Buffer (TBS)

	1000 ml
Glycine	14.4 g
Trizma base	3.03 g

Adjust pH to 8.3.

Destain

De-stain gel 3-4 successive changes of methanol over 1 h.

Store the gel in a plastic bag with some water.

TBS with Tween (TTBS)

(1.M Tris; 0.4% (w/v) SDS)

Tween 20	0.5 ml
TBS	1000 ml

Adjust pH to 7.5.

Luminex Buffer

PBS-TBN

Dulbecco's Phosphate Buffered Saline (D-PBS)	500 ml 4.78 g
Tween-20	100 µl
bovine serum albumin	0.5 g
sodium azide	0.25 g

Make up fresh and use the same day.

Adjust pH to 7.4.

Luria–Bertani (LB) Media

Tryptone	1000 ml 10 g

Yeast extract		5 g
NaCl		10 g

For LB agar, supplement with Agar 14 g l^{-1}.

Dissolve in distilled water and adjust pH to 7.2. Autoclave at 121˚C for 15 min.

Appendix II: Molecular Reference Guide

Concentration of DNA used in cloning plasmid

(formula provided in cloning kit, Invitrogen, Paisley U.K.)

= (size of fragment *(bp)* x 25) / 3931

= (1078 x 25) / 3931

= 6.85 ng ml^{-1}

Table 1: Table for estimating DNA concentration for sequencing (DNA amount selected should be between the range of 50 and 100 fmol.

Size (kilobase	ng for 50	ng for 100
0.4	13	26
0.5	16	33
1.0	33	65
2.0	65	130

Appendix III: Serology Data

Table 2: Antibody titres (-log2+1; ± standard error) of fish artificially infected with *Flavobacterium psychrophilum* obtained from xMap analysis and ELISA.

Fish	ELISA Titre	Sonicated *Fp* xMap Titre	*Fp* LPS xMAP titre
1	9	10	9
2	7	8	8
3	8	8	8
4	7	8	8
5	7	8	8
6	6	7	6
7	7	9	8
8	7	8	8
9	8	9	8
10	7	8	8
11	7	8	-
12	7	8	-
13	7	8	-
14	7	8	-
15	7	8	-
16	7	9	-
17	7	8	-
18	8	9	-
19	7	8	-
20	7	8	-
21 (-ve	4	5	3
22 (-ve	5	5	4
23 (-ve control)	4	4	4

Appendix IV: Serology Data from Chapter 4

Table 3: Raw data obtained from the Luminex™ xMap assay to detect antibodies against *Fp* for all batches of rainbow trout fry from Life Stage III (mean fluorescence intensity titers (MFI)) converted to (-Log2+1) values.

Fish number	Denmark I	UK	Denmark II	USA
1	9	8	8	10
2	8	9	9	9
3	8	8	10	8
4	8	7	9	9
5	7	9	5	9
6	8	9	5	9
7	8	8	9	10
8	8	9	10	9
9	10	8	9	5
10	3	8	9	10
11	8	9	8	8
12	9	8	10	9
13	8	9	9	8
14	8	8	4	10
15	8	9	9	9
16	8	10	9	8
17	8	8	9	9
18	2	4	8	8
19	9	8	10	9
20	8	9	8	9
21	9	8	9	10
22	4	9	8	10
23	8	9	5	9
24	7	9	10	9
25	5	8	9	9
26	8	8	8	9
27	9	9	9	9
28	8	8	9	9
29	8	8	9	9
30	8	9	9	10
31	8	8	9	9
32	9	8	9	9
33	8	8	8	9
34	9	9	8	9
35	8	8	9	3
36	8	9	8	9
37	4	9	9	9
38	9	7	8	10
39	8	8	3	9
40	7	5	9	5
41	8	8	9	9
42	8	8	9	9
43	8	9	9	8
44	8	8	8	9
45	9	9	9	9
46	8	9	9	9
47	8	9	9	10
48	9	8	9	9
49	8	8	9	4
50	8	8	9	9