Diagnosis and Identification of *Aeromonas salmonicida* and Detection of Latent Infections in Carrier Fish

Final Report

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PROJECT TITLE

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ABBREVIATIONS

AAHL	Australian Animal Health Laboratories, VIC, Australia
AFDL	AAHL Fish Diseases Laboratory
ANGIS	Australian National Genomic Information Service
AP	Primers directed against the *vap*A gene of *Aeromonas salmonicida* (Gustafson *et al.* 1992)
A.sal	*Aeromonas salmonicida*
ATCC	American Type Culture Collection
AQIS	Australian Quarrantine and Inspection Service
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine Serum Albumin
CBBA	Coomassie Brilliant Blue Agar
CFU	Colony Forming Unit
dNTP	Deoxy nucleotide triphosphate
DIG	Digoxygenin
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylenediaminetetraacetic acid
GE	Genome Equivalent
HC-PCR	Hybridisation capture PCR
IC	Inhibition control
LAF	Large Animal Facility, AAHL
LDL	Lower Detection Limit
MIY	Primers directed against *A. salmonicida* subsp. *salmonicida* (Miyata *et al.* 1996)
NFHRL	National Fish Health Research Laboratory, WV, USA
NTU	Nephelometric Turbidity Unit
PAAS	Probe Assay *A. salmonicida* plasmid (Hiney *et al.* 1992)
PBSA	0.1M Phosphate Buffered Saline, pH 7.4
PCR	Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid
rDNA	Ribosomal dexoyribonucleic acid
RAPD	Random Amplified Polymorphic DNA
RCNSS	Richard Cronin National Salmon Station, MA, USA
RNA	Ribonucleic acid
RNase	Ribnuclease
SBA	Sheep Blood Agar (Columbia Agar Base, Oxoid CM 331, supplemented with 5% v/v defibrinated sheep's blood)
SDS	Sodium dodecyl sulfate
SFC	Secure Frozen Culture
SIF	Stress Induced Furunculosis
SSC	Standard saline citrate buffer
subsp.	Subspecies
syn.	Synonym
TAE	Tris/acetic acid/EDTA buffer
TBE	Tris/boric acid/EDTA buffer
TE	Tris/EDTA buffer
T_m	Melting temperature, calculated as 4(G+C) + 2(A+T)
TSA	Tryptone Soy Agar, Oxoid CM 13.1
Triton X-100	t-Octylphenoxypolyethoxyethanol
U	Units of enzyme
V	Volts

NON-TECHNICAL SUMMARY

Aeromonas salmonicida is a complex group of bacterial pathogens, many strains of which cause a range of diseases in salmonids and in other fish. *A. salmonicida* subspecies *salmonicida* is the causative agent of furunculosis which is one of the most serious infectious diseases of salmonids and is exotic to Australia. This disease has had a major economic impact on the salmonid aquaculture industries of Europe, North America and Japan. Other isolates of *A. salmonicida* cause a variety of diseases in a wide range of fish, both in freshwater and marine environments. Disease outbreaks involving these other strains are increasing overseas. Many, but not all of these diseases are exotic to Australia. We do not currently have the capability to rapidly and reliably distinguish the highly pathogenic, exotic strains from less harmful enzootic strains.

The major objective of this project was to develop improved procedures for the detection and identification of *A. salmonicida* species and sub-species to enhance our diagnostic capability for this serious pathogen of fish. In addition, the technology would allow differentiation between exotic and enzootic strains.

It is important that any developed technology should undergo extensive validation of its sensitivity and specificity using a representative range of bacterial isolates. A total of 308 isolates of exotic and enzootic strains of *Aeromonas salmonicida* were used in this study. Phenotypic tests, including those in current use for *A. salmonicida* identification, employed in this study were often inadequate with regards to the accurate identification of *A. salmonicida* isolates to both species and subspecies levels. Results demonstrated that the application of phenotypic tests alone are of limited value, and further highlighted the need for improved diagnostic techniques with regards to *A. salmonicida* identification.

It is well-established that molecular techniques such as polymerase chain reaction (PCR) have the potential to provide specific and sensitive diagnostic procedures. In the initial stages of the project the literature was searched for potential PCR primers and/or DNA probes which could be developed into diagnostic reagents. A number of PCR primer sets and nucleic acid probes were identified and were evaluated with respect to their specificity and sensitivity. Following evaluation, using pure bacterial cultures as the target, three PCR primer sets were considered of sufficient specificity and sensitivity to warrant further development.

The PCR tests AP and PAAS appeared to be 100% specific for the species *A. salmonicida* and did not cross-react with any of the non-target organisms (bacterial species other than *A. salmonicida*) used in this study. Combining the results of both AP and PAAS tests offered the best 'coverage' in terms of identifying the target organism, with only 0.6% of *A. salmonicida* isolates being falsely scored as negative. Thus, in combination, these two PCR tests had a sensitivity of 99.4%.

A further test, the MIY PCR, which was purported to be specific for the sub-species *A. salmonicida salmonicida*, was modified and optimised, and to date, its specificity and sensitivity for the sub-species *A. salmonicida* subspecies *salmonicida*, have both been measured at 100%.

Thus the AP, PAAS and MIY PCR tests examined appeared to have a high level of specificity and sensitivity with regards to identifying pure bacterial cultures. These tests could therefore be of immediate benefit with regards to identifying pure bacterial cultures. These tests provide a rapid means of identification when compared to the time-consuming biochemical methods, and a greater degree of reliability than serological techniques. Further project work was undertaken to determine whether these tests could be used to detect bacteria in tissues from fish with experimental infections.

Bacteria were isolated from the mucus, gills, gut, kidney and spleen of experimentally infected brown trout and positively identified by all 3 PCRs – AP, PAAS, and MIY. With regards to the direct detection of *A. salmonicida* in tissues taken from the experimentally infected fish, both the AP and PAAS PCRs yielded positive results when applied to overtly infected mucus, gill, intestine, muscle lesion, spleen and kidney samples. The MIY PCR was less sensitive, and will require some form of pre-enrichment step to improve its performance with regard to direct detection (i.e. no culture step) of the sub-species *A. salmonicida salmonicida*. The next stage of the project was undertaken to determine whether these tests could be used to detect bacteria in tissues from naturally infected fish. Subspecies *A. salmonicida salmonicida* is exotic to Australia and there are no naturally infected salmonid populations in Australia. Therefore this part of the project was carried out overseas in collaboration with the National Fish Health Research Laboratory (NFHRL), Kearneysville, WV, USA. This laboratory has access to salmonid populations which are known to

be naturally infected with *A. salmonicida salmonicida*. Bacteria isolated from these naturally infected fish were successfully identified as *A. salmonicida* using the AP and PAAS PCRs, and as *A. salmonicida* subsp *salmonicida* using the MIY PCR. All results were in agreement with the biochemical tests currently employed by the NFHRL to identify *A. salmonicida* and *A. salmonicida* subsp *salmonicida*. Typically PCR tests yielded results in 1 to 2 days, compared to 7 days for classical biochemical testing, thus demonstrating the suitability of the PCR tests as a replacement for the more time-consuming biochemical tests.

The PCR assays were capable of direct detection of *A. salmonicida* in tissues from fish with overt infections, with mucus, gill and kidney samples most likely to yield a positive result. However, it was demonstrated that bacterial culture was a more reliable and sensitive method for the detection of *A. salmonicida* in covertly infected salmonids than did the direct PCR testing of tissue samples. Thus it is highly recommended that any target pathogen that may be present in the tissues of covertly infected fish are first concentrated by the inclusion of a culture step, as used in this study, prior to PCR screening. Culture alone did not detect *A. salmonicida* in all salmonid populations either, but the development of a medium selective for *A. salmonicida*, and the use of multiple-point sampling regimes, could overcome such problems.

Within the time-constraints of this project, it could not be demonstrated that hybridisation-capture PCR could improve test performance to allow detection of covert infections. However, it is likely that, with further development, such a procedure would be able to enrich the target nucleic acid from tissue samples and effectively enhance the performance of the diagnostic test.

In view of the previous results only a limited number of tissue samples were obtained from both salmonid (marine) and non-salmonid (freshwater) farmed populations in Australia. Results from standard culture technique and the PCR tests demonstrate that the sampled populations were free of detectable *A. salmonicida* infections.

During the course of this project significant advances in our capability for the detection and identification of *A. salmonicida* infections have been made. The use of PCR technology has provided a test which is more rapid than the biochemical tests currently used for the identification of *A. salmonicida*. The PCR tests have undergone extensive evaluation and validation using a broad range of bacterial isolates from all regions of the world. In addition, a further PCR test which is specific for the exotic sub-species *A. salmonicida salmonicida* has been developed and validated. This PCR test provides us with a capability to identify this important sub-species within 2-3 days of obtaining diagnostic specimens.

SECTION 1 INTRODUCTION

1.1 BACKGROUND

1.1.1 *Aeromonas salmonicida* – a threat to aquaculture and natural fish resources in Australia

Aeromonas salmonicida is the aetiologic agent of a broad range of clinical syndromes in a variety of fish species. As such, this pathogen has been the focus of considerable scientific research since it was first recognised (Emmerich and Weibel, 1894). Taxonomically, *Aeromonas salmonicida* is a complex group that includes four officially recognised subspecies: subsp. *salmonicida*, subsp. *achromogenes*, subsp. *smithia* and subsp. *masoucida*. *A. salmonicida* subspecies *salmonicida*, also referred to as **'typical'** *A. salmonicida*, is the causative agent of furunculosis which is one of the most serious infectious diseases of salmonids. This disease continues to have a major economic impact on the salmonid aquaculture industries of Europe, North America and Japan (AQIS, 1999; Bernoth, 1997a). Most salmonids are affected by this disease, with Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) being highly susceptible.

The remarkable phenotypic variation displayed by this group, and the difficulties of assigning many new isolates to the existing taxonomy based on phenotype (Austin *et al.*, 1989) has lead to a proliferation of disease reports referring to so-called **'atypical'** strains. Overseas, the frequency of disease outbreaks attributable to these strains is increasing (Wiklund and Dalsgaard, 1998). Atypical *A. salmonicida* has been reported as causing a variety of diseases in a wide range of fish, both in freshwater and marine environments (Pederson *et al.*, 1996).

Salmonid aquaculture in Australia has seen a marked increase in production over the last 20 years, particularly in the Atlantic salmon industry in Tasmania. Recent isolation of *A. salmonicida* from marine fish species, such as the greenback flounder and Atlantic salmon, serve to show that there are natural reservoirs of *A. salmonicida* in the Australian marine environment. In addition, given the practical problems associated with identifying new isolates and differentiating these from exotic strains such as *A. salmonicida* subsp. *salmonicida*, it is vital that we develop rapid and sensitive diagnostic procedures for this pathogen. Advances in molecular diagnostics can provide a solution to the problems and shortcomings of convential bacteriological procedures reliant on the analysis of phenotype. Molecular diagnostics in general, and PCR in particular, provide a rapid means of identifying and differentiating bacterial isolates that may display fastidious cultural properties or phenotypic properties that would hinder their otherwise timely identification.

1.1.2 *Aeromonas salmonicida* in Australia

Australia is currently free of typical *Aeromonas salmonicida*, the causative agent of furunculosis. However, some atypical strains of *A. salmonicida* are considered enzootic. Goldfish ulcerative disease (GUD), caused by an atypical strain, was first recognised in Australia in 1974 at a commercial goldfish farm in Victoria. Its source was traced to imported goldfish from Japan (Trust *et al.*, 1980). In recent years other atypicals has been reported in both freshwater fish species eg silver perch and in marine fish species eg greenback flounder and Atlantic salmon (Whittington *et*

al., 1995). The incidence of disease outbreaks appears to be increasing and poses a significant threat to both aquaculture and natural fisheries over a wide geographic range within south-eastern Australia. It is essential that the technology to differentiate between exotic strains (particularly typical *A. salmonicida*) and enzootic strains be developed.

In countries where furunculosis is enzootic a number of antibacterial agents such as oxytetracycline and oxolinic acid are licensed for use in aquaculture. However, chemotherapy is expensive and involves lengthy withdrawal times. There are also a number of risks associated with drug use, including possible re-infection after treatment (Hiney *et al.*, 1997), induction of carrier fish and induction of drug-resistant bacterial variants (Aoki, 1997). The efficacy of the currently available injected vaccines is yet to be established. The most effective control measure to date is the destruction of the infected fish population and disinfection of the infected site. Given that the establishment of furunculosis in Australian salmon farms could herald the end of this country's salmon industry, the best strategy for Australian fisheries is surely one of avoidance (McKelvie *et al.*, 1994).

1.1.3 *Aeromonas salmonicida* – a diagnostic nightmare

Members of the genus *Aeromonas* are Gram negative, facultative anaerobes, and fall within the family Vibrionacea. *A. salmonicida* is psychrophilic and non-motile, and these two traits are traditionally used to distinguish it from other aeromonad species. However, the taxonomy of this particular species is complicated by the great phenotypic heterogeneity that it displays between isolates, and the current disagreement over the nomenclature of isolates (Austin and Austin, 1999). There are 4 described subspecies of *A. salmonicida* plus a number of isolates simply described as 'atypical' but not assigned to any particular subspecies. Atypical isolates are so phenotypically diverse as to make their identification by classical means very challenging; biochemical testing often yields conflicting results. In addition to these problems, the high degree of homology between the major immunogenic components (the surface A-layer protein and the outer-membrane lipopolysaccharide) of *A. salmonicida* isolates means that polyclonal sera cannot be used to differentiate between strains or even subspecies (Crane and Bernoth, 1996). Thus the identification of *A. salmonicida* remains problematic.

1.1.4 Latent infections and carrier fish

Furunculosis poses a very serious exotic disease threat, especially to our growing salmonid aquaculture industries. The threat is further complicated by the fact that the bacterium can be present as clinically inapparent or '**covert**' infections. There are two important dimensions to be considered with respect to covert infections, these being **latency** i.e. the disease only becomes clinically apparent when the carrier has been stressed, and **carriage** i.e. the covertly infected host disseminates the bacterium to other fish and the environment.

A. salmonicida is responsible for a range of clinical syndromes, many of which are precipitated by conditions of stress such as elevated water temperature, over-crowding, trauma and poor water quality. Infections may remain covert indefinitely but may become overt pending environmental

conditions and their affect on carriers (Hiney *et al.*, 1997). Thus latently infected fish play a significant role in the epizootiology of the disease.

The range of known carriers of *A. salmonicida* is extensive, increasing the opportunities for transmission of the microorganism to susceptible fish, including via authorised importation of live fish and/or untreated fish products into Australia. Furthermore, there is no control on access to sea-cage sites by wild populations of fish attracted by the abundance of food. Such populations could act as an additional source of carriers.

Carriers can be extremely difficult to detect, due to the fact that *A. salmonicida* is likely to be present only in low numbers, and its primary location within the host remains unknown. All of these factors lead to the possibility that *A. salmonicida* subsp. *salmonicida* could enter Australia undetected, establish covert infections in fish, and become widely disseminated before the first clinical case of furunculosis is reported.

1.2 NEED

1.2.1 *Aeromonas salmonicida* and salmonid aquaculture

It is well established that *A. salmonicida* has had a significant impact on salmonid aquaculture in North America, Europe and Japan (Bernoth, 1997a). Unless better methods of control are developed, then salmonid farming industries may well contract in countries where furunculosis is enzootic (Munro and Hastings, 1993). In light of these current perceptions, the best strategy for Australian fisheries is surely one of avoidance. In order to facilitate this however, improved diagnostic procedures aimed at the rapid and precise identification of *A. salmonicida*, especially in carrier fish, must be developed.

1.2.2 Improved diagnosis of *Aeromonas salmonicida* isolates

Identification of *A. salmonicida* based on traditional methods is not only time consuming and problematic, it is also currently incapable of differentiating strains exotic to Australia from Australian enzootic strains. The ability to differentiate between these strains is vital given the broadening host range in our domestic freshwater environment and the recent marine isolations from flounder and Atlantic salmon in Tasmania.

Molecular techniques, which target the genetic information of an organism and are therefore highly specific, may have advantages over the more traditional modes of bacterial identification which rely upon phenotypic characteristics. DNA probes and PCR technology will be investigated in an effort to increase the speed and accuracy of the identification of *Aeromonas salmonicida*, and also in an effort to distinguish the exotic typical strains from the enzootic atypical strains.

1.2.3 Detection of latent infection in carriers

It is clear that covertly infected fish, which harbour *A. salmonicida*, play a critical role in the epidemiology of furunculosis, and probably other related diseases, in cultured salmonid and non-salmonid fish (Hiney *et al.*, 1997). The current standard method for detecting carriers is to culture from kidney and spleen of fish subjected to the Stress-Induced Furunculosis (SIF) assay. This assay involves the injecting the fish with corticosteroids and then exposing them to elevated

temperatures for 2 to 3 weeks (Bullock and Stuckey, 1995). This process requires large numbers of fish to be sacrificed, is resource-intensive, time-consuming, and expensive. Moreover, ELISA technology does not appear to improve the sensitivity of detection above that of normal bacterial isolation methods.

However, molecular probes are being developed for the diagnosis and identification of *A. salmonicida*, and could increase the probability of detecting carriers. There are reports of probes which appear to differentiate this species from the other Aeromonad species. In addition to the hybridization approach, detection of latent infections may also be greatly enhanced by the use of the polymerase chain reaction (PCR). Both approaches would address the most significant problems presented to the diagnostician i.e. the identification of covert infections. The further development of a molecular taxonomic scheme may also allow the precise identification of isolates of *A. salmonicida*.

1.3 OBJECTIVES

1.3.1 Original objectives

1. Undertake molecular characterisation of a range of exotic and enzootic *A. salmonicida* subspecies including correlation with biochemical, serological and pathogenic features.
2. Identify genus-, species- and subspecies-specific properties such as nucleotide sequences with potential for diagnostic use.
3. Develop diagnostic procedures using molecular technology.
4. Validation of molecular diagnostic procedures using experimental infections carried out in the microbiologically secure aquarium facility at AAHL.
5. Preliminary survey of wild and farmed populations of fish and shellfish in S.E. Australia in collaboration with NSW, South Australia, Tasmania and Victoria.

1.3.2 Revised objectives

Based on information available after the commencement of the project it became apparent that the original project objects should be modified slightly to take advantage of recent research results from other laboratories. In addition, as the project proceeded and results were reported at international scientific meetings, other research groups showed a keen interest in collaboration. It was deemed that such collaborations would have significant benefits for the project and were pursued.

The revised objectives are summarised below.

1. Characterisation of a comprehensive reference collection of major strains, both exotic and enzootic, of *A. salmonicida* subspecies.
2. Identify published but unvalidated nucleotide sequences with potential for diagnostic use.
3. Develop diagnostic procedures using molecular technology.

4. Validation of molecular diagnostic procedures using experimental infections carried out in the microbiologically secure aquarium facility at AAHL.
5. Validation of the molecular diagnostic procedures developed at AAHL using naturally infected populations of salmonids: a short-term collaborative project with the National Fish Health Research Laboratory, West Virginia, U.S.A.
6. Determine whether the use of hybridisation-capture PCR would enhance the sensitivity of the test to allow detection of covert infections.
7. Field study: a preliminary survey of wild and farmed populations of freshwater and marine fish

SECTION 2 METHODS

2.1 Bacterial Cultures

2.1.1 The AFDL *A. salmonicida* collection

In order to undertake extensive *in vitro* validation and determination of the specificity and sensitivity of the PCR tests, the AAHL Fish Diseases Laboratory (AFDL) collected a wide variety of *A. salmonicida* isolates from around the world. The collection was comprised of type and reference cultures from recognised culture collections, and a range of clinical and laboratory strains originating from as large a host and geographic range as possible. We gratefully acknowledge the contributions of the various laboratories listed in Table 2.1. The distribution of these isolates according to their country of origin and host species are as outlined in Tables 2.2 and 2.3. Approximately 85% of the isolates in the AFDL *A. salmonicida* collection are exotic to Australia.

Table 2.1 List of Contributors to the AFDL *A. salmonicida* collection

Contributor	Laboratory
	AFDL
	AFHRL, Benalla
Mrs. J. Petrie	Agriculture and fisheries Dept, Aberdeen,Scotland
	American Type Culture Collection
Dr. Steiner Høie	Central Veterinary Laboratory, Oslo, Norway
Dr. Harry Kalnins	Commonwealth Serum Laboratories
Dr. Inger Dalsgaard	Danish Institute for Fisheries and Marine Research
Dr. Ted Meyers	Department of Fish and Game, Alaska, USA
Mr. L D. Ashburner	Dept. of Conservation Forests and Lands, Victoria
Dr. E.B. Shotts	Dept. of Microbiology, University of Georgia, USA
Dr. Jeremy Carson	Fish Health Unit, Mt. Pleasant Lab.,DPIWE, Tas.
Dr. R. Whittington	Elizabeth MacArthur Agricultural Institute, NSW
Dr. M. Hiney	Fish Disease Group, University College Galway, Ireland
Dr. Chris Rogers	Fish Diseases Laboratory, CEFAS, Weymouth, UK
Prof. Brian Austin	Herriot Watt University, Scotland
Dr. Christian Michel	Insititut National de la Recherche Agronomique
Dr. O. Haenen	Inst for Animal Science and Health, The Netherlands
	Institute for Experimental Pathology, University of Iceland
Dr. Stuart D. Miller	Institute of Aquaculture, University of Stirling, Scotland
Dr Tom Wiklund	Institute of Parasitology, ABO Akademi Uni., Finland
	National Collection of Industrial and Marine Bacteria, Scotland
Dr Rocco Cipriano	National Fish Health Research Laboratory, WV, USA
Dr. R.B. Callinan	NSW Dept. of Fisheries, RVL Wollongbar
Dr. T.P.T. Evelyn	Pacific Biological Station, Nanaimo, BC, Canada
Prof. Takashi Aoki	Tokyo University of Fisheries, Japan
Dr. P. Rintamaki	University of Oulu, Finland

Table 2.2 Distribution of *A. salmonicida* isolates according to country of origin

Country of Origin	# Isolates
Australia	45
Continental Europe	120
Iceland	21
Japan	8
North America	35
UK and Ireland	70
Unknown	9
Total number of isolates	308

Table 2.3 Distribution of *A. salmonicida* isolates according to host species

Host species	# Isolates	Host species	# Isolates
Salmonids (11 spp)	148	Minnow	1
Blenny	2	Pike	4
Bream (2 spp)	2	Plaice	2
Carp (2 spp)	6	Roach	2
Cod	5	Rudd	4
Dab	3	Sablefish	1
Eel (2 spp)	4	Silver perch	1
Flounder (3 spp)	25	Striped trumpeter	3
Goldfish	40	Turbot	3
Grayling	2	Unknown/Misc.	44
Haddock	1	Whiting	3
Lingcod	1	Wolf Fish	1

2.1.2 Cultures other than *A. salmonicida*

A variety of bacterial cultures, listed in Table 2.4, were employed to test the specificity of the PCR assays. Isolates included representatives of all *Aeromonas* DNA hybridisation groups (Huys and Swings, 1999), and a range of common fish pathogens.

Table 2.4 Bacterial isolates used as negative controls in PCR screening.

Isolate Name	Isolate No.
Yersinia ruckeri	ATCC 29473
Carnobacterium piscicola	ATCC 35586
Edwardsiella ictaluri	85:10067-1A
Enterococcus seriolicida	ATCC 49156
Aeromonas hydrophila subsp. *hydrophila*	ATCC 7966
Aeromonas caviae	ATCC 15468
Aeromonas media	ATCC 33907
Aeromonas eucrenophila	ATCC 23309
Aeromonas veronii biovar *veronii*	ATCC 35624
Aeromonas veronii	ATCC 35941
Aeromonas schubertii	ATCC 43700
Aeromonas trota (syn. *A. ichtiosmia*)	ATCC 49657
Aeromonas sobria	ATCC 43979
Aeromonas veronii biovar *sobria*	ATCC 9071
Aeromonas jandaei	ATCC 49568
Flexibacter maritimus	NCIMB 2154
Flexibacter ovolyticus	NCIMB 13127
Photobacterium phosphoreum	NCIMB 1282
Vibrio ordalii	NCIMB 2167
Vibrio tubiashii	NCIMB 1340
Vibrio anguillarum (syn. *Listonella anguillarum*)	ATCC 19264
Vagococcus salmoninarum	NCFB 2777
Vibrio alginolyticus	ATCC 17749
Vibrio harveyi	ATCC 14126
Vibrio vulnificus	ATCC 27562
Vibrio fluvialis	ATCC 33809
Vibrio natriegens	ATCC 14048

2.1.3 Maintenance of bacterial strains

All bacterial isolates were stored in MicroBank™ vials (Pro-Lab Inc) at -80^{o}C as per manufacturer's instructions. When required, isolates were grown on 5% Sheep Blood Agar (SBA) and incubated aerobically at 22^{O}C for 2-4 d.

2.2 Biochemical Characterization

A biochemical profile was obtained for each isolate, in order to confirm the isolate's identity as *Aeromonas salmonicida*, and to group the isolate according to subspecies. The profile consisted of the following tests: Gram stain; pigment production on TSA; cytochrome oxidase; motility; growth on SBA at 37°C; indole; methyl red; Voges-Proskauer; arginine dihydrolase (Moeller); nitrate reduction; aesculin hydrolysis; gas production (1% carbohydrate in OF basal medium) from D-glucose; and acid production from L-arabinose, D-galactose, maltose, D-mannitol, sucrose, and trehalose. Media was prepared according to the manufacturer's instructions and tests were conducted according to Balows *et al.* (1993) except that cultures were incubated at 22°C. Tests were examined 1, 2, 3, 5, 7, 10 d post-inoculation. Terminal end-product tests were evaluated 4 - 10 d post-inoculation.

2.3 *In Vitro* Molecular Methods

2.3.1 Genomic DNA extraction

Aeromonas salmonicida isolates were grown aerobically on TSA plates for 3 days at 22°C. Genomic DNA was extracted using the DNAzol® reagent (Life Technologies™), the QIAmp® Tissue Kit (Qiagen), or the Puregene® DNA Isolation Kit (Gentra Systems) as per the manufacturer's instructions. The Puregene® Kit became the method of choice due to its better yields and ease of use. The extracted genomic DNA (typically 5 μL of a final extraction volume of 100 μL) was assessed by electrophoresis on 1% agarose gels supplemented with 0.5 μg mL^{-1} ethidium bromide. Electrophoresis was carried out at 10 V cm^{-1} for 1 h using 1x TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0).

2.3.2 Design of 15e4 primers

Primers 15e4F and 15e4R (Table 2.5) were designed using the Primer Designer V 2.0 package (Scientific and Educational Software© 1991), to yield a 182 bp PCR product from the RAPD-generated DNA fragment '15e4' described by Oakey *et al.* (1998) and purported to be specific for all members of the species *A. salmonicida*.

2.3.3 PCR protocols

PCRs were performed in 0.2 mL thin walled PCR tubes (Quantum Scientific Pty Ltd) in a GeneAmp® 9600 thermal cycler (Perkin Elmer-Cetus). The primer sequences used in the different PCRs are listed in Table 2.5. Each 25 μL PCR contained 2.5 μL of 10x reaction buffer, and the four deoxynucleotide triphosphates adenine, thymine, guanine and cytosine at a final concentration of 0.2 mM each. The amounts of the specific primers, *Taq* DNA Polymerase (Promega Corporation) or Platinum™ *Taq* DNA polymerase (Life Technologies™), and final concentration of $MgCl_2$ are summarised in Table 2.6. All PCRs had an initial denaturation time of 1 min, with main cycling conditions as outlined in Table 2.6, followed by a final extension time of 3 min.

Table 2.5 Summary of PCR primer sets examined in this study

PCR	Primer	Primer Sequence (5' → 3')	Target Species	Primer Target	Reference	Product Size
PAAS primary	PAAS1	CGT TGG ATA TGG CTC TTC CT	*Aeromonas salmonicida*	plasmid borne	O'Brian *et al.*, 1994	423 bp
	PAAS2	CTC AAA ACG GCT GCG TAC CA				
PAAS nested	PAAS4	AGG TAA GTC TAT TAG GTT CG	*Aeromonas salmonicida*	plasmid borne	Mooney *et al.*, 1995	278 bp
	PAAS5	CAA TGT GAA AAA GGA AGG CG				
AP	AP1	GGC TGA TCT CTT CAT CCT CAC CC	*Aeromonas salmonicida*	*vap*A gene	Gustafson *et al.*, 1992	421 bp
	AP2	CAG AGT GAA ATC TAC CAG CGG TGC				
MIY	MIY1	AGC CTC CAC GCG CTC ACA GC	*Aeromonas salmonicida* subsp. *salmonicida*	RAPD fragment	Miyata *et al.*, 1996	512 bp
	MIY2	AAG AGG CCC CAT AGT GTG GG				
15e4	15e4F	CTG GAT GGA GAG CAA TCG CA	*Aeromonas salmonicida*	RAPD fragment	Oakey *et al.*, 1998	182 bp
	15e4R	GGT CTA TCA CGC TGT CGT CA				
16SU	27F	GAG TTT GAT CCT GGC TCA G	All Bacteria	16S rDNA	Dorsch & Stackebrandt, 1992	1500 bp
	1492R	TAC GGY[a] TAC CTT GTT ACG ACT T	(universal primers)			
16Sa	16S af	TTT CGC GAT TGG AT GAA	*Aeromonas salmonicida*	16S rDNA	Carson, 1998	261 bp
	16S ar	TTG ACA CGT ATT AGG CGC CA				
16Sb	16S bf	GGC CTT TCG CGA TTG GAT GA	*Aeromonas salmonicida*	16S rDNA	Høie *et al.*, 1997	271 bp
	16S br	TCA CAG TTG ACA CGT ATT AGG CGC				
ASA1	ASA1-1	GGT TAC AGC GAG CAG GAG CG	*Aeromonas salmonicida*	RAPD fragment	I. Hirono, 1997 [pers. comm.]	360 bp
	ASA1-2	ACT GAG CCA TGC CTT GCG GG				
ASA4	ASA4-1	AAC AGT ACG CCG ACC GCC TC	*Aeromonas salmonicida*	RAPD fragment	I. Hirono, 1997 [pers. comm.]	378 bp
	ASA4-2	GCA CTG GCA TCC CGA GAG C				

[a] Y=C:T at a ratio of 1:1.

Template DNA was added in the amount 1-10 ng unless otherwise specified. In the case of the PAAS nested PCR, 1 µL of product from the PAAS primary PCR product was used as template. Genomic DNA from the *A. salmonicida* isolate SFC 291 was used as a positive control, sterile distilled water served as the negative control.

Products were visualised on 1.5% agarose gels supplemented with 0.5 µg mL^{-1} ethidium bromide. Electrophoresis was carried out at 12 V cm^{-1} using 1x TBE buffer (10 mM Tris, 8.3 mM boric acid, 0.1 mM EDTA, pH 8.6).

Table 2.6 Summary of variable PCR parameters used in this study

PCR	Reagent Concentrations			Cycling Conditions			
	$MgCl_2$ (mM)	Primer (pmoles)	Units *Taq* polymerase	No. Cycles	Denaturation	Annealing	Extension
PAAS	2.5	8	0.25	30	95°C 30 sec	57°C 30 sec	72°C 1min 30 sec
AP	2.5	8	0.25	30	95°C 30 sec	57°C 30 sec	72°C 1min 30 sec
MIY	1.5	16	1.20	35	94°C 30 sec	68°C for 1 min 30 sec	
15e4	2.0	8	0.50	30	95°C 15 sec	58°C 15 sec	72°C 1min
16SU	1.5	9	0.50	28	94°C 30 sec	49°C 30 sec	72°C 1 min 30 sec
16Sa	1.4	8	0.50	29	94°C 30 sec	60°C 30 sec	72°C 2min
16Sb	3.0	8	1.00	40	94°C 30 sec	54°C 30 sec	72°C 1min
ASA1	1.5	10	0.60	30	95°C 30 sec	58°C 30 sec	72°C 1min
ASA4	1.5	10	0.60	30	95°C 30 sec	58°C 30 sec	72°C 1min

2.3.4 *In vitro* PCR specificity

The specificity of the various assays was determined by screening genomic DNA prepared from the bacterial isolates listed in Table 2.4 with the PCRs listed in Table 2.5. When non-specific reactions occurred, resulting in extraneous bands of product as determined by gel electrophoresis, the stringency of the PCR was increased. This was achieved by increasing the annealing temperature by 1 – 2°C, until reaching the theoretical T_m of the primers. If the primers continued to cross-react with the non *A. salmonicida* control templates, the PCR was deemed to be non-specific.

2.3.5 *In vitro* PCR sensitivity

The sensitivity of those assays demonstrated to be 100% specific was determined by PCR screening of the AFDL collection of *A. salmonicida* isolates. Each *A. salmonicida* genomic DNA sample was screened 4 times by each PCR. A PCR was deemed positive based on product size, as determined by agarose gel electrophoresis and as compared to known standards. If an *A. salmonicida* DNA sample yielded a negative result, it was subsequently re-tested. If a sample was negative in all of AP, PAAS, and MIY systems, it was tested with universal primers specific for the 16S ribosomal RNA gene i.e. the '16SU' PCR. A negative result in the latter PCR indicated that the DNA preparation was recalcitrant to amplification. In such cases, the source bacterium was re-cultured, and the DNA isolated and re-tested. If the DNA was amplified in the 16SU PCR, the

negative results obtained by the A.sal species-specific PCR assays were confirmed as truly negative.

2.3.6 Screening of *A. salmonicida* plasmids with the PAAS3 probe

Plasmid extraction. *Aeromonas salmonicida* growth conditions were as for genomic DNA extraction. Plasmids were extracted using either the method of Kado and Liu (1981), or the BRESAspin™ Plasmid Mini Kit (GeneWorks Pty Ltd) as per manufacturer's instructions.

Plasmid blots. The *A. salmonicida* plasmid extracts were denatured by incubation in a boiling water bath for 10 minutes, then blotted onto nylon membranes (Boehringer Mannheim) using the method of Reed and Mann (1985).

DIG labelling. The 3' end labelling reaction was performed with 100 pmol of the PAAS3 oligonucleotide 5'-GCTAGCCAACTCTCTTTCCA-3' (O'Brien *et al.*, 1994), 1 nmol digoxygenin-11-ddUTP (Boehringer Mannheim) and terminal deoxynucleotidyl transferase (Boehringer Mannheim) according to the manufacturer's instructions.

Hybridization conditions. DNA filters were prepared as outlined above, and prehybridization was performed in Hybaid tubes containing 20 mL hybridization solution (5 x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, and 1% (w/v) skim milk powder). The membrane was prehybridized for 1 h in a Hybaid oven at 10^{o}C below the probe's theoretical T_m of 60^{o}C. The hydridization solution was discarded and replaced with 3 mL fresh hybridization solution containing 12.5 pmol of DIG-labelled probe. Hybridization proceeded overnight, and the membrane was then washed three times for 15 min in 4 x SSC-0.1% SDS at hybridization temperature.

Chemiluminescent detection. Detection of digoxigenin-labelled oligonucleotide was performed by using alkaline phosphatase-labelled anti-digoxigenin Fab antibody fragments (Boehringer Mannheim) and the chemiluminescent alkaline phosphatase substrate CDP-Star™ (Boehringer Mannheim) according to the suppliers recommended procedure. The membrane was exposed to Lumi-film (Boehringer Mannheim) for 1 min. After detection, the filter was washed at increasingly higher temperatures to assay probe specificity under stringent conditions, and the filter re-detected after each wash. To remove the probe, the filter was washed three times for 15 min with a boiling 0.1 x SSC-0.1% SDS solution.

2.3.7 SDS-PAGE and Western blotting

a. Preparation of samples

A. salmonicida isolates for electrophoresis were cultured on Columbia Blood Agar Base (Oxoid CM331), supplemented with 5% defibrinated sheep's blood. Cultures were incubated for 48 hours at 25°C in air prior to harvest and processing. The cells were washed 3 times in sterile PBSA. Approximately 100 mg of bacteria were transferred to a microfuge tube containing 1 mL sterile PBSA after repetitive pipetting the cells were sedimented at 13,000 x *g* for 1 minute. The supernatant was discarded and the washing steps repeated twice more. The remaining pellet was then resuspended in 1 ml of SDS-glycerol buffer containing 10% glycerol, 2.3% SDS, 0.0625 M

Tris/HCl pH 6.8, 5% mercaptoethanol. Following incubation at 100°C for 10 minutes insoluble material was removed from the samples by centrifugation at 13,000 x *g* for 5 minutes. 600 µL of the supernatant was then transferred to a new tube and frozen at -20°C prior to electrophoresis.

b. Electrophoresis

Bacterial proteins were analysed by Western blot following electrophoresis using the buffer system of Laemmli (1970). Bacterial proteins were resolved in 0.75 mm X 8 cm 12% acrylamide gels overlayed with a 2.5 cm 4.75% stacking gel. Piperazine di-acrylamide was substituted for N,N'—methylene—bis—acrylamide on a weight for weight basis, the final concentration of cross—linker being 2.67% with respect to total acrylamide. Gels were run at 10 mA for 10 minutes followed by 50 mA constant current until the dye front reached the bottom of the resolving gel. For electrophoresis, approximately 5 – 20 µL of sample was loaded in each well, the respective volumes having been previously estimated to give equal staining intensity in each lane.

c. Transfer

Following electrophoresis, proteins were transferred to 0.22 µm nitrocellulose sheets (NitroPure, Micron Separations Inc. # WP2HY00010) at 30 V for 16 hours at 4°C using the buffer system of Towbin *et al.* (1979).

d. Western blot

The transferred bacterial proteins were probed with hyper-immune polyclonal antisera raised against *A. salmonicida* A protein (supplied by Dr. Guri Eggset, Marin Bioteknologi i Tromsø, Norway). The membranes were blocked for 30 minutes with SM solution (3% w/v skim milk in 0.01M Tris-HCl pH 7.4, 0.15M NaCl) at room temperature. The blocking solution was discarded and the membranes were reacted with the sera diluted 1:500 in SM solution and the membranes incubated at room temperature on a slow-speed flat-bed orbital shaker for 1 hour. The membranes were than given two washes of 5 minutes with 0.01M Tris-HCl pH 7.4, 0.15M NaCl, 0.05% v/v Tween 20, followed by a final wash of 10 minutes in 0.01M Tris-HCl pH 7.4, 0.15M NaCl (no Tween 20). All membranes were then incubated with freshly prepared anti-rabbit, HRPO conjugate (Silenus RAH) diluted at 1:500 with SM solution using the same incubation conditions used for the primary antibody step. The membranes were washed again as described previously and developed using 4-chloro-1-naphthol as substrate at room temperature for 15-30 minutes to obtain optimal staining with a minimum of background. The substrate was prepared by dissolving 60 mg 4-chloro-1-naphthol in 20 ml methanol on ice; just before use this was mixed with 100 ml in 0.01M Tris-HCl pH 7.4, 0.15M NaCl to which 60µl of 30% v/v H_2O_2 had been previously added.

2.3.8 DNA Sequencing

Product (typically 50 ng DNA) from the 16SU PCRs were sequenced with the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) using the 1110f primer (GCAACGAGCGCAACCC), purified using the QIAquick PCR Purification Kit (Qiagen), and run on

an ABI model 377 automated sequencer (PE Applied Biosystems). The resultant sequences were compared against the GenBank database using BLAST (Altschul *et al.*, 1990) to determine their general taxonomic affiliation.

2.4 Seeded Tissue Studies

2.4.1 Preparation of bacterial inocula

A. salmonicida isolates were chosen for the seeding experiments on the basis that they were positive by the AP, PAAS, and MIY PCRs, and had been subject to minimal passage. An overnight culture of *A. salmonicida* (SFC 291 or SFC 166) grown on SBA at room temperature was used to prepare a cell suspension of 115 NTU's in 15 mL sterile PBSA as determined by the Hach nephalometer. The number of cells present in the suspension was calculated by either drop plate counts (Miles and Misra, 1938) or direct counts using the WSI Counting Chamber (Weber Scientific International Ltd). Aliquots of dilutions of the suspension ranging from 10^8CFU/mL to 10^4CFU/mL were added to the tissue samples.

2.4.2 Preparation of fish tissues

The following tissues were obtained from uninfected rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) for use in the seeding experiments: mucus, gill, spleen, kidney and intestine. Mucus was obtained by scraping the side of the fish with a sterile scalpel blade, the other tissues were aseptically excised from the necropsied fish. Mucus was weighed and then diluted 1:1 with sterile PBS, mixed thoroughly with a pipette, and aliquoted in 100 µL lots. The other tissues were divided into 25 or 50 mg aliquots. All aliquots were stored in sterile 1.5 mL Eppendorf tubes at –20°C.

2.4.3 Extracting DNA from seeded and unseeded salmonid tissues

Several methods were trialled in an effort to identify the most efficacious means of extracting DNA suitable for amplification from fish tissue. Seeded tissue samples generally consisted of a 50 µL aliquot of an *A. salmonicida* cell suspension (Section 2.4.1) and 50 mg of frozen fish tissue being added to 200 µL of sterile distilled water in a 1.5 mL Eppendorf tube, then macerated with a Kontes Pellet Pestle® (Edwards Scientific), vortexed, and processed using one of the methods outlined below. Unseeded samples were processed in the same manner and contained fish tissue, but no *A. salmonicida* cells. Typically, 1 µL of a fish tissue DNA extract, or 1 µL of a 1:20 dilution of the extract, would be used as template in a PCR.

a. Boiling method

The mix was boiled for 10 min, cooled on ice and centrifuged for 5 min at 6,000 x *g* to pellet cellular debris. The resultant supernatant was diluted 1:50 with sterile distilled water prior to use in the PCR.

b. Proteinase K method

Proteinase K was added to a final concentration of 200 µg/mL and the mixture incubated at 55°C for 3 h. The mix was then boiled for 10 min, cooled on ice and centrifuged for 5 min at 6,000 x *g* to pellet cellular debris. The resultant supernatant was diluted 1:50 with sterile distilled water prior to use in the PCR.

c. Triton x-100 method (Agersborg et al.*, 1997)*

A 200 µL aliquot of 2% Triton X-100 (BioRad®) was added, the mixture vortexed, and held at room temperature for 10 min. The mix was then boiled for 10 min, cooled on ice and centrifuged for 5 min at 6,000 x *g* to pellet cellular debris. The resultant supernatant was diluted 1:50 with sterile distilled water prior to use in the PCR.

d. Chelex and Triton method (Khan and Cerniglia, 1997).

A 200 µL aliquot of 5% chelex-100 resin (BioRad® Biotechnology Grade, Sodium Form) was added, the mixture was vortexed, and held at 56°C for 10 min. Next a 200 µL aliquot of 0.1% Triton X-100 (BioRad®) was added. The mix was boiled for 10 min, cooled on ice and then centrifuged for 5 min at 6,000 x *g* to pellet cellular debris. The resultant supernatant was diluted 1:50 with sterile distilled water prior to use in the PCR.

e. Chelex method

A 200 µL aliquot of 5% chelex-100 resin (BioRad® Biotechnology Grade, Sodium Form) was added, the mixture vortexed, boiled for 10 min, cooled on ice and then centrifuged for 5 min at 6,000 *g* to pellet cellular debris. The resultant supernatant was diluted 1:50 with sterile distilled water prior to use in the PCR.

f. Enzymatic/chemical lysis method

A 50 µL suspension of *A. salmonicida* cells and 750 µL of 1 x SSC was added to 20 mg of frozen fish tissue in a 2.0 mL Eppendorf tube and the mix ground with a Kontes Pellet Pestle® (Edwards Scientific). A 20 µL aliquot of lysozyme (0.1 mg/mL) was added and the mix held at 60°C for 30 min. Next 7 µL of 20% SDS and 20 µL of proteinase K (10 mg/mL) were added and the mixture held at 60°C for at least 3 h, followed by the addition of 3 µl RNase A and incubation at 37°C for 1 h.

Next 800 µL of buffer (1 x TE) equilibrated phenol was added, the mixture vortexed, and the phases separated by centrifugation at 12,000 x *g* for 5-10 min. The aqueous phase was transferred to a centrifuge tube containing an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), vortexed, and centrifuged as before. A final extraction in an equal volume of chloroform:isoamylalcohol (24:1) was performed. The aqueous phase was transferred to a clean centrifuge tube and the DNA precipitated by the addition of one-tenth volume 3M sodium acetate (pH 4.5) and a double volume of cold 100% ethanol and incubated at -70°C for 1 h, or -20°C overnight. Nucleic acids were pelleted by centrifugation at 12,000 x *g* for 20 min. The pellet was washed with cold 70% ethanol, air-dried and dissolved in 100 µL sterile HPLC grade water.

g. Puregene® Kit (Gentra Systems)

DNA was extracted using the Puregene® Kit (Gentra Systems). Chilled Lysis Solution (600 µL) was added to 50 mg fish tissue mixed with a 50 µL suspension of *A. salmonicida* cells in a 1.5 mL Eppendorf tube, and the mix macerated with a Kontes Pellet Pestle® (Edwards Scientific). Then 6 µL of proteinase K (10 mg/mL) was added and the mix incubated at 60°C overnight. RNase A was added, the tube inverted 25 times to mix the contents, and then incubated at 37°C for 1 h. After cooling to room temperature, 200 µL of chilled Protein Precipitation Solution was added, the mixture vortexed and centrifuged at 13,000 x *g* for 3 min to pellet cellular debris. The supernatant was transferred to a fresh eppendorf tube containing 600 µL 100% isopropanol, the contents gently mixed by inverting the tube 50 times, and then centrifuged at 13,000 x *g* for 1 min to pellet the DNA. The pellet was washed with 600 µL 70% ethanol, then air dried, resuspended in 100 µL DNA Hydration Solution and stored at 4°C. Extracts were diluted 1:20 in sterile distilled water prior to PCR.

h. The QIAmp® Tissue Kit (Qiagen)

DNA was extracted using the QIAmp® Tissue Kit (Qiagen) according to the manufacturer's instructions.

2.4.4 Determination of the Lower Detection Limit (LDL) of the AP, PAAS and MIY PCRs

a. Calculation of the LDL of the PCRs based on purified DNA preparations

Genomic DNA was extracted from SFC262 using the Puregene® Kit and quantitated using the GeneQuant II RNA/DNA calculator (Pharmacia Biotech). Serial dilutions of the DNA, ranging from 20 ng to 20 fg, were used as template directly in the AP, PAAS and MIY PCR assays (Section 2.3.3). Alternatively, the *A. salmonicida* DNA was mixed with 1-2 µg aliquots of brown trout tissue DNA (extracted from 25 mg of either mucus, kidney, spleen, intestine or gill material using the Puregene® Kit as outlined in Section 2.4.3), and then amplified in the AP, PAAS and MIY PCRs.

b. Calculation of the LDL of the PCRs based on addition of A. salmonicida whole cell preparations

Whole cell suspensions of *A. salmonicida* were prepared as described in Section 2.4.1, and 50 µL aliquots of cells suspensions ranging from 10^7 CFU/mL to 10^3 CFU/mL were added to 25-50 mg lots of fish tissues which had been prepared as outlined in Section 2.4.2. DNA was extracted using the Puregene® Kit (Section 2.4.3) and amplified using the AP, PAAS and MIY PCRs (Section 2.3.3).

c. Methods employed to improve the LDL of the PCRs

The addition of various co-solvents to the PCRs (such as 400 ng/PCR BSA or Life Technologies™ PCRx Enhancer Solution), as well as alterations to the cycle number, length of cycles, and primer and enzyme concentrations were trialled in an effort to improve the LDLs of the PCRs.

2.5 *A. salmonicida* overt infection in fish held at AAHL

All fish infection experiments were conducted in the biological isolation area of AAHL, at an ambient temperature of 18°C.

2.5.1 Development of a model of infection in goldfish

Goldfish (*Carassius auratus*) weighing at least 10 g were anaesthetised with benzocaine (10mg/L) and inoculated with 50 µL of saline containing graded doses of 10^4 and 10^1 cells of *A. salmonicida* (SFC 1) by injection into the abdominal cavity. Fish were then transferred to separate aquaria containing 70 L aerated freshwater and monitored daily for clinical signs of disease – external lesions, lethargy, unnatural swimming or other behaviours. Fish displaying any of these signs were euthanased by benzocaine overdose (100 mg/L) and processed as outlined in Section 2.5.4. Uninfected control fish (n=10) were injected intraperitoneally with 50µL sterile saline and maintained under the same conditions as the infected fish. Seven days after the initial exposure, any surviving fish were euthanased by anaesthetic overdose and sampled.

2.5.2 Generation of overt infection by intraperitoneal inoculation

Brown trout (*Salmo trutta*) were first anaesthetised with benzocaine (10mg/L). Each fish received between 10^6 and 10^4 cells of *A. salmonicida* (SFC 262) in 100 µL of sterile saline by injection into the abdominal cavity. Fish were then transferred to separate 70 L aquaria and monitored daily for clinical signs of disease – gross lesions, lethargy, abnormal swimming behaviour. Fish displaying any signs were euthanased by benzocaine overdose (100 mg/L). Uninfected control fish (n=10), injected intraperitoneally with 100µL sterile saline only, were housed in a separate tank in the same room and maintained under the same conditions as the infected fish. Ten days after the initial exposure, surviving fish were euthanased by anaesthetic overdose and sampled.

2.5.3 Generation of overt infection by bath exposure

Brown trout were placed in 50L of water containing 10^5 CFU/ml of *A. salmonicida* for 30 min. Fish were then transferred to 70 L aquaria and monitored daily for clinical signs of disease – gross lesions, lethargy, abnormal swimming behaviour. Fish displaying any of these clinical signs were to be euthanased by benzocaine overdose (100 mg/L). Unexposed control fish (n=10) were housed in a separate tank in the same room and maintained under the same conditions as the exposed fish. Ten days after the initial exposure, surviving fish were euthanased by anaesthetic overdose and sampled.

2.5.4. Identification of *A. salmonicida* from experimentally infected fish tissue

Material from the brown trout tissues (mucus, gill, kidney, spleen and intestine) was inoculated onto SBA plates and incubated for 48 h at 20°C. Colonies suspected to be *A. salmonicida* were then either picked using a sterile pipette tip and used directly as template in the PCR mix, or subcultured and DNA extracted using the Puregene® Kit to provide a source of template for the AP, PAAS, and MIY PCRs.

Alternatively, the Puregene® Kit was used to directly extract DNA from 50 mg aliquots of fish tissue which had been aseptically excised from the necropsied fish. This DNA was then used as template in the AP, PAAS and MIY PCR assays (as outlined in Section 2.3.3).

2.6 *A. salmonicida* overt infection in salmonids held at the NFHRL, WV, USA

2.6.1 Generation of overt infection by bath exposure

Labrador and Nauyuk strains of Arctic char *Salvelinus alpinus* (*n* = 18) were placed in water containing 10^5 CFU/mL *A. salmonicida* for 30 min. Fish were then transferred to 50 L aquaria, maintained at 14°C, and monitored daily for clinical signs of disease – lesions, lethargy, colour change, abnormal swimming behaviour. Fish displaying any signs were euthanased by benzocaine overdose (100 mg/L). Unexposed control fish (n=10) were housed in a separate tank in the same room and sampled at the end of the experiment.

2.6.2 Generation of overt infection by stress induction

Rainbow trout (*n* = 20), unvaccinated Atlantic salmon (*n* = 20) and vaccinated Atlantic salmon (*n* = 24) from three separate populations of salmonids maintained at the National Fish Health Research Laboratory, Leetown, and suspected to be covertly infected with *A. salmonicida* were subjected to the SIF (stress induced furunculosis) assay. This assay was conducted according to the method of McCarthy (1977) except the fish were injected intraperitoneally with 0.1 mL of prednisolone 21-hemisuccinate (Sigma Chemical Co.) rather than prednisolone 21-acetate, at a rate of 20 mg/kg fish. The fish were held at 18°C for up to 9 days. Each day the aquaria were checked and dead fish removed for processing as described in Sections 2.6.3 and 2.6.4. After day 8 or 9, all surviving fish were euthanased, and the sacrificed rainbow trout and vaccinated Atlantic salmon were then processed for detection of the pathogen.

2.6.3 Cultural identification of *A. salmonicida* from fish tissue obtained from the SIF test

Bacteria were isolated from the infected fish tissues and identified according to the method of Cipriano *et al.*, 1992. Tissues (mucus, gill, spleen, kidney, intestine) were aseptically removed in the following manner: mucus was collected by passing a sterile scalpel along the lateral surface of each fish. A portion of the gills, spleen, posterior third of the kidney, and a 1 cm section of the intestine were removed. Material from the tissues was used to inoculate CBBA (TSA, Difco, supplemented with 0.01% w/v CBB, R-250, Bio-Rad Laboratories) and incubated for 48 h at 20°C. Dark blue colonies were confirmed as *A. salmonicida* subsp. *salmonicida* if they yielded gram-negative, non-motile bacilli that produced brown pigment on TSA, yielded an alkaline-over-acid reaction in triple sugar iron agar (Difco), fermented glucose, were positive for cytochrome oxidase, liquified gelatine, but were negative for indole and ornithine decarboxylase. Microbiological tests were conducted as described by MacFaddin (1980).

2.6.4 PCR identification of *A. salmonicida* from tissue obtained from Bath Exposure and SIF experiments

DNA was extracted from 50 mg aliquots of tissue using the Puregene® Kit as previously described (Section 2.4.3), and used as template in the AP and PAAS PCR assays.

2.7 Detection of *A. salmonicida* in covertly infected fish tissue

A variety of fish (Table 2.7) suspected of being covertly infected with *A. salmonicida* were euthanased in tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington, USA). Tissues were collected as described above and each sample was halved, with one portion being used for direct PCR testing, the other portion being used for the cultural identification and enumeration of *A. salmonicida*.

Table 2.7 Covertly infected fish used in this study

Salmonid species	Source	Comments
Atlantic salmon	Raceway 3, Richard Cronin National Salmon Station, Sunderland, MA, USA	3+ reconditioned kelts. Positive for *A. salmonicida* by both culture and ELISA.
Atlantic salmon	Raceway 5 & 6, Richard Cronin National Salmon Station, Sunderland, MA, USA	2+ excess domestic stock, receiving run-off water from infected reconditioned kelts. Mortalities recorded. *A. salmonicida* culture positive.
Brown trout	Bennington, VT, USA	1+, furunculosis outbreak and *A. salmonicida* culture positive 6 months ago. Ongoing history of mortalities.
Rainbow trout	NFHRL, Leetown, WV, USA	Established carrier population. *A. salmonicida* culture positive.
Atlantic salmon	NFHRL, Leetown, WV, USA	Established carrier population. *A. salmonicida* culture positive.

2.7.1 Cultural identification and enumeration of *A. salmonicida* from covertly infected fish

Bacteria were isolated from the fish tissues, enumerated and identified according to the method of Cipriano *et al.*, 1992. Tissue samples (mucus, gill, spleen, kidney, intestine) were collected, weighed and diluted 1:10 (w/v) in sterile phosphate-buffered saline (pH 7.2), and mixed by repeated expulsion through a 1.0 mL pipette. Serial $\log_{10}$ dilutions were prepared in PBSA. Aliquots (10 µL) of each dilution were drop plated onto Coomassie Brilliant Blue agar (TSA, Difco, supplemented with 0.01% CBB, R-250, Bio-Rad Laboratories). The CBB plates were incubated for 48 h at 20°C, and bacteria were quantified as colony-forming units (CFU) in dilutions containing 10 – 30 colonies. Dark blue colonies were confirmed as *A. salmonicida* subsp. *salmonicida* according to the test results previously outlined in Section 2.6.3.

2.7.2 PCR identification of *A. salmonicida* from covertly infected fish tissue

DNA was extracted from 50 mg aliquots of tissue using the Puregene® Kit as previously described (Section 2.4.3), and used as template in the AP, PAAS and MIY PCR assays.

2.8 Hybridisation-capture PCR (HC-PCR)

2.8.1 Preparation of Hybridisation Capture Probe

The hybridisation capture PCR was based on a biotinylated probe for the *A. salmonicida vap*A (A-layer) gene and was used in conjunction with the AP PCR (Gustafson *et al.*, 1992).

The design of the capture probe was based on a 396 base pair region adjacent to the AP primer binding site (Appendix 6) and was produced by PCR of *A. salmonicida* DNA (Isolate 1107/1B) using biotinylated primers (Life Technologies™). Cycle parameters were based on the T_m of the primers and previous results obtained with the GeneAmp® 9600 thermal cycler (Perkin Elmer-Cetus). Mg^{2+} concentration was optimised by titration of Mg^{2+} between 1 and 4mM. Each 25 µl reaction contained approximately 10 ng of DNA, 2.5 µL of 10x reaction buffer (Life Technologies™), 1.5 µL of 50 mM $MgCl_2$, 0.25 µL of each amplification primer (approx. 0.4 µM) (forward primer AS 1f 808 GbioTTTCCGAAGGTTTCTTG and reverse primer AS 1r1203 CbioTCGTCCTTGAAGTAGTTATAGG), all four deoxynucleotide triphosphates at 0.2 mM each, and 0.5 U (0.1 µL) of Platinum™ Taq DNA Polymerase (Life Technologies™). Reaction mixes were held for 2 minutes at 94°C and amplified for 30 cycles using a GeneAmp® 9600 thermal cycler, with denaturation for 30 sec at 94°C, annealing for 30 sec at 60°C, and elongation for 60 sec at 72°C. The 396 base pair amplicon (biotinylated probe) was checked by electrophoresis (10 V cm^{-1}) using a 1% (w/v) agarose gel using TBE buffer and supplemented with approximately 0.5 µg mL^{-1} ethidium bromide. The biotinylated probe was then purified using a QIAquick PCR Purification Kit (Qiagen) according to manufacturers instructions.

The purified probe was again checked by electrophoresis (as above) and quantitated spectrophotometrically using a GeneQuant II DNA calculator (Pharmacia Biotech). The purified capture probe was then stored at -20°C prior to use.

2.8.2 Determination of Threshold of Detection of detection PCR (AP PCR)

DNA was isolated from a young actively growing culture (72 hrs) of *A. salmonicida* isolate 1107/1B on SBA using the Puregene® Kit as previously described (Section 2.4.3). The freshly prepared DNA stock solution was quantitated spectrophotometrically using a GeneQuant II DNA calculator and stored at -20°C for use. Dilutions of target DNA ranging in concentration from 100 to 0.1 pg/µL were prepared from the DNA stock. In each case an AP PCR reaction volume of 25 µL was used and 1 µL of each dilution was added as a source of template. All PCR reactions were performed in duplicate. Following PCR, reaction products were assessed visually after electrophoresis of 10 µL of product (10 V cm^{-1}) using a 1% (w/v) agarose gel using TBE buffer and supplemented with approximately 0.5 µg mL^{-1} ethidium bromide. The last lane containing a plainly visible band in a photograph of the gel was taken as the end-point.

2.8.2 Hybridisation Capture

Two hybridisation capture methods were evaluated, a hybridisation capture using a direct coupled probe and hybridisation capture by sequential hybridisation, streptavidin binding and magnetic capture.

2.8.3 Hybridisation Capture Using Direct Coupled Probe

In this procedure the capture probe was directly coupled to para-magnetic beads via the biotin-streptavidin complex before addition to the DNA sample. The probe/DNA complex was then separated and concentrated by magnetic capture prior to AP PCR of the target DNA.

a. Preparation of Direct Coupled Probe

The double stranded capture probe was checked by electrophoresis, as previously described. M-280 Streptavidin coated Dynabeads® (Dynal) were supplied as a suspension containing 6-7 x 108 Dynabeads® per ml (10 mg/ml), suspended in phosphate buffered saline (PBS) pH 7.4, containing 0.1% BSA and 0.02% NaN_3. In order to remove azide the beads were washed by resuspending the Dynabeads® with gentle shaking of the vial to obtain a homogeneous suspension, 50 µL of the suspension was added to a tube containing 1 mL of PBSA, 0.5% BSA. The beads were then captured and concentrated in a Dynal magnet assembly (MPC-1, Dynal) for 1-2 minutes. The supernatant was aspirated with a pipette. The tube was then removed from the magnet and the washing step repeated once. The beads were then resuspended in a final volume of 500 µl of PBSA, 0.5% BSA. This equalled 1 mg/ml in 500 µL, or 500 µg of beads. To this was added 50 – 250 ng of labelled DNA probe, prepared from the frozen stock. The amount of DNA probe used was based on the DNA saturation concentration of the beads (up to 200 pmoles of biotinylated single stranded oligonucleotide), this was decreased 5 fold in order to limit the selective capture of un-annealed primer strands. Finally, the unlabelled strand was removed by washing in 0.1M NaOH for 10 minutes, followed by a second brief wash for 30 seconds in the same buffer. The sample was then neutralised and washed in 5mM Tris HCl, pH 8.0, 0.5mM EDTA, 1M NaCl, 0.5% BSA for 1 to 2 minutes. The final preparation was resuspend in the same neutralisation wash buffer.

b. Hybridisation Capture by Direct Coupled Probe

All hybridisation capture reactions were carried out in sterile microcentrifuge tubes. In each case the DNA was suspended in 6X SSPE / 0.1%SDS (900mM NaCl, 60mM $NaH_2PO_4.2H_2O$, 6mM EDTA, pH 7.4, SDS 0.1% w/v) buffer with a final volume adjusted to 500 µL. 10 µL of bead linked capture probe (as previously described above) was added to each DNA sample, ie. 5-25 ng probe per 500 µL sample. The samples were then boiled for 5 minutes and transferred immediately to a water bath at 65°C. After overnight incubation (17 to 19 hours) the bead complex was captured for 1 to 2 minutes on a particle concentrator (magnet), and resuspend in 30 µL of molecular biology grade water.

2.8.4 Hybridisation Capture by Sequential Hybridisation, Streptavidin Binding and Magnetic Capture

DNA samples were prepared in volumes up to 450 µL in 6X SSPE / 0.1%SDS (900mM NaCl, 60mM $NaH_2PO_4.2H_2O$, 6mM EDTA, pH 7.4, SDS 0.1% w/v) hybridisation buffer. To this was added 50 µL containing 20 pmol of capture probe, to a final volume of 500 µL. The amount of purified probe added to the mix was calculated as 50% of the binding capacity of the Streptavidin coated beads according to the manufacture's specifications. The sample was then boiled in a water bath for 5 minutes and immediately transferred to a water bath at 65°C and incubated overnight (17 to 19 hours) at 65°C. The samples were then cooled to room temperature over a 1 hour period. 15 µL of washed Dynabeads® (previously described above) M-280 were added and the samples mixed gently at room temperature for 2 hours on a flat bed orbital shaker. The bead complex was captured for 1 to 2 minutes on a particle concentrator (magnet), and washed gently twice with 950 µL PBS / 0.5% BSA, concentrating the beads for 1 minute after each wash. The beads were then resuspended in 10-30 µL of molecular biology grade water.

2.8.5 PCR Detection

The PCR detection system was the AP system previously outlined in the PCR protocols section 2.3.3. In each case 10 to 15 µL of the bead/complex suspension was added directly to the PCR mix, the amount of water used in master mix preparation was adjusted in order to maintain the correct concentrations of the other components.

2.8.6 Determination of Lower Limits of Detection of HC-PCR System Using Direct Coupled Probe

Dilutions of DNA between 1 ng/µL and 1 pg/µL (1000, 500, 100, 50, 10, 5 and 1 pg/µL) were prepared in 6X SSPE / 0.1%SDS (900mM NaCl, 60mM NaH2PO4.2H2O, 6mM EDTA, pH 7.4, SDS 0.1% w/v) buffer with a final volume adjusted to 500 µL. This sample was then examined using the HC-PCR procedure outlined in Section 2.8.3.

2.8.7 Determination of Lower Limits of Detection of HC-PCR System Using Sequential Hybridisation, Streptavidin Binding and Magnetic Capture

Dilutions of DNA between 1 ng/µL and 1 pg/µL (1000, 500, 100, 50, 10, 5 and 1 pg/µL) were prepared in 450 µL volumes of 6X SSPE / 0.1%SDS, as above. This sample was then examined using the HC-PCR procedure described in Section 2.8.4.

2.9 Preliminary survey of wild and farmed populations of freshwater and marine fish

Mucus and kidney samples were collected from goldfish (*n* = 20) from Boolarra, Victoria and from Atlantic salmon (*n* = 20) from Macquarie Harbour, Tasmania. DNA was extracted from 25 mg aliquots of kidney tissue and 100 µl aliquots of mucus using the Puregene® Kit as previously described (Section 2.4.3). These extracts were used as template in the AP and PAAS PCR assays. Material from the mucus and kidney tissues was also streaked onto SRB plates and incubated for 48 h at 20°C. Any colonies suspected to be *A. salmonicida* were to be either picked

using a sterile pipette tip and used directly as template in the PCR mix, or subcultured and DNA extracted using the Puregene® Kit to provide a source of template for the AP, PAAS, and MIY PCRs.

SECTION 3 DETAILED RESULTS

3.1 OBJECTIVE: Characterisation of a comprehensive reference collection of major strains, both exotic and enzootic, *of A. salmonicida* subspecies

A total of 243 *A. salmonicida* isolates from the AFDL reference collection were characterised using a panel of 20 phenotypic tests as summarised in Section 2.2. Numeric taxonomic analysis of the results was undertaken in collaboration with Prof. Brian Austin (Department of Biological Sciences, Heriot-Watt University, Edinburgh), an internationally recognised expert on the taxonomy of Aeromonadaceae. Results from 14 of the biochemical tests were forwarded to Prof. Austin for analysis by a computer software program developed in his laboratory for the identification of *A. salmonicida*. Results from this analysis grouped the isolates into 30 phenotypic clusters (Appendix 1), reflecting the broad phenotypic diversity of the genus. Hence on the basis of phenotypic testing alone it was not possible to accurately assign all the isolates to a recognised subspecies.

An additional 65 isolates were later submitted to the AFDL *A. salmonicida* collection and were confimed as *A. salmonicida* on the basis of their phenotypic and biochemical characteristics. A total of 308 confirmed isolates of *A. salmonicida* was used for PCR test validation.

3.2 OBJECTIVE: Identify published but unvalidated nucleotide sequences with potential for diagnostic use

During the course of the project, six PCR-based tests and one probe-based test targeting *A. salmonicida*, and one PCR test targeting *A. salmonicida* subsp. *salmonicida*, were reported (Gustafson *et al.*, 1992, Hiney *et al.*, 1992, Mooney *et al.*, 1995, Carson, 1998, Høie *et al.*, 1996, Miyata *et al.*, 1996, Hirono, 1997, Oakey *et al.*, 1998). Primer sets were obtained for the PCR tests, and a set of primers designed against Oakey *et al.*'s probe 15e4 (Section 2.3.2), and optimisation of these tests initiated.

Although two other sets of *A. salmonicida* primers have been reported in the literature - one targeting the glycerophospholipid-cholesterol acyltransferase (GCAT) gene, and the other targeting the serine protease gene (Austin *et al.*, 1998). These PCR primers had been demonstrated not to be specific for *A. salmonicida*, and were not included in this study.

3.3 OBJECTIVE: Develop diagnostic procedures using molecular technology

3.3.1 *In vitro* specificity of PCR tests targeting *A. salmonicida*

The PAAS (Hiney et al., 1992) and AP (Gustafson *et al.*, 1992) PCR tests did not produce any false positive reactions with the 27 non-target bacterial DNA extracts (Table 2.4). Therefore these PCRs were considered to be 100% specific for their target, *A. salmonicida*.

The other proposed *A. salmonicida* PCRs were shown to be non-specific, i.e. cross-reactions occurred with DNA from non-target organisms, even when the test conditions were manipulated to

increase the stringency (Table 3.1). These tests could not be validated and were therefore omitted from further study.

Table 3.1 Non-specific PCR tests

PCR test	Non-target organism detected
16Sa (Carson, 1998)	*A. hydrophila* ATCC 7966
16Sb (Høie *et al.*, 1997)	*A. hydrophila* ATCC 7966, *A. media* ATCC 33907, *A. veronii* biovar *sobria* ATCC 9071, *A. sobria* ATCC 43979
15e4 (based on Oakey *et al.*, 1998)	*A. hydrophila* ATCC 7966, *A. eucrenophila* ATCC 23309, *Aeromonas* sp. ATCC 35941, *A. caviae* ATCC 15468, *A. veronii* biovar *veronii* ATCC 35624
ASA1 (Hirono, 1997)	*A. hydrophila* ATCC 7966, *A. bestiarum*, *A. media* ATCC 33907, *A. veronii* biovar *sobria* ATCC 9071, *A. sobria* ATCC 43979, *A. veronii* biovar *veronii* ATCC 35624, *Aeromonas* sp. ATCC 35941, *A. jandaei* ATCC 49568
ASA2 (Hirono, 1997)	*A. hydrophila* ATCC 7966, *A. bestiarum*, *A. media* ATCC 33907, *A. veronii* biovar *sobria* ATCC 9071, *A. sobria* ATCC 43979, *A. veronii* biovar *veronii* ATCC 35624, *Aeromonas* sp. ATCC 35941, *A. jandaei* ATCC 49568

After extensive laboratory trials, further evaluation of the nested primer set of Mooney *et al.* (1996) was abandoned due to contamination problems which consistently resulted in false positive results. This would prove troublesome for a standard diagnostic test. It is noteworthy that such difficulties are common with nested PCR tests and unless their performance and application is extensively evaluated, they should not be used for diagnostic testing.

3.3.2 *In vitro* sensitivity of PCR tests targeting *A. salmonicida*

a. Determining the in vitro *sensitivity of the PCRs*

The sensitivity of a diagnostic test is defined as the proportion of true positive results that a test reliably identifies, as compared to another technique. In the context of this study, biochemical and phenotypic characterisation was used as the gold standard for identification (Bernoth, 1997b).

The AP and PAAS (primary) PCRs, which had been previously shown to be specific for *A. salmonicida*, were used to screen the AFDL *A. salmonicida* library (n = 308) in order to determine their sensitivity *in vitro* (summary given in Table 3.2, full results in Appendix 2).

Table 3.2 *In vitro* sensitivity of the *A. salmonicida*-specific PCRs

PCR test	Sensitivity (n = 308)
AP	93.3%
PAAS	93.0%
AP + PAAS	**99.4%**

Based on the AFDL collection of *A. salmonicida* isolates, the AP test and the PAAS test yielded 6.7% and 7.0% false negatives, respectively. When used in combination, these two tests yielded

0.6% false negatives. The 'false negative' isolates were further examined in order to determine the reason(s) for these results.

b. Determining why the in vitro *sensitivity of the specific PCRs was less than 100%*

PAAS PCR. As the primer binding sites for the PAAS PCR are plasmid bourne, plasmid DNA was isolated from the PAAS PCR-negative isolates (Fig 3.1) and probed with the PAAS 3 probe (Section 2.3.6) in order to determine whether the target primer site was present.

The PAAS 3 probe hybridised with a band, approximately 6 kb in size, in the plasmid profiles of SFCs 190 and 308 (as indicated by the arrow in Fig 3.1), indicating that the plasmid target site was present in these two isolates. Failure of the PAAS 3 probe to hybridise with the remaining plasmid extracts demonstrated the isolates SFC 20, 21, 22, 123, 164, 168, 170, 187, 188, 191, 192, 197, 198, 213, 222, 226, 229, 259, 480, 484, 491, 725, and 728 lacked the target plasmid:.

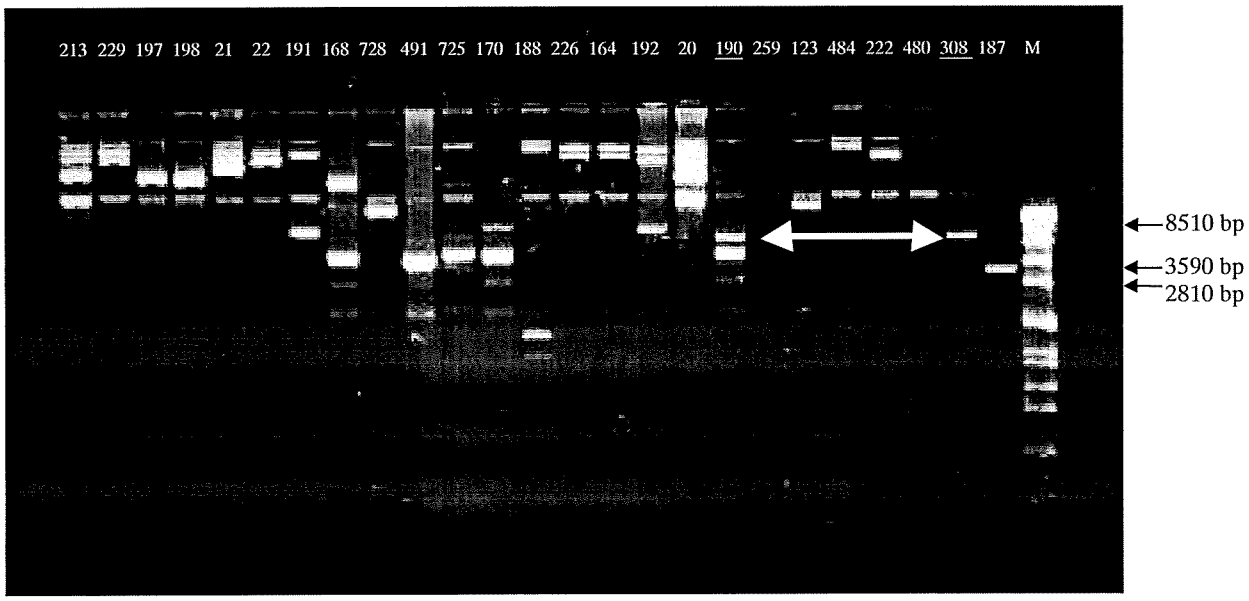

Fig 3.1 Plasmids extracted from the previously PAAS PCR-negative *A. salmonicida* isolates using the BRESAspin™ Plasmid Mini Kit (GeneWorks Pty Ltd). *A. salmonicida* SFC isolate numbers are marked above their corresponding plasmid profiles. The arrow indicates the plasmid bands of the two isolates (underlined) that hybrized with the PAAS 3 probe. 'M' denotes the molecular weight marker SPP-1 DNA/*Eco*R1.

AP PCR. Further studies using the Western blotting technique with the A-protein-specific antiserum were undertaken in order to determine whether the *vap*A gene, which contains the AP PCR primer sites, was functional and being expressed. It was demonstrated that AP PCR-positive isolates did not necessarily express the A protein e.g. SFC 36 (ATCC 14172). Also, some AP PCR-negative isolates could continue to produce the A protein eg SFCs 308, 317, 481, 483, 484 (Fig 3.2).

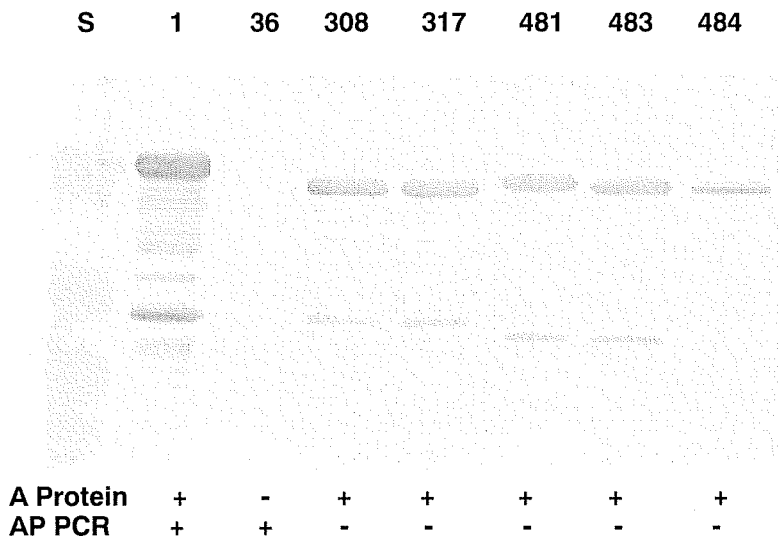

Fig 3.2 Western blot of protein profiles of selected *A. salmonicida* isolates, demonstrating that there is not a direct correlation between the presence of A protein and the AP PCR result. 'S' denotes the protein standard.

These results indicate that not all *A. salmonicida* isolates contain the AP PCR priming sequences.

3.3.3 PCR test targeting *A. salmonicida* subspecies *salmonicida*

During initial studies with the Miyata primer set (Miyata *et al.*, 1996) using the published thermocycling conditions difficulties were encountered with respect to both the sensitivity and the reproducibility of the assay. However, following optimisation of the PCR parameters (conditions as outlined in Table 2.6), the MIY PCR was found to be specific for its target subspecies, *A. salmonicida* subsp. *salmonicida* (Fig 3.3). This test correctly identified all isolates from the AFDL collection previously identified as *A. salmonicida* subsp. *salmonicida*. This included all isolates submitted as 'typical' and presumed to be *A. salmonicida* subsp. *salmonicida*, and 85 of the 128 isolates which were submitted with no description but were found to be indole-negative. Isolates which are negative for indole production are usually, but not always *A. salmonicida* subsp. *salmonicida*. Using the optimised conditions the test did not produce false positive results with any of the negative control extracts, any other *A. salmonicida* subspecies, or any from the collection that was considered 'atypical' (Appendix 2).

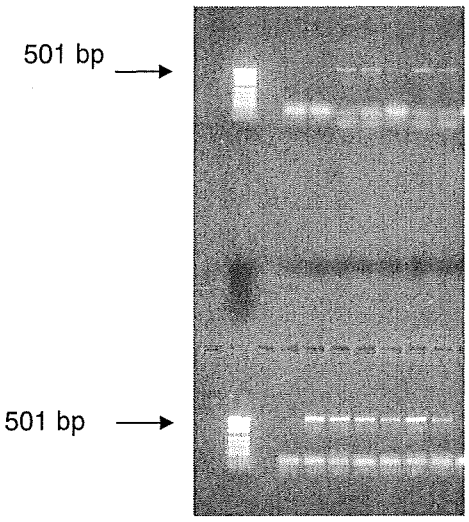

Fig 3.3 Effect of cycling conditions on the *A. salmonicida* subsp. *salmonicida* PCR (Miyata *et al.* 1996). The PCR products in the top lane of the gel are a result of employing a 60°C annealing temperature (published conditions). The PCR products in the lower lane of the gel are a result of employing a 68°C annealing temperature (optimised conditions). DNA extracted from *A. salmonicida* subsp *salmonicida* was the template used for both PCRs. The molecular weight marker is pUC19/*Hpa*II, the first reaction in each row is the negative control (water only).

3.3.4 Sequencing

A. salmonicida isolates which were negative by all PCR tests or were motile (a phenotypic trait inconsistent with the accepted description of the genus), were subjected to partial 16S rDNA sequencing to confirm their identity. The 16S rRNA genes were amplified using the 16SU PCR and partially sequenced using the 1100f primer (Dorsch and Stackebrandt, 1992). The general taxonomic affiliation and likely source of the sequences, as outlined in Table 3.3, was determined using the BLAST tool available on ANGIS.

Table 3.3 Partial sequence analysis of selected isolates

SFC	Comments	PCR results			Likely Source
		AP	PAAS	MIY	
187	negative by all 3 PCRs	-	-	-	*A. salmonicida*
189	negative by all 3 PCRs	-	-	-	*A. salmonicida*
301	motile *A. salmonicida*	-	+	-	*A. salmonicida*
302	motile *A. salmonicida*	+	+	-	*A. salmonicida*
762	negative by all 3 PCRs	-	-	-	*Yersinia ruckeri*

These results indicate that sequencing of the 16S rRNA gene can be useful in determining the taxonomic affiliation of an isolate that is negative by all 3 PCR tests.

3.3.5 Preparation of seeded tissues with known bacterial inocula

A standard procedure for seeding tissues *in vitro* was developed (Section 2.4). In an effort to determine the exact number of *A. salmonicida* cells being used to seed fish tissues, plate counts prepared from the seeding suspensions were compared with direct counts using a WSI counting chamber. It was shown that plate counts were underestimating the number of bacteria present by about one order of magnitude compared to the direct counts. This is most likely due to *A. salmonicida*'s tendency to autoagglutinate in suspension (Bernoth, 1990).

3.3.6 Extracting DNA from seeded and unseeded salmonid tissues

Initial work on template extraction techniques revealed that the enzymatic/chemical lysis method yielded better quality DNA and also led to the best lower limit of detection compared to the boiling, proteinase K, Triton X-100, chelex and triton, or chelex methods. The Puregene® Kit was trialled as another means of DNA extraction, and was found to equal the enzymatic/chemical lysis method in terms of yield and purity of the DNA preparations, as well as the PCR LDLs. Since the Puregene® Kit was simpler and faster to use, it became the method of choice for all future DNA extractions.

3.3.7 Determination of the LDL (lower limit of detection) of the PCRs

Tissues excised from disease-free brown trout were seeded with varying numbers of *A. salmonicida* cells (Section 2.4.4.b). Total DNA was extracted using the Puregene® Kit and used as template in the AP, PAAS and MIY PCRs. At this stage the AP and PAAS PCRs were able to reliably detect only between 10^6 - 10^7 *A. salmonicida* CFUs per gram of fish tissue. The MIY PCR could only detect 10^7 - 10^8 *A. salmonicida* CFUs per gram of fish tissue. These relatively high LDLs, which were unacceptable for a diagnostic PCR, clearly illustrated the need for further optimization of the PCRs.

To this end, cocktails of purified *A. salmonicida* DNA and fish tissue DNA were prepared and used as template in the AP, PAAS and MIY PCRs (Section 2.4.4.a). Initially the LDLs for the AP and PAAS PCRs were approximately 20 ng *A. salmonicida* DNA in the presence of 1 µg fish tissue DNA. The addition of various co-solvents such as 400 ng/PCR BSA, or Life Technologies™ PCRx Enhancer Solution, did not improve the LDLs of the PCRs. However, alterations to the cycle number, length of cycles, and primer and enzyme concentrations did enhance the performance of the AP and PAAS PCRs (Fig 3.4).

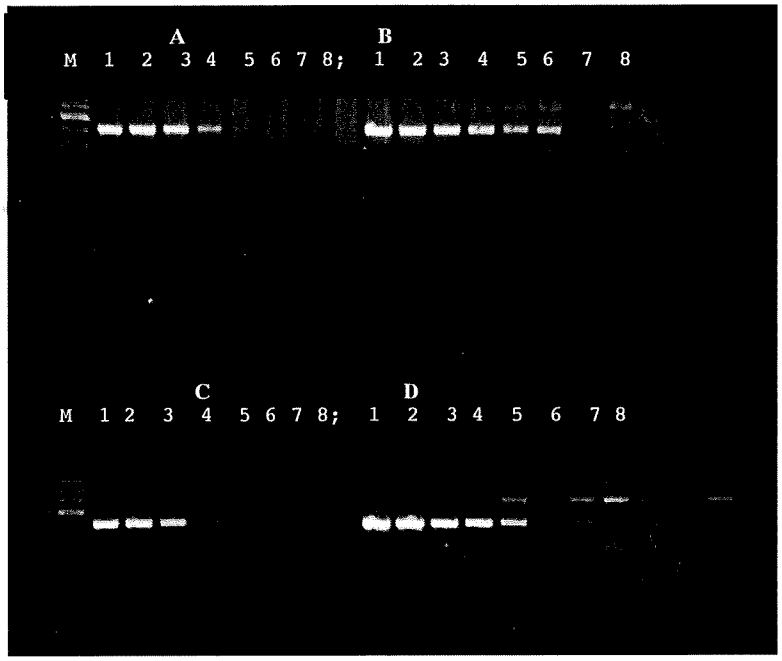

Fig 3.4 Lanes A1-A8 and C1-C8 = AP PCR, lanes B1-B8 and D1-D8 = PAAS PCR. Lanes A8, B8, C8 and D8 are the negative controls (no target DNA). Lanes A1-A7, B1-B7, C1-C7 and D1-D7 contain *A. salmonicida* DNA in the following amounts: 2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 0 fg (negative control) per PCR. Lanes B1-B7 and D1-D7 contain *A. salmonicida* DNA in the following amounts: 20 ng, 2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 0 fg (negative control) per PCR. Reactions C1-C8 and D1-D8 also contain 1 µg brown trout kidney DNA per PCR. 'M' denotes the molecular weight marker pUC19/*Hpa*II.

The inclusion of large amounts of non-target fish tissue DNA (1 to 2 µg) led to a decrease in the LDL of the PCRs by about 1 order of magnitude (Table 3.4). When cocktails of low amounts of target DNA (<20 pg) and high levels of non-target DNA (>1 µg) were tested with the PAAS PCR, some non-specific bands were found to occur (example given in Fig 3.4). However, these extraneous bands were of a different size to the desired product. In these cases the target band could be excised from the gel and sequenced to confirm identity.

We were unable to improve the performance of the MIY PCR to the same extent as the AP and PAAS PCRs. The LDL of the MIY PCR for target DNA alone was in the range of 2 – 20 ng DNA per reaction. Addition of large amounts of non-target fish tissue DNA (1 to 2 µg) once again led to a decrease in the LDL of the PCRs by about 1 order of magnitude ie 20 – 200 ng DNA per reaction. Based on these findings, the MIY PCR is unlikely detect *A. salmonicida* in covertly infected fish tissues. A pre-enrichment step should overcome this problem.

The improved PAAS and AP PCR assays were applied to whole cell seeded preparations (example given in Fig 3.5). The LDL for the PAAS PCR was found to be approximately 10^3 CFU/g fish tissue, whilst the LDL for the AP PCR was approximately 10^4 CFU/g (Table 3.4). The LDL for the MIY PCR, however, was in the range 10^6 - 10^7 CFU/g.

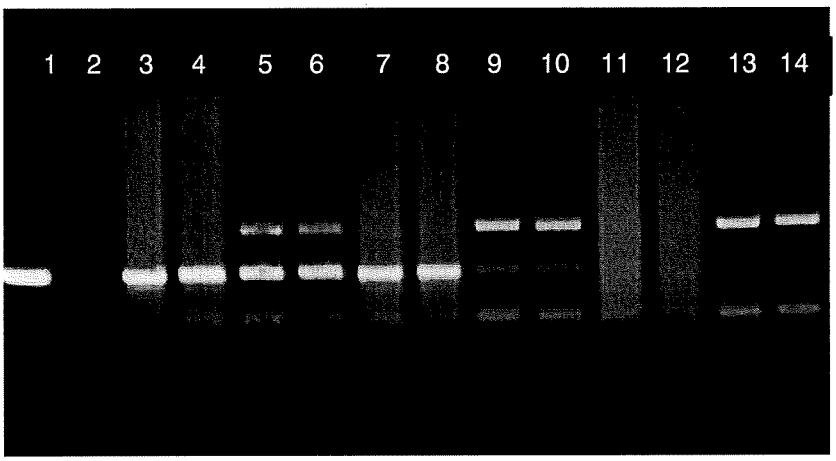

Fig 3.5 PAAS PCR of kidney tissue seeded with whole cell preparations of *A. salmonicida*. Lane 1 is the positive PCR control (template was 200 pg *A. salmonicida* DNA) and lane 2 is the negative control (no *A. salmonicida* DNA added to the PCR). The templates used in lanes 3-4, 7-8, and 11-12 were extracts prepared from 25 mg brown trout kidney tissue seeded with 2 x 10^3, 2 x 10^2 and 2 x 10^1 *A. salmonicida* CFUs respectively. The templates used in lanes 5-6, 9-10, and 13-14 were amplified from 1:20 dilutions of extracts prepared from 50 mg brown trout kidney tissue seeded with 2x10^3, 2x10^2 and 2x10^1 *A. salmonicida* CFUs, respectively.

Table 3.4 Final LDLs of the *A. salmonicida* PCRs

PCR	Range of LDL of PCRs		
	Target template only	Target template + 1 µg fish tissue DNA	CFU/g tissue
PAAS	2 pg – 200 fg	20 pg – 2 pg	10^4 - 10^3
AP	20 pg - 2 pg	200 pg – 20 pg	10^5 - 10^4
MIY	20 ng – 2 ng	200 ng – 20 ng	10^7 - 10^6

3.4 OBJECTIVE: Validation of molecular diagnostic procedures using experimental infections carried out in the microbiologically secure aquarium facility at AAHL

3.4.1 Detection of *A. salmonicida* in experimentally infected goldfish

Overt infection was successfully generated in goldfish, with fish dying from day two to day five post-inoculation. Suspected *A. salmonicida* colonies isolated onto SBA from infected goldfish tissue (skin lesions, kidney, spleen) were positive by both the AP and PAAS PCRs. All isolates were MIY PCR negative – this result was expected as an atypical *A. salmonicida* (SFC 1) was used to generate the infection.

3.4.2 Detection of *A. salmonicida* in experimentally infected brown trout

Attempts to generate overt infection in brown trout via bath challenge with *A. salmonicida* SFC 262 were unsuccessful. However, i.p. injection with 10^6 CFU of the same bacterium generated overt infection in brown trout, with fish dying from day three to five post-inoculation.

a. Culture-based identification of A. salmonicida in experimentally infected fish

Bacteria were readily isolated on SBA from the mucus, gills, intestine, kidney and spleen of dead fish. Isolates were positive by the AP (example given in Fig 3.6), PAAS, and MIY PCRs. Amplifying directly from the bacterial colony (i.e. aseptically transferring a small amount of isolate to the PCR mix itself) was successful 90% of the time. Extracting DNA from the isolate with the Puregene® Kit prior to PCR ensured a 100% success rate.

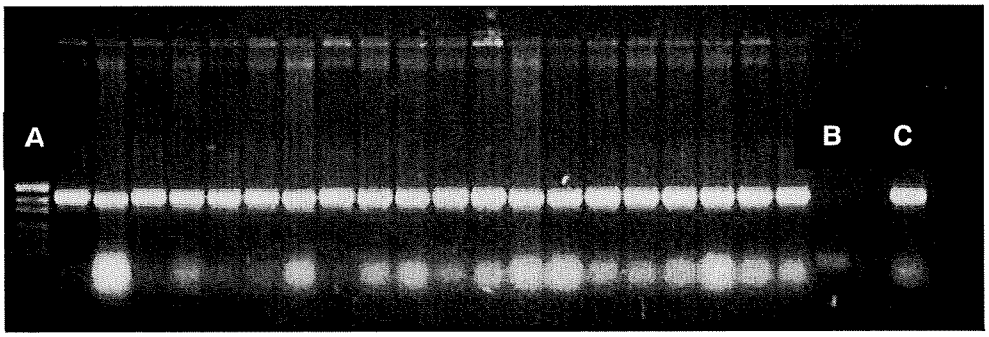

Fig 3.6 AP PCR of isolates cultured from the organs of brown trout which had been experimentally infected with *A. salmonicida* (SFC 262). 'M' denotes the molecular weight marker pUC19/*Hpa*II, 'B' denotes the negative control (no *A. salmonicida* DNA) and 'C' denotes the positive control (1 ng *A. salmonicida* DNA added to the PCR).

b. Direct PCR detection of A. salmonicida in experimentally infected fish

A variety of methods (Table 3.5) were used to extract DNA directly from the tissues of dead fish (25 mg aliquots of mucus, gill, intestine, kidney or spleen material), and again the Puregene® Kit extraction method become the technique of choice. It was also found that using Platinum™ *Taq* (Life Technologies™), as opposed to *Taq* DNA Polymerase (Promega Corporation), significantly improved the performance of all 3 PCR systems (Table 3.5, example given in Fig. 3.7) and so became the DNA polymerase of choice.

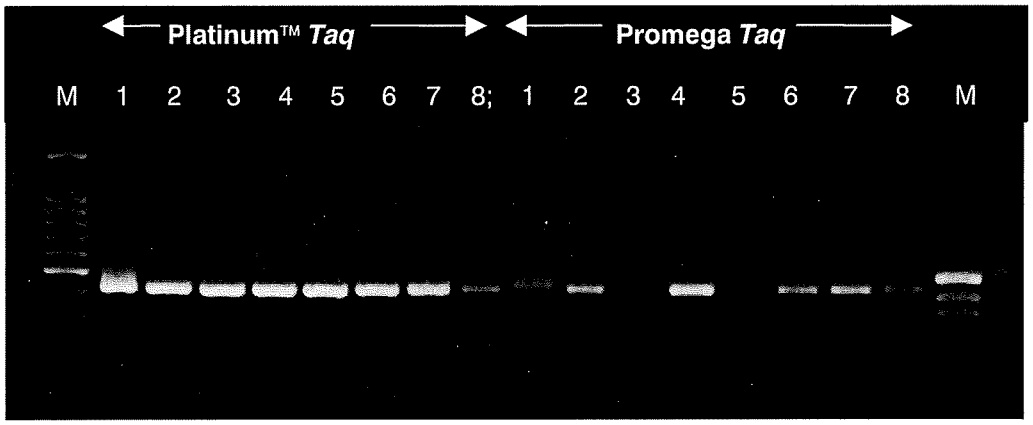

Fig 3.7 PAAS PCR of extracts prepared from experimentally infected brown trout kidney tissue, using two different DNA polymerases. The template material was prepared as per Table 3.5. 'M' denotes the molecular weight marker pUC19/*Hpa*II.

Table 3.5 Comparison of the effect of using different extraction methods and DNA polymerases on the amplification of target *A. salmonicida* DNA from kidney tissue from experimentally infected brown trout.

Template Preparation		PCR					
		AP		PAAS		MIY	
#	Method	Prom[a]	Plat[b]	Prom	Plat	Prom	Plat
1	Boiling (Section 2.4.3 a)	(+)[c]	+	(+)	+	-	-
2	As in #1, diluted 1:20	(+)	+	(+)	+	-	-
3	Puregene® Kit (Section 2.4.3 g)	-	+	-	+	-	(+)
4	As in #3, diluted 1:20	+	+	+	+	(+)	+
5	QIAmp® Kit (Section 2.4.3 h)	+	+	-	+	-	+
6	As in #5, diluted 1:20	(+)	+	(+)	+	-	+
7	Chelex / triton (Section 2.4.3 d)	(+)	+	(+)	+	-	-
8	As in #7, diluted 1:20	(+)	+	(+)	+	-	-

[a] Prom = *Taq* DNA polymerase (Promega Corporation)

[b] Plat = Platinum™ *Taq* (Life Technologies™)

[c] (+) = a weakly positive reaction

A comparison of the results obtained using the AP and PAAS PCRs to directly amplify *A. salmonicida* DNA from Puregene® Kit prepared extracts from experimentally infected tissues is given in Table 3.6. Due to the poorer LDL of the MIY PCR when applied to direct tissue detection, this PCR was not routinely used to screen tissue extracts.

Table 3.6 Direct PCR detection of *A. salmonicida* in tissues taken from experimentally infected brown trout.

Template Source DNA	Template Dilution	Template Volume (µl)	PCR Result	
			AP	PAAS
mucus	-	1	-	+
mucus	1:10	1	+	+
gill	-	1	+	+
gill	1:20	1	+	+
intestine	-	1	+	+
intestine	1:20	1	+	+
lesion	-	1	+	+
lesion	1:20	1	+	+
spleen	-	1	+	-
spleen	1:20	1	+	+
kidney	-	1	+	+
kidney	1:20	1	+	+
mucus IC*	-	1	+	+
gill IC	-	1	+	+
intestine IC	-	1	+	+
intestine IC	1:20	1	+	+
kidney IC	-	1	+	+
kidney IC	1:20	1	+	+
spleen IC	-	1	+	+
spleen IC	1:20	1	+	+
+ve control (20 ng *A. salmonicida*)	-	1	+	+
-ve control (water)	-	1	-	-

IC = inhibition control ie 20 ng *A. salmonicida* DNA added to PCR

3.5 OBJECTIVE: Validation of the molecular diagnostic procedures developed at AAHL using naturally infected populations of salmonids: a short-term collaborative project with the National Fish Health Research Laboratory, West Virginia, U.S.A.

3.5.1 Generation of overt infection in salmonids held at the NFHRL, WV, USA

Overt infection was successfully generated in Arctic char via the bath challenge (Section 2.6.1), and in covertly infected rainbow trout and Atlantic salmon (both vaccinated and unvaccinated) via the stress-induced furunculosis (SIF) assay (Section 2.6.2). The history of mortality due to stress-induced furunculosis is listed in Table 3.7. On day 8 postadministration, the remaining 16 rainbow trout were euthanased, and on day 9 postadministration, the remaining 4 unvaccinated Atlantic salmon and 8 remaining vaccinated Atlantic salmon were euthanased.

Table 3.7 History of SIF mortalities in salmonids held at the NFHRL

Day (postadministration SIF)	***Salmonid Test Group***		
	Rainbow trout (n = 20)	***Unvaccinated Atlantic salmon (n = 20)***	***Vaccinated Atlantic salmon (n = 24)***
1	0	0	0
2	0	1	3
3	0	6	6
4	1	7	4
5	1	2	2
6	2	0	1
7	0	0	0
8	n/a	0	0
Total # SIF Mortalities	4	16	16

3.5.2 Culture-based identification of *A. salmonicida* in overtly infected fish tissues

Bacteria were isolated on CBBA from the tissues of all necropsied fish and subjected to a battery of biochemical tests in order to establish their identity (Section 2.6.3). The AP & PAAS combined PCR tests were then used to confirm the identity of suspected *A. salmonicida* isolates, and the MIY PCR test to confirm the identity of suspected *A. salmonicida* subsp *salmonicida* isolates. All results were in agreement with the biochemical tests currently employed by the NFHRL to identify *A. salmonicida* and *A. salmonicida* subsp *salmonicida*.

It should be noted that the PCR took approximately four weeks to establish in the NFHRL, due to inter-laboratory differences. However, once established, PCR of pure cultures was faster than phenotypic screening. Typically PCR tests yielded results in 1 to 2 days, compared to 7 days for classical biochemical testing.

3.5.3 Direct PCR detection of *A. salmonicida* in overtly infected fish tissues

Mucus, gill, intestine, kidney and spleen samples were taken from the overtly infected (diseased) fish. DNA was extracted directly from the tissue samples using the Puregene® Kit and screened using the AP, PAAS and MIY PCR tests.

All mortalities obtained prior to day 8 post-administration of the SIF assay were PCR positive for all tissue samples. Overall, at least 1 tissue sample was PAAS- and AP-PCR positive for 95% of all fish tested (Appendix 5). Only 1 vaccinated Atlantic salmon, and 3 rainbow trout, were PCR negative by all tissue samples - these 4 fish were from amongst those destroyed at the termination of the SIF assay. Mucus was the tissue most likely to yield a PCR positive result, intestine was the tissue least likely to yield a PCR positive result (Table 3.8). No tissue sample that was positive for *A. salmonicida* by PCR screening was negative for *A. salmonicida* by culture on CBBA.

Table 3.8 Proportion of tissues from overtly infected salmonids demonstrated, by PCR, to be infected with *A. salmonicida*.

Tissue	*Salmonid Test Group*			
	Arctic char	*Rainbow trout*	*Atlantic salmon (vaccinated)*	*Atlantic salmon (unvaccinated)*
Mucus	100%	80%	75%	100%
Gill	39%	60%	71%	100%
Spleen	27%	35%	75%	100%
Kidney	44%	40%	79%	100%
Intestine	11%	35%	71%	100%

The presence of *A. salmonicida* subsp. *salmonicida*, where determined by PCR, correlated with results based on the biochemical testing of isolates and by IFAT of mucus and kidney samples taken from the rainbow trout.

3.5.4 Collection of samples from covertly infected salmonids, USA

As the Atlantic salmon in Raceway 3, Richard Cronin National Salmon Station (RCNSS) MA., were part of the ongoing New England Salmon Restoration Program, they could not be lethally sampled. Therefore, mucus samples only were taken from these fish (n = 100).

Samples (mucus, gill, spleen, kidney, intestine) were collected from the Atlantic salmon in Raceways 5 and 6 at the RCNSS, and the brown trout from Bennington, VT. These fish were believed to be covertly infected on the basis that *A. salmonicida* had been cultured from these populations within the six months prior to sampling (Table 2.7).

Samples (mucus, gill, intestine, kidney and spleen) were also collected from the rainbow trout and Atlantic salmon (unvaccinated) populations held at the NFHRL, WV. These fish had been demonstrated to be covertly infected with *A. salmonicida* by the application of the stress-induced furunculosis (SIF) assay to a proportion of each population (Table 3.7).

In all cases the tissue samples were divided into 2 equal aliquots, with one aliquot being tested for *A. salmonicida* using the culture system of Cipriano *et al.* (1994a), and the other aliquot being tested directly with the PCRs developed in Australia, based on the PAAS, AP and MIY primers.

3.5.5 Cultural identification and enumeration of *A. salmonicida* from covertly infected fish tissue

A variety of bacteria, including *A. salmonicida*, were isolated from the 100 mucus samples taken from the Atlantic salmon in Raceway 3 of the RCNSS, MA., and subsequently biochemically identified (Appendix 3, Table 1).

A. salmonicida was not isolated from the Atlantic salmon held at the RCNSS in Raceway 5 (Appendix 4, Table 1) or Raceway 6 (Appendix 4, Table 2), or from the brown trout located at Bennington (Appendix 4, Table 3).

A. salmonicida was isolated from the rainbow trout (Appendix 4, Table 4) and the unvaccinated Atlantic salmon (Appendix 4, Table 5) located at the NFHRL, WV.

The total bacterial load of the various samples was calculated, and the level of any *A. salmonicida* present enumerated. The levels of *A. salmonicida* determined to be present in the tissues ranged from 8.3×10^2 to 2×10^6 CFU/g. Some of the other bacteria isolated from the lethally sampled salmonids were also biochemically identified, but not enumerated (Appendix 3, Table 2) in order to provide information on the range of flora present on the fish at the time of sampling.

The number and type of *A. salmonicida* culture-positive tissues found in the lethally sampled fish (*n* = 40) were: 8 mucus, 9 gill, 3 kidney, 2 spleen and 2 intestine (Table 3.9). Six fish were culture-positive by gill samples only, four fish were culture-positive by mucus samples only. No fish was culture-positive based solely on kidney, spleen or intestine samples.

The AP & PAAS combined PCR tests were then used to confirm the identity of *A. salmonicida* isolates, and the MIY PCR test to confirm the identity of *A. salmonicida* subsp *salmonicida* isolates. Results were in agreement with the biochemical tests currently employed by the NFHRL to identify *A. salmonicida* and *A. salmonicida* subsp *salmonicida* (Table 3.9). Typically PCR tests yielded results in 1 to 2 days, compared to 7 days for classical biochemical testing.

The PCR assays were also used to screen a variety of bacteria isolated from the samples, including *Pseudomonas diminuta*, *Pseudomonas pseudoalcaligenes, Pseudomonas fluorescens, Aeromonas hydrophila, Commomonas tarrigania, Shewanella putrefasciens, Acinetobacter* sp, *Staphylococcus* sp. and *Moraxella* sp. No false positive reactions occurred, further demonstrating the specificity of the PCR tests.

3.5.6 Direct PCR detection of *A. salmonicida* from covertly infected fish tissue

DNA was extracted from the tissue samples taken from the covertly infected fish using the Puregene® Kit and screened using the AP, PAAS and MIY PCR tests. All tissue samples taken from those fish demonstrated to be negative for *A. salmonicida* by culture onto CBBA i.e. the Atlantic salmon from Raceways 5 and 6 at the RCNSS, MA, and the brown trout from Bennington, VT, were negative by direct PCR screening.

Results from the direct PCR screening of tissue samples taken from fish demonstrated to be positive for *A. salmonicida* by culture onto CBBA are given in Table 3.9. No tissue sample that was positive for *A. salmonicida* by PCR screening was negative for *A. salmonicida* by culture on CBBA. These results show that culture was more reliable and sensitive than direct PCR for the detection of *A. salmonicida* in covertly infected fish (Table 3.10).

Table 3.9 Comparison of PCR testing of *A. salmonicida* isolates and tissue extracts of covertly infected fish

Salmonid species	Location	Fish #	Tissue	*A.sal* cfu/g	PCR of Isolates			PCR of Tissues		
					AP	PAAS	MIY	AP	PAAS	MIY
Atlantic salmon 3+	Raceway 3, RCNSS, MA	18	Mucus	2.0E+03	+	+	+	-	-	-
		23	Mucus	7.7E+03	+	+	+	-	-	-
		56	Mucus	8.3E+02	+	+	+	-	-	-
		63	Mucus	5.0E+03	+	+	+	-	-	-
		66	Mucus	1.1E+03	+	+	+	-	-	-
		78	Mucus	1.0E+03	+	+	+	-	-	-
		97	Mucus	2.5E+03	+	+	+	-	-	-
		100	Mucus	1.4E+04	+	+	+	-	-	-
Rainbow trout	NFHRL, WV	5	Mucus	1.3E+04	+	+	+	-	-	-
			Gill	6.7E+04	+	+	+	-	-	-
			Spleen	1.7E+05	+	+	+	-	-	-
			Kidney	**4.0E+05**	+	+	+	+	+	-
			Intestine	3.9E+04	+	+	+	-	-	-
		18	Spleen	**2.0E+06**	+	+	+	+	+	(+)[a]
			Kidney	1.9E+05	+	+	+	-	-	-
			Intestine	1.8E+05	+	+	+	-	-	-
		19	Mucus	**1.3E+05**	+	+	+	+	+	(+)
			Gill	1.3E+04	+	+	+	-	-	-
		20	Mucus	1.9E+04	+	+	+	-	-	-
Atlantic salmon (unvaccinated)	NFHRL, WV	1	Gill	2.0E+03	+	+	+	-	-	-
		2	Mucus	7.1E+04	+	+	+	-	-	-
		5	Mucus	5.0E+04	+	+	+	-	-	-
			Kidney	6.3E+03	+	+	+	-	-	-
		7	Mucus	**2.0E+05**	+	+	+	-	+	-
		9	Gill	2.9E+04	+	+	+	-	-	-
		11	Gill	5.0E+04	+	+	+	-	-	-
		15	Gill	2.5E+04	+	+	+	-	-	-
		17	Gill	**1.0E+05**	+	+	+	+	+	-
		18	Gill	1.0E+04	+	+	+	-	-	-
		19	Mucus	**2.9E+05**	+	+	+	+	+	-
		20	Mucus	2.3E+04	+	+	+	-	-	-
			Gill	6.0E+04	+	+	+	-	-	-

[a](+) denotes an extremely weak positive reaction

Table 3.10 Comparison of cultural isolation of *A. salmonicida* Vs direct PCR detection of *A. salmonicida* in tissues sourced from covertly infected salmonids

Source of covertly infected tissue	Positive by culture	Positive by direct PCR
Atlantic salmon, Raceway 3, RCNSS, MA (n = 100)	8%	0%
Rainbow trout, NFHRL, WV (n = 20)	20%	15%
Atlantic salmon (unvaccinated), NFHRL, WV (n = 20)	55%	15%

3.6 OBJECTIVE: Determine whether the use of hybridisation-capture PCR would enhance the sensitivity of the test to allow detection of covert infections

Two hybridisation capture methods were evaluated. Both methods relied on a DNA hybridisation probe for a region on the *vapA* (A-protein) gene of *A. salmonicida*, adjacent to the AP PCR priming sites. Following hybridisation of the probe with *A. salmonicida* target DNA in the sample and subsequent magnetic capture, the capture probe/DNA complex was screened by AP PCR.

The first method utilised a biotinylated probe directly coupled via covalently bound streptavidin to the surface of a paramagnetic polymer coated microbead. These probe-coated beads were added directly to purified DNA samples. Following a period of incubation, during which time the probe binds to the target DNA, the bead/DNA complex is "captured" with a magnet and washed prior to PCR. In the second method the same biotinylated probe was used as in the first technique, but in this case the biotinylated probe was added directly to the DNA sample. After incubation and binding to the target DNA, streptavidin coated paramagnetic beads were added to the samples and the DNA bound probe allowed to complex with the beads. Finally the beads were captured and concentrated magnetically and subjected to the same PCR.

In order to reliably compare the relative threshold of detection of direct PCR and the HC-PCR procedure the threshold of detection was determined for one set of DNA samples and PCR reagents. The results of AP PCR using DNA template ranging from 100 to 0.1 pg are shown in Figure 3.8 and summarised in Table 3.11. While it was sometimes possible to see very faint bands in the lanes from reactions containing 1.0 and 0.5 pg of DNA template, these were not of sufficient brightness to be confident of their presence. The lower limit of detection in this case determined to be between 1 and 5 pg. This result is consistent with the result of 2 to 20 pg obtained using Isolate SFC 262 (Table 3.4).

Table 3.11 Summary of results for AP PCR detection of DNA from *A. salmonicida* isolate 1107/1B.

Sample No.	DNA template (pg)	Visible band	Result
1	100.0	Yes	+
2	50.0	Yes	+
3	10.0	Yes	+
4	5.0	Yes	+
5	1.0	Very Weak	-
6	0.5	Very Weak	-
7	0.1	No	-
8	0.0	No	-

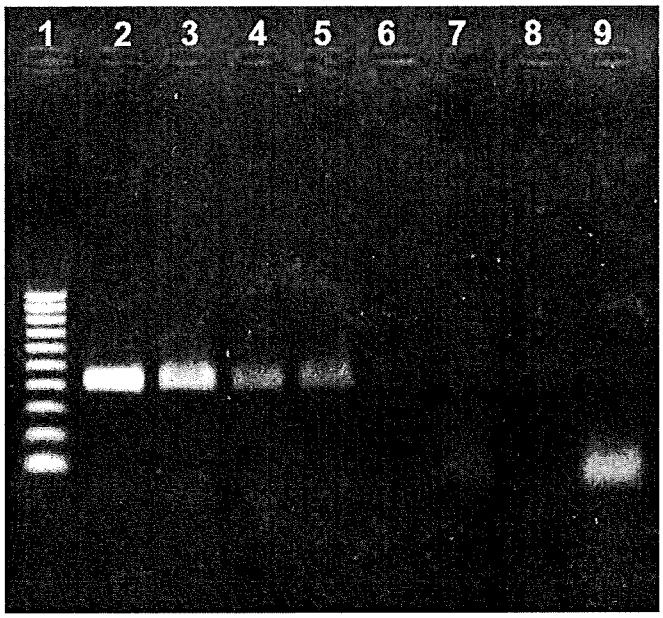

Fig 3.8 Detection Limits of AP PCR using *A. salmonicida* 1107/1B DNA. Lane 1 contains the 100bp marker, Lanes 2 - 8 contain *A. salmonicida* DNA in the following amounts: 100, 50, 10, 5, 1, 0.5, 0.1 fg, Lane 9 contains the negative control.

3.6.1 Optimisation of Mg^{2+} concentration and Production of Hybridisation Capture Probe

Although the PCR conditions first selected for probe production appeared to work well, the Mg^{2+} concentration of the reaction was optimised in order to increase the potential yield of the reaction. Four concentrations of Mg^{2+} were examined using 2 DNA template concentrations. Following electrophoresis of PCR products a Mg^{2+} concentration of 3mM was chosen for probe production (Figure 3.9). A prominent single band of approximately 400 bp (396 bp expected) was consistently observed in gels (Figure 3.10).

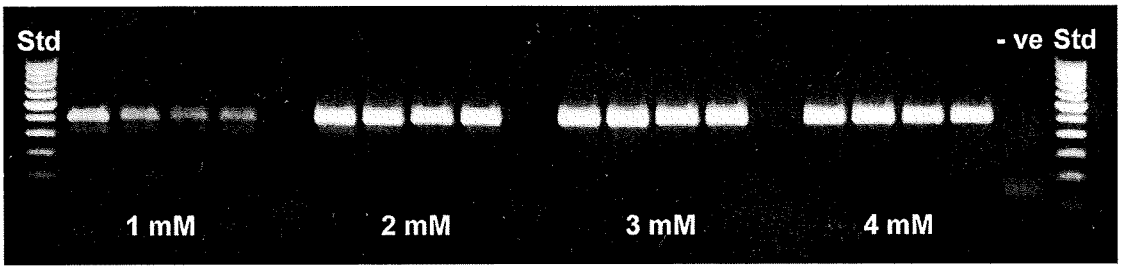

Fig 3.9 Effect of various Mg^{2+} concentrations in Probe Production PCR.

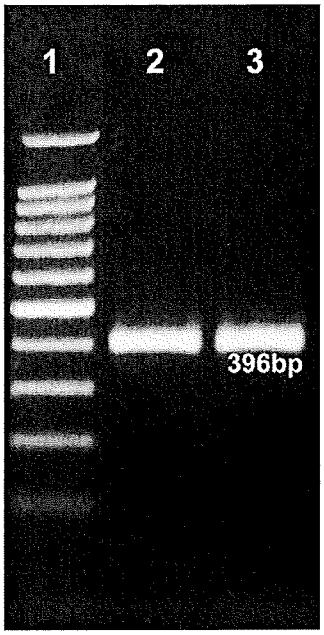

Fig 3.10 Production of hybridisation capture probe.

3.6.2 Lower Limits of Detection of HC-PCR Using Coupled Probe

DNA samples ranging in concentration from 1 ng/µL to 1 pg/µL were tested. No reaction products were observed, even at the lowest DNA dilutions. It was decided on this basis to try the indirect hybridization capture method using sequential hybridization followed by streptavidin binding and magnetic capture.

3.6.3 Determination of Lower Limits of Detection of HC-PCR System Using Sequential Hybridization, Streptavidin Binding and Magnetic Capture

DNA dilutions of 1000, 500, 100, 50, 10, 5 and 1 pg/µL were examined using the indirect HC-PCR procedure described in Section 2.8.4. A clear positive result was only obtained in the presence of relatively large amounts of target DNA. The lower limit of detection, even in free solution without the presence of potentially interfering host DNA, was only between 1,000 and 500 pg/µL (Fig 3.11).

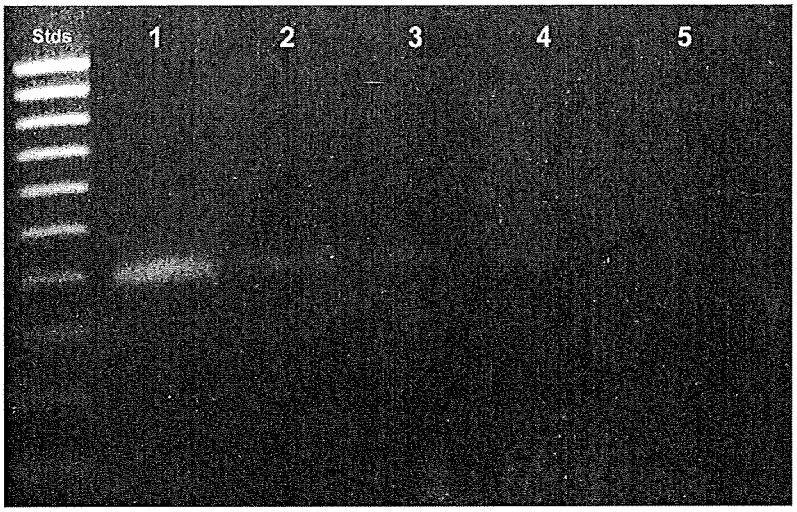

Fig 3.11 Indirect HC-PCR of *A. salmonicida* 1107/1B DNA. Lane 1 1000 pg/µL, Lane 2 500 pg/µL, Lane 3 100 pg/µL Lane 4 50 pg/µL and Lane 5 10 pg/µL.

3.7 OBJECTIVE: Field study: a preliminary survey of wild and farmed populations of freshwater and marine fish

A. salmonicida was not isolated from any of the tissue samples taken during the course of the survey, nor were any tissue samples positive by direct screening with the AP and PAAS PCR assays.

SECTION 4 DISCUSSION

4.1 OBJECTIVE: Characterisation of a comprehensive reference collection of major strains, both exotic and enzootic, *of A. salmonicida* subspecies

An extremely broad phenotypic diversity was expressed by the *A. salmonicida* isolates, with 30 separate clusters being recognised (Appendix 1). It was observed that the phenotypic tests employed in this study were often inadequate with regards to the accurate identification of *A. salmonicida* isolates to both species and subspecies levels. For example, isolates SFC 301 and 302 were motile, and isolates SFC 201, 203 and 469 were negative by the cytochrome oxidase test (Appendix 1). Such results should have excluded these isolates from the species *A. salmonicida*, yet other tests including PCR (Appendix 2) and sequencing (Table 3.3) demonstrated that these isolates were indeed *A. salmonicida*. There have been previous reports in the literature of such anomalies within this species e.g. Chapman *et al.*, 1991; McIntosh and Austin, 1991; Teska *et al.*, 1992; Wiklund *et al.*, 1994; Pederson *et al.*, 1996. This again demonstrates that the application of phenotypic tests alone are of limited value, and highlights the need for improved diagnostic techniques with regards to *A. salmonicida* identification.

4.2 OBJECTIVE: Identify published but unvalidated nucleotide sequences with potential for diagnostic use

A number of PCR primer sets and nucleic acid probes were identified in the literature as warranting further study (Section 3.2). Only a limited number of bacteria had been screened in such studies, and therefore further evaluation of these tests was required. It is important to remember that non-culture-based methods such as PCR provide us with an indication or sign that the target organism is present, as opposed to isolating the viable disease-causing agent itself. Hence they are referred to as 'proxy' methods or measurements, because they only indirectly indicate the presence of the target organism in a sample (Hiney, 1997). One crucial, but often overlooked, aspect of proxy measurements is that their application must be validated i.e. the extent to which the technique can be legitimately used for a specified purpose must be thoroughly investigated (Hiney and Smith, 1998).

Diagnosis of *A. salmonicida* in Australia would have far-reaching consequences, and it was therefore vital that the candidate PCRs identified by this study were rigorously and systematically evaluated. The validation protocol used in this study was based on the framework proposed by Hiney and Smith (1998) and involved the following four phases:

1. *in vitro* testing of pure cultures of bacteria
2. seeded tissue studies
3. experimentally generated infection studies
4. field trials

4.3 OBJECTIVE: Develop diagnostic procedures using molecular technology

The first stage of developing the diagnostic procedures involved using the candidate PCRs to screen the bacterial isolates in AFDL's reference collection. The key issues to be considered here are specificity i.e. the proportion of true positive results, and sensitivity i.e. the proportion of true negative results (Bernoth, 1997b).

4.3.1 *In vitro* specificity of PCR tests targeting *A. salmonicida*

It was decided to test the *in vitro* specificity of the PCR tests first, on the basis that only those tests that were 100% specific for their target would be further investigated and validated. Screening of the negative control organisms demonstrated the following PCRs proved to be non-specific: 16Sa, 16Sb, 15e4, ASA1 and ASA2 (Table 3.1) and these were therefore excluded from further study.

The primer sets 16Sa and 16Sb were both designed *a priori* and target the 16S rRNA gene (Høie *et al.*, 1996; Carson, 1998). This gene is a particularly useful and popular PCR target site due to its ubiquitous nature in prokaryotes, and the presence of both highly conserved and more variable regions of nucleotides. These properties of the 16S rRNA gene have been exploited to facilitate the rapid identification of organisms based on PCR product size and sequence (Fox *et al.*, 1980). However, *Aeromonas* is a relatively recently evolved genus (Ruimy *et al.*, 1994) and, as such, sequence variation is probably too low to permit reliable discrimination between species (Stackebrandt and Geobel, 1994). It is of interest to note the similarity between the 16Sa and 16Sb primer sets (Table 2.5) – although independently derived, they occur in the same region of the 16S rRNA gene, highlighting the constraints of designing PCR primers to a known DNA target when there is a paucity of differential sequence information (Carson, 1998). The lack of specificity observed in this study for these two primer sets, particularly with regard to other *Aeromonas* species, was probably due to the high degree of homology of the 16S rDNA between these species. The difference between the number of cross-reactions observed for each primer set is most likely a function of the PCR conditions. As the 16Sa test has a higher annealing temperature, a lower concentration of magnesium in the reaction mix, and a lower number of cycles (Table 2.6), it is the more stringent of these two tests and far less likely to cross-react with closely related species.

In contrast to the 16S PCRs, the 15e4, ASA1 and ASA2 PCR tests were generated by screening RAPD profiles of *A. salmonicida* DNA extracts, identifying common bands, sequencing these and then designing primers. This 'random' approach, while capable of generating species or sub-species specific primers - the MIY PCR primers were also generated in this manner (Miyata *et al.*, 1996) - does require extensive verification, particularly as the target site is unknown and therefore no assumptions can be drawn regarding the distribution of the target site amongst unrelated organisms. It should also be noted that although the 15e4 probe appeared specific for its target species (Oakey *et al.*, 1988), the primers designed in this study based on that probe were not. This highlights the difficulty of designing specific primers when only a limited amount of sequence data is available. Development of PCR-based tests was the primary goal of this study due to the lower detection limits afforded by PCR compared to probing.

The remaining putative *A. salmonicida*-specific PCR tests (AP and PAAS) appeared to be 100% specific and did not cross-react with any of the non-target organisms listed in Table 2.4. These tests were selected for further validation. An ideal diagnostic test would be both 100% specific and 100% sensitive but this is an unlikely event in the real world, and so the next phase involved determining the *in vitro* sensitivity of the tests.

4.3.2 *In vitro* sensitivity of PCR tests targeting *A. salmonicida*

a. Determining the in vitro *sensitivity of the PCRs*

The sensitivity of the AP and PAAS PCRs was established by screening the AFDL *A. salmonicida* library (*n* = 308) (summary given in Table 3.2, full results in Appendix 2). Combining the results of both AP and PAAS tests offered the best 'coverage' in terms of identifying the target organism, with only 0.6% of *A. salmonicida* isolates being falsely scored as negative.

b. Determining why the in vitro *sensitivity of the specific PCRs was less than 100%*

PAAS PCR. The failure of the PAAS primer set to identify 100% of the *A. salmonicida* isolates appeared to be related to the primer target site which had previously been shown to occur on a 6.4 kb cryptic plasmid (Mooney *et al.*, 1995) and was believed to be present in approximately 90% of *A. salmonicida* isolates (Powell, 1997, pers. comm.). Thus the plasmid profiles of those isolates found to be PAAS PCR-negative were probed with the PAAS3 nucleic acid probe (O'Brien *et al.*, 1994) to determinethe presence or absence of the primer target site in 21 isolates. Extracts of 2 of these isolates were demonstrated to contain a unique band, approximately 6 kb in size (Fig 3.1) that did hybridise with the PAAS probe. When DNA was re-extracted from these 2 isolates using the Puregene® Kit (Gentra Systems) as opposed to the original method based on use of the DNAzol® reagent (Life Technologies™), they were found to be PAAS PCR-positive. This finding highlights the importance of using a reliable method of DNA extraction prior to PCR.

AP PCR. The failure of the AP primer set to identify all of the *A. salmonicida* isolates also appeared to be related to the primer target site. The AP primers target a region of the *vap*A gene, which encodes a unique subunit protein (the "A-protein") of the A-layer of *A. salmonicida* (Chu *et al.*, 1991). It was demonstrated that AP PCR-positive isolates did not necessarily express the A protein e.g. SFC36, Fig 3.2. This phenomenon had previously been observed by Gustafson *et al.*, 1992. In addition to this, it was also demonstrated that some of the AP PCR-negative isolates were still able to express A protein e.g. SFC308, Fig 3.2. This result may be due to a mutation that occurs within the priming site, but still leaves the gene functional.

4.3.3 PCR test targeting *A. salmonicida* subspecies *salmonicida*

Difficulties experienced during the initial setup of the MIY PCR highlighted the problems that can sometimes arise with the transfer of PCR methodology between laboratories. After an initial optimisation period, the test was successfully used in the laboratory. The optimised conditions were forwarded to Dr Steinar Høie (Central Veterinary Laboratory, Norway) who had also

experienced difficulties with the original published parameters but found that the new conditions, as devised by AFDL, performed well in his laboratory (Høie, pers. comm.). To date, based on results from this and other projects, the MIY PCR appears to be specific for *A. salmonicida* subsp. *salmonicida*.

4.3.4 Seeded tissue studies

Once the PCR tests had been validated using a large number of purified *A. salmonicida* DNA preparations, the next phase of validation, i.e. the seeded tissue studies, was undertaken. Most data available regarding the trialling of PCRs to detect infectious material involves the use of seeded tissue i.e. healthy tissue which is "spiked" with a known pathogen titre, calculated by, for example, plate counts. Use of seeded tissues alone to evaluate a PCR test would be inadequate and misleading, as there are numerous fundamental differences between infected tissues which harbour a pathogen and healthy tissue seeded with a laboratory-grown bacterium. For example the expressed phenotype of the bacterium may differ (Fernandez *et al.*, 1995; Garduno and Kay, 1995). The physical and chemical accessibility of the pathogen may be quite different, and additional PCR inhibitors could be present in the infected tissue. However, bearing these constraints in mind, experiments involving seeded tissue proved to be a useful starting point with regards to developing protocols.

Serial dilutions of cocktails of purified *A. salmonicida* DNA and fish tissue (typically kidney) DNA were prepared and used as template in the PCRs. The initial results were disappointing, with LDLs of 20 ng *A. salmonicida* DNA per 1 µg fish tissue DNA. Using the approximation that 5 fg DNA is equivalent to 1 cell or 1 genome equivalent (GE) (Gustafson *et al.*, 1992), then 20 ng *A. salmonicida* DNA represents $4x10^6$ cells. In our experience, 100 mg of fish tissue yielded at least 1 - 2 µg fish tissue DNA. Thus a LDL of $4x10^6$ cells per 1 µg fish tissue DNA would be equivalent to approximately $8x10^7$ cells per gram of tissue. Indeed, when disease-free brown trout tissues were seeded with a known number of *A. salmonicida* cells at this phase of the project, the LDL of the PCRs varied from $10^6 - 10^8$ CFU/g fish tissue (Section 3.3.7). This limit would be inadequate for the direct detection of the pathogen given reports ranging from 10^1-10^7 CFU *A. salmonicida*/g fish tissue (e.g. Nomura *et al.*, 1993; Cipriano *et al.*, 1994a).

In an effort to improve the PCR detection limits, the addition of various co-solvents such as BSA (Kreader, 1996) was trialled, and components of the PCR mix and cycle conditions manipulated. Alterations to the number of cycles, length of cycles, and primer and enzyme concentrations did enhance the performance of the AP and PAAS PCRs. Unfortunately, the MIY PCR was not improved to the same extent. Using these new conditions (Table 2.6), the LDLs of the PCRs (Table 3.4) were determined for:

1. purified target DNA only
2. purified target DNA plus 1 µg fish tissue DNA
3. whole cell suspensions of *A. salmonicida* added to 50 mg fish tissue.

The PAAS PCR had the lowest detection limit with regards to purified target template only, being in the range 2 pg to 200 fg. The superior performance could be due to the target site being plasmid-borne, as plasmids can occur in multiple copies within the cell (Hardy, 1986), thus providing more initial template DNA for the PCR. Hiney *et al.* (1992) reported a PAAS detection limit of 10 fg DNA, but it must be pointed out that this was using the less stringent $MgCl_2$ concentration of 3.5 mM, compared to the 2.5 mM $MgCl_2$ concentration used in this study (we opted for a lower $MgCl_2$ concentration in order to maximise the specificity of the PCR).

Addition of either fish tissue or fish tissue DNA to the reaction mix was found to have an inhibitory effect on all of the PCRs. This type of interference has been reported previously (e.g. Gustafson *et al.*, 1992; Høie *et al.* 1997) and is a major limitation of the direct PCR detection of a target pathogen in infected tissue samples. It was also noted that weak non-specific bands occurred in the PCRs when high levels of non-target DNA (>1 µg) were included in the reaction mix. This result may be due to large concentrations of non-target DNA 'out-competing' the target DNA (which has higher homology with the primers, but occurs much less frequently) with regards to primer binding sites, particularly in the first few rounds of amplification. However, these non-specific bands were not deemed to be a problem as they differ in size to the desired product, and also because sequencing the PCR product would be used as part of the diagnostic procedure to confirm identity of the product.

Gustafson *et al.* (1992) had reported a LDL of 10^4CFU/g when the AP PCR was applied to fish tissue samples seeded with known amounts of *A. salmonicida*, and our results fall within this limit. However, our LDL for the PAAS PCR of 10^4-10^3 CFU/g tissue does not reach the 200 GE/g sample reported by O'Brien *et al.*, 1994. It must be realised however that the latter study involved a different matrix - either filtered effluent or faecal matter - both of which may differ in the degree of inhibitory effect they have on the PCR compared to fish tissue. More importantly, their measurement of the LDL was based on a comparison of PCR yield with that obtained when using pure DNA as template. Given the non-linear kinetics of PCR, such a comparison could be wildly inaccurate, and could more than account for the differences in the reported LDL.

Mooney *et al.* (1995), using the nested PAAS PCR (which we abandoned in this study due to problems with false positive results), reported a LDL of 100 GE/fish. However, as Bernoth (1997b) points out, this statement is misleading as the 100 GE limit was in fact per 10–100 µL of blood sample, and so actually equates to $10^3 - 10^4$ GE/mL blood. It is therefore considered that the PCR LDLs calculated in this study are a realistic measure of the performance of the PCRs, as applied to seeded tissue samples.

The LDLs of the AP and PAAS PCRs are approaching the theoretical limit of direct PCR detection, based on the figure of $2x10^3$ cells g^{-1} of fish tissue as calculated by Carson (1998). The only means of improving upon this figure is to concentrate the target DNA prior to PCR, perhaps via a pre-enrichment step (Gustafson *et al.*, 1992) or use of a technique such as hybridisation-capture.

4.3.6 Summary

The AP, PAAS and MIY PCR tests examined appeared to have a high level of specificity and sensitivity with regards to identifying pure bacterial cultures. These tests could therefore be of immediate benefit with regards to identifying pure bacterial cultures. These tests provide a rapid means of identification when compared to the time-consuming biochemical methods, and a greater degree of reliability than serological techniques.

Given these results, it was decided not to pursue the development of a novel test for *A. salmonicida*. Rather, it was viewed as more appropriate to continue using the published tests, but to apply them now to the detection of the pathogen in infected tissue.

The seeded tissue studies permitted further optimisation of the experimental diagnostic procedures, including the development of a standard procedure for the extraction of total DNA from fish tissue seeded with *A. salmonicida*, and the empirical determination of the LDLs of the three candidate PCRs.

The *A. salmonicida* subsp. *salmonicida*-specific MIY PCR was unable to reach the detection limits of the optimised AP and PAAS *A. salmonicida* species-specific PCRs, which had LDLs of 10^5 - 10^4 CFU/g seeded tissue and 10^4 - 10^3 CFU/g seeded tissue respectively.

It is important to note that these LDL values can not be extrapolated to determine the performance of the PCRs in the case of infected tissues. Use of seeded tissues does not take into consideration a number of vital factors - particularly the distribution, both in time and space, of the target organism in the host tissue. It was therefore of paramount importance that all tests which were based on the study of seeded tissues were further validated using tissues from infected animals. The next phase of the study therefore dealt with the examination of tissues obtained from experimentally infected fish.

4.4 OBJECTIVE: Validation of molecular diagnostic procedures using experimental infections carried out in the microbiologically secure aquarium facility at AAHL

A key factor in this phase of the project was the experimental generation of *A. salmonicida* infections in fish. There are three main options with regards to administering *A. salmonicida* with the purpose of infecting fish (Bricknell, 1995):

1. injection
2. bath exposure
3. co-habitation

These options vary in their reproducibility and authenticity e.g. i.p. injection of *A. salmonicida* is a highly reproducible means of incurring overt infection but is hardly authentic, whereas the co-habitation of infected and uninfected fish mimics the natural route of infection but is often irreproducible and does not always lead to acute disease (Smith, 1998, pers. comm). In this study, only the i.p. injection of *A. salmonicida* successfully generated experimental infection in brown trout, which is unfortunate because this method probably least resembles the process of natural infection. However, this still enabled progression beyond the seeded tissue studies, as it provided

the target pathogen the opportunity to reproduce within the host, possibly allowing alterations of phenotype to occur. This disease state then provided the study with a 'non-sterile incurred matrix' (Hiney, 1997) in which the bacterium was embedded, as opposed to simply mixing healthy tissue and a laboratory grown strain of *A. salmonicida*.

Bacteria were isolated on SBA from the mucus, gills, gut, kidney and spleen of the experimentally infected brown trout and positively identified by all 3 PCRs – AP, PAAS, and MIY.

With regards to the direct detection of *A. salmonicida* in tissues taken from the experimentally infected fish, both the AP and PAAS PCRs yielded positive results when applied to overtly infected mucus, gill, intestine, muscle lesion, spleen and kidney samples (Table 3.6). The MIY PCR however was less sensitive, and it appears this PCR will definitely require some form of pre-enrichment step to improve its performance.

4.4.1 Summary

The AP and PAAS PCR tests were successful when applied to experimentally infected fish - the 'non-sterile incurred microcosm' (Hiney, 1997). One should bear in mind however, that injecting fish with a laboratory-grown bacterium is still quite artificial. The performance of the PCRs still had to be evaluated using naturally infected fish – the last phase of the validation process.

4.5 OBJECTIVE: Validation of the molecular diagnostic procedures developed at AAHL using naturally infected populations of salmonids: a short-term collaborative project with the National Fish Health Research Laboratory, West Virginia, U.S.A.

4.5.1 Overtly infected tissue samples

Bath challenge with *A. salmonicida* generated overt infection in Arctic char. There are a number of possible reasons why this technique was successful at the NFHRL and not at the AAHL (Section 3.4.2), including differences in the virulence of the bacterial isolate used and differences in the susceptibility of the host species (Arctic char vs. brown trout) to infection with *A. salmonicida*.

The stress-induced furunculosis (SIF) assay were used to generate overt infection in covertly infected rainbow trout and Atlantic salmon held at the NFHRL, thereby confirming that these populations were indeed covertly infected.

Bacteria isolated from these fish were successfully identified as *A. salmonicida* using the AP and PAAS PCRs, and as *A. salmonicida* subsp *salmonicida* using the MIY PCR. All results were in agreement with the biochemical tests currently employed by the NFHRL to identify *A. salmonicida* and *A. salmonicida* subsp *salmonicida*. Typically PCR tests yielded results in 1 to 2 days, compared to 7 days for classical biochemical testing, thus demonstrating the suitability of the PCR tests as a replacement for the more time-consuming biochemical tests.

The AP and PAAS PCRs were also used to directly detect *A. salmonicida* in tissue samples taken from the overtly diseased fish. Cipriano *et al.* (1992; 1994b; 1996a,c,d) have previously reported levels of *A. salmonicida* in overtly infected fish, including Atlantic salmon, ranging from 10^3 – 10^7 CFU/g, and even as high as 10^9 CFU/g, in both kidney and mucus samples. Given that the

developed PCRs cover at least part of this range, it was not surprising to find that direct PCR detection of overtly infected tissues was possible in many instances. The fact that mucus yielded PCR products more often than other tissues, especially intestine, could be a reflection of numerous factors e.g. the pathogen occurs in higher numbers in some tissues compared to others, or some types of diseased tissues may inhibit the PCR more than others.

4.5.2 Covertly infected tissue samples

A. salmonicida was detected by culture in Atlantic salmon held in Raceway 3 at the RCNSS, and rainbow trout and Atlantic salmon populations held at NFHRL (the latter two groups had been previously demonstrated to be covertly infected by the SIF assay). The pathogen load ranged between 8.3×10^2 and 2×10^6 CFU/g tissue sample. These levels are higher than those reported by Nomura *et al.* (1993) of 10^2 CFU/g in pink salmon (*Oncorhynchus gorbuscha*) and 10^1 CFU/g kidney in chum salmon (*Oncorhynchus keta*), but are within the range reported by other researchers, as cited by Hiney *et al.* (1997).

A. salmonicida was cultured from predominately gill and mucus samples, with only 3 of the 15 culture-positive, lethally-sampled salmonids (n = 40) having systemic infections i.e. kidney and/or spleen were culture-positive. However, 2 of these systemically-infected fish were not culture-positive by either gill or mucus samples, demonstrating the importance of sampling more than one tissue site when attempting the culture of *A. salmonicida* from covertly infected fish (Bernoth, 1997b). The predominance of the external surfaces of the fish as the site of pathogen carriage is in keeping with the findings of Cipriano *et al.* (1994a; 1996a,d). The PCR assays correctly determined the identity of the *A. salmonicida* isolates in a shorter time than that required by the biochemical tests, again demonstrating their usefulness with regards to identifying cultured bacteria.

Direct PCR testing detected the presence of *A. salmonicida* in the rainbow trout and Atlantic salmon populations held at the NFHRL, but to a lesser degree than culture. The PCR assays also completely failed to yield positive results for the Atlantic salmon held in Raceway 3 at the RCNSS, probably due to the pathogen only being present at levels outside the detection limits of these tests. From the results obtained, it would appear that, for covertly infected tissues, the level of detection for the AP and PAAS PCRs was approximately 4×10^5 CFU/g sample (there are instances where the PCRs could detect fewer CFUs than this, but detection at lower levels was not consistent). In no instance was a sample PCR-positive but culture-negative, nor was any particular sample site always PCR-negative, suggesting that the main constraint on direct detection were the LDLs of the respective PCR assays. It is interesting to note that the LDL of the PCRs does not equate to those levels determined by the seeded tissue studies. This result emphasises the fact that, when attempting to determine the performance of an assay, one cannot simply extrapolate from laboratory-based studies, but rather test performance must be determined empirically.

The failure to detect *A. salmonicida* by either culture or PCR in the Atlantic salmon held in Raceways 5 and 6 at RCNSS, or the brown trout from Bennington (Section Appendix 4, Tables 1 –

3) could be due to a number of factors. Firstly, as the LDL of culture is estimated to be around 10^2 CFU/g tissue sample (Cipriano, 1997), the occurrence of the target pathogen at levels lower than this would produce false negative results. Secondly, only single-point inspections were performed on these populations and it has been noted in the past that such inspections can produce erroneous results (Cipriano *et al.*, 1994a), possibly as a result of the proposed transient nature of covert infections (Scallan *et al.*, 1993). It has been found that sampling at multiple intervals over time can provide greater accuracy with regards to the cultural detection of *A. salmonicida* in covertly infected salmonids (Cipriano *et al.*, 1997), but unfortunately it was not possible to incorporate such a sampling regime within the bounds of this study. These issues may have been resolved by use of the SIF assay, which has been reported as being more reliable than culture, and to provide the most definitive single-point assay for the detection of covert *A. salmonicida* infections (Cipriano *et al.*, 1997). However, it was not possible to conduct the SIF assay on these salmonid populations. Thirdly, the prevalence of the pathogen may have been at such low levels that a larger sample size was required e.g. if the disease prevalence was as low as 1%, then at least 100 fish would have to be sampled to yield just 1 positive result.

4.5.3 Summary

Hiney and Smith (1998) in their discussion on the validation of proxy methods state that the "major role [of validation] is in providing rational grounds for rejecting techniques whose application to environmentally derived matrices has been shown to be invalid". Following their recommended guidelines proved to be extremely useful with regards to the systematic evaluation of the methods developed in this study. As the primary aim of this study was to use PCR to establish the presence/absence of *A. salmonicida*, as opposed to predicting the consequences of the presence of the pathogen, it was pertinent to use the principle of comparative validation (Hiney and Smith, 1998), i.e., performance of the PCRs was compared to previously validated and accepted 'gold standard' techniques. Thus each phase of the study eliminated those PCR assays demonstrated to be invalid on the basis of their specificity, sensitivity, and finally their lower limit of detection, as compared to culture, and (finally) the SIF assay.

This study demonstrated that the PCR technology developed at the AAHL could be used to replace the time-consuming biochemical testing currently used to confirm the identity of *A. salmonicida* isolates cultured from both overtly and covertly infected salmonids.

The PCR assays were capable of direct detection of *A. salmonicida* in overtly infected fish tissues, with mucus, gill and kidney samples most likely to yield a positive result.

It was demonstrated that culture was a more reliable and sensitive method for the detection of *A. salmonicida* in covertly infected salmonids than did the direct PCR testing of tissue samples. It is highly recommended that any target pathogen that may be present in the tissues of covertly infected fish are first concentrated by the inclusion of a culture step, as used in this study, prior to PCR screening. Culture alone did not detect *A. salmonicida* in all salmonid populations either, but the development of a medium selective for *A. salmonicida*, and the use of multiple-point sampling regimes, could overcome such problems.

4.6 OBJECTIVE: Determine whether the use of hybridisation-capture PCR would enhance the sensitivity of the test to allow detection of covert infections

The threshold of detection in mixed DNA samples derived from tissues may be substantially increased by non-target DNA or inhibitory substances co-purified in the sample. This was borne out in this study where the LDL of the AP PCR was found to be 1 order of magnitude higher when examining DNA samples derived from tissues. In order to detect lower concentrations of *A. salmonicida* DNA in these samples and possibly allow the detection of covertly infected fish, 2 hybridisation capture methods were evaluated. Hybridisation of target DNA followed by selective capture and PCR has the potential to both concentrate target DNA present at very low concentrations in a sample and to remove PCR inhibitors. However, the results obtained in this study show there was no advantage in using hybridisation capture (in its current form) as a method of DNA preparation prior to PCR.

4.7 OBJECTIVE: Field study – a preliminary survey of wild and farmed populations of freshwater and marine fish

Neither culture nor direct PCR screening detected either typical or atypical *A. salmonicida* in the fish surveyed in this study. These results could indicate that *A. salmonicida* is totally absent from these populations, or alternatively, that *A. salmonicida* is present but at levels below the threshold of detection of culture. The prevalence of the pathogen may also be so low that an increased number of fish need to be sampled. Future surveys could be greatly improved by the development of a medium selective for *A. salmonicida*, and the use of multiple-point sampling regimes.

SECTION 5 BENEFITS

There is a broad host range for the bacterial pathogen *Aeromonas salmonicida*. In Australia, this bacterial pathogen has been isolated from a number of marine and freshwater fish species including goldfish, Atlantic salmon, silver perch and greenback flounder. To date, only "atypical" subspecies of *A. salmonicida* have been isolated from diseased fish. The "typical" subspecies, *A. salmonicida salmonicida*, which is the causative agent of classical furunculosis in salmonids is exotic to Australia and is recognised as a major threat to the salmonid industry in Australia. Rapid and accurate diagnosis is crucial, particularly for industries which produce those fish species susceptible to infection by *A. salmonicida*. Early detection of the pathogen would significantly enhance the success of any control measures deemed necessary in the event of a disease outbreak. Differential diagnosis between "typical" and "atypical" subspecies is essential.

The technology developed during the course of this project allows detection and identification of *A. salmonicida* in a matter of a few days. Previous to the development of PCR-based techniques such identification may have required 1-2 weeks. It is anticipated that, in the event of a disease outbreak, the development of these improved methods for the detection and identification of *A. salmonicida* will reduce the potential damage to the affected industry directly; potential benefits may include reduction in the loss of stock and in the increased work-load brought on by a disease outbreak. The use of these specific and sensitive techniques to demonstrate the absence of pathogens is also likely to enhance our international trade position. It is difficult to quantify the potential benefit in dollar terms since the availability of rapid and accurate diagnostic procedures is only one aspect impacting on the management of emergency diseases and the ultimate cost of the disease outbreak to the affected industry.

Thus the benefits and beneficiaries are the same as those stated in the original application and include salmonid and non-salmonid aquaculture industries as well as natural fisheries in the southern regions of Australia. The States involved in these industries are New South Wales, Western Australia, South Australia, Victoria and Tasmania. The State Departments responsible for providing veterinary services to these industries will also benefit by the availability of these improved diagnostic procedures.

SECTION 6 INTELLECTUAL PROPERTY

The procedures developed during the course of this project are extensions of previously published data and therefore it is unlikely that any intellectual property has been generated.

SECTION 7 FURTHER DEVELOPMENT

It is expected that the technology developed during the course of this project will form part of an Australian Standard Diagnostic Technique (SDT) for the detection and identification of *Aeromonas salmonicida* in fish. A series of SDTs is currently being developed for aquatic animal diseases as part of AQUAPLAN and will become available through this process. In addition, it is anticipated that training in fish bacteriology will be available to fish disease diagnosticians through a series of training workshops such as that provided by FRDC Project Number 00/149.

Copies of the Final Report will be made available to those State diagnostic laboratories with particular interest in detection and identification of *Aeromonas salmonicida*.

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SECTION 9 DISTRIBUTION

Copies of the Final Report will be distributed to the following organisations.

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Agersborg, A., Dahl, R. and Martinez, I. (1997) Sample preparation and DNA extraction procedures for polymerase chain reaction identification of *Listeria monocytogenes* in seafoods. *International Journal of Food Microbiology* **35**(3):275-280.

Altschul, S. F., Gish, W., Miller, W., Meyers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**:403-410.

Aoki, T. (1997) Resistance plasmids and the risk of transfer. In *Furunculosis: Multidisciplinary Fish Disease Research*. Bernoth, E.-M., Ellis, A. E., Midtlyng, P. J., Olivier, G. and Smith, P., Editors. Academic Press, London. p. 433-440.

AQIS (1999) Risk Management: salmonids. In *Import risk analysis on non-viable salmonids and non-salmonid marine finfish, July 1999* Commonwealth of Australia, Canberra. p. 140-181.

Austin, B., Austin, D. A., Dalsgaard, I., Gudmundsdottir, B. K., Høie, S., Thornton, J. M., Larsen, J. L., O'Hici, B. and Powell, R. (1998) Characterization of atypical *Aeromonas salmonicida* by different methods. *Systematic and Applied Microbiology* **21**(1):50-64.

Austin, B. and Austin, D. A., eds. (1999) *Bacterial Fish Pathogens: Disease in Farmed and Wild Fish*. 3rd ed. Springer-Praxis, London.

Austin, D. A., McIntosh, D. and Austin, B. (1989) Taxonomy of fish associated *Aeromonas* spp., with the description of *Aeromonas salmonicida* subsp. *Smithia* subsp. nov. *Systematic and Applied Microbiology* **11**:277-290.

Balows, E., Hausler, W.J., Herrmann, K.L., Isenberg, H.D. and Shadomy, H.J., eds. (1993) *Manual of Clinical Microbiology*. 5th ed. American Society for Microbiology, Washington, D.C.

Bernoth, E.-M. (1990) Autoagglutination, growth on tryptone-soy-Coomassie-agar, outer membrane protein patterns and virulence of *Aeromonas salmonicida* strains. *Journal of Fish Diseases* **13**:145-155.

Bernoth, E.-M. (1997a) Diagnosis of furunculosis: the tools. In *Furunculosis: Multidisciplinary Fish Disease Research*. Bernoth, E.-M., Ellis, A. E., Midtlyng, P. J., Olivier, G. and Smith, P., Editors. Academic Press, London. p. 98-158.

Bernoth, E.-M. (1997b) Furunculosis: the history of the disease and of disease research. In *Furunculosis: Multidisciplinary Fish Disease Research*. Bernoth, E.-M., Ellis, A. E., Midtlyng, P. J., Olivier, G. and Smith, P., Editors. Academic Press, London. p. 1-20.

Bricknell, I. R. (1995) A reliable method for the induction of experimental furunculosis. *Journal of Fish Diseases* **18**:127-133.

Bullock, G. L. and Stuckey, H. M. (1975) *Aeromonas salmonicida*: detection of asymptomatically infected trout. *The Progressive Fish-Culturist* **37**:237-239.

Carson, J. (1998) Development of molecular probes for use in bacterial disease diagnosis and

health monitoring of farmed and wild finfish in Australia. In *Final Report on FRDC Project 93/128* Fisheries Research and Development Corporation, Canberra.

Chapman, P. F., Cipriano, R. C. and Teska, J. D. (1991) Isolation and phenotypic characterization of an oxidase-negative *Aeromonas salmonicida* causing furunculosis in coho salmon (*Oncorhynchus kisutch*). *Journal of Wildlife Diseases* **27**(1):61-67.

Chu, S., Cavaignac, S., Feutrier, J., Phipps, B. M., Kostrzynska, M., Kay, W. W. and Trust, T. J. (1991) Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. *Journal of Biological Chemistry* **266**(23):15258-15265.

Cipriano, R. C., Ford, L. A., Teska, J. D. and Hale, L. E. (1992) Detection of *Aeromonas salmonicida* in the mucus of salmonid fishes. *Journal of Aquatic Animal Health* **4**:114-118.

Cipriano, R. C., Ford, L. A., Schachte, J. H. and Petrie, C. (1994a) Evaluation of mucus as a valid site to isolate *Aeromonas salmonicida* among asymptomatic populations of lake trout (*Salvelinus namaycush*). *Biomedical Letters* **49**:229-233.

Cipriano, R. C., Ford, L. A. and Jones, T. E. (1994b) Relationship between resistance of salmonids to furunculosis and recovery of *Aeromonas salmonicida* from external mucus. *Journal of Wildlife Diseases* **30**(4):577-580.

Cipriano, R. C., Bullock, G. L. and Noble, A. (1996a) Nature of *Aeromonas salmonicida* carriage on asymptomatic rainbow trout maintained in a culture system with recirculating water and fluidized sand biofilters. *Journal of Aquatic Animal Health* **8**:47-51.

Cipriano, R. C., Bullock, G. L., Schill, W. B. and Kretschmann, R. (1996b) Enhanced culture of *Aeromonas salmonicida* from covertly infected rainbow trout following administration of the stress induced furunculosis test. *Biomedical Letters* **54**:105-112.

Cipriano, R. C., Ford, L. A., Nelson, J. T. and Jenson, B. N. (1996c) Monitoring for early detection of *Aeromonas salmonicida* to enhance antibiotic therapy and control furunculosis in Atlantic salmon. *The Progressive Fish-Culturist* **58**:203-208.

Cipriano, R. C., Ford, L. A., Teska, J. D., Schachte, J. H., Petrie, C., Novak, B. M. and Flint, D. E. (1996d) Use of non-lethal procedures to detect and monitor *Aeromonas salmonicida* in potentially endangered or threatened populations of migrating and post-spawning salmon. *Diseases of Aquatic Organisms* **27**:233-236.

Cipriano, R. C., Ford, L. A., Smith, D. R., Schachte, J. H. and Petrie, C. J. (1997) Differences in detection of *Aeromonas salmonicida* in covertly infected salmonid fishes by the stress-inducible furunculosis test and culture-based assays. *Journal of Aquatic Animal Health* **9**:108-113.

Cipriano, R. C. (1997) Strategies for management of furunculosis in Atlantic salmon effected by non-lethal detection of *Aeromonas salmonicida*: a review. *Bulletin of the European Association of Fish Pathologists* **17**(6):215-219.

Crane, M. S. J. and Bernoth, E.-M. (1996) Molecular biology and fish disease diagnosis: current

status and future trends. *Recent Advances in Microbiology* **4**:41-82.

Dorsch, M. and Stackebrandt, E. (1992) Some modifications in the procedure of direct sequencing of PCR amplified 16S rDNA. *Journal of Microbiological Methods* **16**:271-279.

Emmerich, R. and Weibel, E. (1894) Über eine durch Bacterien erzeugte Seuche unter den Forellen. *Archiv für Hygiene* **21**:1-21

Fernandez, A. I., Perez, M. J., Rodriguez, L. A. and Nieto, T. P. (1995) Surface phenotypic characteristics and virulence of Spanish isolates of *Aeromonas salmonicida* after passage through fish. *Applied and Environmental Microbiology* **61**(5):2010-2012.

Fox, G., Stackebrandt, E., Hespell, R., Gibson, J., Maniloff, J., Dyer, T., Wolfe, R., Balch, W., Tanner, R., Magrum, L., Zablen, L., Blakemore, R., Gupta, R., Bonen, L., Lewis, B., Stahl, D., Luehrsen, K., Chen, K. and Woese, C. (1980) The Phylogeny of Prokaryotes. *Science* **209**:457-463.

Garduno, R. A. and Kay, W. W. (1995) Capsulated cells of *Aeromonas salmonicida* grown *in vitro* have different functional properties than capsulated cells grown in vivo. *Canadian Journal of Microbiology* **41**(10):941-945.

Gustafson, C. E., Thomas, C. J. and Trust, T. J. (1992) Detection of *Aeromonas salmonicida* from fish by using polymerase chain reaction amplification of the virulence surface array protein gene. *Applied and Environmental Microbiology* **58**(12):3816-3825.

Hardy, K. (1986) *Bacterial Plasmids*. 2nd ed. Van Nostrand Reinhold Co. Ltd., England.

Hiney, M., Dawson, M. T., Heery, D. M., Smith, P. R., Gannon, F. and Powell, R. (1992) DNA probe for *Aeromonas salmonicida. Applied and Environmental Microbiology* **58**(3):1039-1042.

Hiney, M. (1997) How to test a test: methods of field validation for nonculture-based detection techniques. *Bulletin of the European Association of Fish Pathologists* **17**(6):245-250.

Hiney, M. P., Smith, P. and Bernoth, E.-M. (1997) Covert *Aeromonas salmonicida* infections. In *Furunculosis: Multidisciplinary Fish Disease Research*. Bernoth, E.-M., Ellis, A. E., Midtlyng, P. J., Olivier, G. and Smith, P., Editors. Academic Press, London. p. 54-97.

Hiney, M. P. and Smith, P. R. (1998) Validation of Polymerase Chain Reaction-based techniques for proxy detection of bacterial fish pathogens: framework, problems and possible solution for environmental applications. *Aquaculture* **162**:41-68.

Høie, S., Heum, M. and Thoresen, O. F. (1996) Detection of *Aeromonas salmonicida* by polymerase chain reaction in Atlantic salmon vaccinated against furunculosis. *Fish and Shellfish Immunology* **6**:199-206.

Høie, S., Heum, M. and Thoresen, O. F. (1997) Evaluation of a polymerase chain reaction-based assay for the detection of *Aeromonas salmonicida* ss *salmonicida* in Atlantic salmon *Salmo salar. Diseases of Aquatic Organisms* **30**:27-35.

Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T. and Williams, S.T., eds. (1994) *Bergey's*

Manual® of Determinative Bacteriology. 9th ed. Williams and Wilkins, Baltimore.

Humphrey, J.D., Lancaster, C.E., Gudkovs, N. and Copland, J.W. (1987) The disease status of Australian salmonids: bacteria and bacterial diseases. *Journal of Fish Diseases* **10**:403-410.

Humphrey, J. D. and Ashburner, L. D. (1993) Spread of the bacterial fish pathogen *Aeromonas salmonicida* after importation of infected goldfish, *Carassius auratus*, into Australia. *Australian Veterinary Journal* **70**(12):452.

Huys, G. and Swings, J. (1999) Evaluation of a fluorescent amplified fragment length polymorphism (FAFLP) methodology for the genotypic discrimination of *Aeromonas* taxa. *FEMS Microbiology Letters* **177**:83-92.

Kado, C. I. and Liu, S.-T. (1981) Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology* **145**:1365-1373.

Khan, A. A. and Cerniglia, C. E. (1997) Rapid and sensitive method for the detection of *Aeromonas caviae* and *Aeromonas trota* by polymerase chain reaction. *Letters in Applied Microbiology* **24**:233-239.

Kreader, C. A. (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Applied and Environmental Microbiology* **62**(3):1102-1106.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of macrophage T4. *Nature* **227**:680-685.

MacFaddin, J. E. (1980) *Biochemical tests for the identification of medically important bacteria*. 2nd ed. Williams and Wilkins, Baltimore, Maryland.

McCarthy, D. H. (1977) Some ecological aspects of the bacterial fish pathogen - *Aeromonas salmonicida. Society for Applied Bacteriology, Symposium on Aquatic Microbiology* **6**:299-324.

McIntosh, D. and Austin, B. (1991) Atypical characteristics of the salmonid pathogen *Aeromonas salmonicida. Journal of General Microbiology* **137**(Pt 6):1341-1343.

McKelvie, L., Reid, C. and Haque, M. (1994) Economic Impact of Salmonid Diseases (furunculosis and infectious haematopoietic necrosis [IHN]). In *DPIE (1997) Salmon Import Risk Analysis, Final Report December 1996* Australian Government Publishing Service, Canberra.

Miles, A. A. and Misra, S. S. (1938) The estimation of the bactericidal power of the blood. *Journal of Hygiene* **38**:732-749.

Miyata, M., Inglis, V. and Aoki, T. (1996) Rapid identification of *Aeromonas salmonicida* subspecies *salmonicida* by the polymerase chain reaction. *Aquaculture* **141**:13-24.

Mooney, J., Powell, E., Clabby, C. and Powell, R. (1995) Detection of *Aeromonas salmonicida* in wild Atlantic salmon using a specific DNA probe test. *Diseases of Aquatic Organisms* **21**:131-135.

Munro, A. L. S. and Hastings, T. S. (1993) Furunculosis. In *Bacterial Diseases of Fish*. Inglis, V., Roberts, R. J. and Bromage, N. R., Editors. Blackwell Scientific Publications, Oxford. p. 122-142.

Nomura, T., Yoshimizu, M. and Kimura, T. (1993) An epidemiological study of furunculosis in salmon propagation in Japanese rivers. *Fisheries Research* **17**:137-146.

O'Brien, D., Mooney, J., Ryan, D., Powell, E., Hiney, M., Smith, P. R. and Powell, R. (1994) Detection of *Aeromonas salmonicida*, causal agent of furunculosis in salmonid fish, from the tank effluent of hatchery-reared Atlantic salmon smolts. *Applied and Environmental Microbiology* **60**(10):3874-3877.

Oakey, H. J., Ellis, J. T. and Gibson, L. F. (1998) The development of random DNA probes specific for *Aeromonas salmonicida. Journal of Applied Microbiology* **84**(1):37-46.

Pedersen, K., Dalsgaard, I. and Larsen, J. L. (1996) Characterization of atypical *Aeromonas salmonicida* isolates by ribotyping and plasmid profiling. *Journal of Applied Bacteriology* **80**(1):37-44.

Reed, K. C. and Mann, D. A. (1985) Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Research* **13**(20):7207-7221.

Ruimy, R., Breittmayer, V., Elbaze, P., Lafay, B., Boussemart, O., Gauthier, M. and Christen, R. (1994) Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium, Aeromonas* and *Plesiomonas* deduced from small-subunit rRNA sequences. *International Journal of Systematic Bacteriology* **44**:16-26.

Scallan, A., Hickey, C. and Smith, P. (1993) Evidence for the transient nature of stress inducible asymptomatic *Aeromonas salmonicida* infections of Atlantic salmon. *Bulletin of the European Association of Fish Pathologists* **13**(6):210-212.

Schotts, E. B. (1994) Furunculosis. In *Blue Book, Version 1. Suggested Procedures for the Detection and Identification of Certain Fishfish and Shellfish Pathogens*. Thoesen, J. C., Editor American Fisheries Society, Fish Health Section.

Stackebrandt, E. and Goebel, B. (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology* **44**:46-49.

Svendsen, Y. S., Dalmo, R. A. and Bogwald, J. (1999) Tissue localization of *Aeromonas salmonicida* in Atlantic salmon, Salmo salar L., following experimental challenge. *Journal of Fish Diseases* **22**(2):125-131.

Teska, J. D., Cipriano, R. C. and Schill, W. B. (1992) Molecular and genetic characterization of cytochrome oxidase-negative *Aeromonas salmonicida* isolated from coho salmon (*Oncorhynchus kisutch*). *Journal of Wildlife Diseases* **28**(4):515-520.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**:4350-4354.

Trust, T.J., Khouri, A.G., Austen, R.A. and Ashburner, L.D. (1980) First isolation in Australia of

atypical *Aeromonas salmonicida*. *FEMS Microbiology Letters* **9**:39-42.

Whittington, R. J., Djordjevic, S. P., Carson, J. and Callinan, R. B. (1995) Restriction endonuclease analysis of atypical *Aeromonas salmonicida* isolates from goldfish *Carassius auratus*, silver perch *Bidyanus bidyanus*, and greenback flounder *Rhombosolea tapirina* in Australia. *Diseases of Aquatic Organisms* **22**:185-191.

Wiklund, T., Dalsgaard, I., Eerola, E. and Olivier, G. (1994) Characteristics of 'atypical', cytochrome oxidase-negative *Aeromonas salmonicida* isolated from ulcerated flounders (*Platichthys flesus* (L.)). *Journal of Applied Bacteriology* **76**(5):511-520.

Wiklund, T. and Dalsgaard, I. (1998) Occurrence and significance of atypical *Aeromonas salmonicida* in non- salmonid and salmonid fish species: a review. *Diseases of Aquatic Organisms* **32**(1):49-69.

SECTION 11 APPENDICES

Appendix 1: Biochemical test results and numerical taxonomic cluster assigned to *A. salmonicida* isolates.

SFC	Biochemical Test Results														Cluster
	Pigment	N_2 reduction	Arginine dihydrolase	Indole	Methyl red	Voges-Proskauer	Aesculin hydrolysis	Acid from arabinose	Acid from galactose	Gas from glucose	Acid from maltose	Acid from mannitol	Acid from sucrose	Acid from trehalose	
1	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
2	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
3	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
4	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
5	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
6	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
7	+	+	+	-	+	-	-	-	+	-	+	+	+	-	1
8	+	+	+	+	-	-	-	-	-	-	+	+	+	-	5
9	+	+	-	+	-	-	-	-	+	-	+	+	+	-	1
10	-	+	+	-	+	-	-	-	-	-	-	-	+	-	12
11	-	+	+	+	+	-	-	-	+	-	+	+	+	-	1
12	-	+	-	+	-	-	-	-	+	-	+	+	+	-	1
13	-	+	-	+	+	-	-	-	+	-	+	+	+	-	1
14	-	+	+	-	+	-	-	-	+	-	+	+	+	-	1
15	+	+	-	-	+	-	-	-	+	-	+	+	+	-	1
16	+	+	-	-	+	-	-	-	+	-	+	+	+	-	1
17	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
18	+	+	-	+	-	-	-	-	+	-	+	+	+	-	nd
19	-	+	-	+	+	-	-	-	-	-	+	+	+	-	5
20	-	+	-	-	+	-	-	-	+	-	+	+	+	-	1
21	-	+	-	+	+	-	-	-	+	-	+	+	+	-	1
22	-	+	-	-	+	-	-	-	-	-	+	+	+	-	5
23	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
24	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
25	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
26	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
27	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
28	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
29	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
30	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
31	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
32	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
33	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
34	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
35	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
36	+	+	+	-	+	-	+	+	-	+	+	+	-	+	21
97	-	-	+	nd	nd	nd	nd	-	+	-	+	-	+	+	3
105	+	+	-	+	+	-	-	+	+	-	+	+	+	-	1
106	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
120	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1

SFC	Biochemical Test Results														Cluster
	Pigment	N_2 reduction	Arginine dihydrolase	Indole	Methyl red	Voges-Proskauer	Aesculin hydrolysis	Acid from arabinose	Acid from galactose	Gas from glucose	Acid from maltose	Acid from mannitol	Acid from sucrose	Acid from trehalose	
121	-	+	+	+	+	-	-	-	+	-	+	+	+	+	2
122	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
123	-	+	+	+	+	+	+	+	+	-	+	+	+	+	25
153	+	+	-	-	+	-	+	+	+	+	+	+	+	+	24
154	+	+	-	-	+	-	+	+	+	+	+	+	-	+	20
155	+	+	-	-	+	-	+	+	+	+	+	+	-	+	20
156	-	+	-	-	+	-	+	+	+	+	+	+	-	+	20
157	-	+	-	-	+	-	+	+	+	+	+	+	-	-	19
158	-	+	-	-	+	-	+	+	+	+	+	+	-	+	20
159	+	+	-	-	+	-	+	+	+	+	+	+	-	+	20
160	+	+	-	-	+	-	+	+	+	+	+	+	-	+	20
161	+	+	-	+	+	-	-	-	+	-	+	+	+	+	2
162	+	+	-	+	+	-	+	-	+	-	+	+	+	+	2
163	+	+	-	+	+	-	+	-	+	-	+	+	+	+	2
164	+	+	-	+	+	-	+	-	+	-	+	+	+	+	2
165	+	+	-	-	+	-	+	+	+	+	+	+	+	+	24
166	+	+	+	-	+	-	+	+	+	+	+	+	+	+	24
167	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
168	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
169	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
170	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
171	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
172	+	+	+	-	+	-	+	+	+	+	+	+	-	+	nd
173	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
174	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
175	+	+	+	-	+	-	+	+	+	+	+	+	-	-	19
176	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
177	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
178	+	+	nd	-	+	-	+	+	+	+	+	+	-	+	20
179	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
180	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
186	-	+	+	-	+	-	+	-	+	-	+	+	+	-	1
187	+	+	-	-	+	-	+	-	+	-	+	+	+	+	2
188	-	+	+	-	+	-	-	-	+	-	+	+	+	+	2
189	-	+	-	-	+	-	+	-	+	-	+	+	+	+	2
190	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
191	+	+	+	+	+	-	+	-	+	-	+	+	+	+	2
192	+	+	+	+	+	-	+	-	+	-	+	+	+	+	2
193	+	+	-	+	+	-	+	-	+	+	+	+	+	+	27
194	-	+	+	+	+	-	-	-	+	-	+	+	+	-	1
195	-	+	+	+	+	-	-	-	+	-	+	+	+	-	1
196	-	+	+	+	+	-	-	-	+	-	+	+	+	-	1
197	-	+	+	+	+	-	-	-	+	-	+	+	+	-	1
198	-	+	+	+	+	-	-	-	+	-	+	+	+	-	1

SFC	Biochemical Test Results														Cluster
	Pigment	N_2 reduction	Arginine dihydrolase	Indole	Methyl red	Voges-Proskauer	Aesculin hydrolysis	Acid from arabinose	Acid from galactose	Gas from glucose	Acid from maltose	Acid from mannitol	Acid from sucrose	Acid from trehalose	
199	-	+	+	+	+	-	-	-	+	-	+	+	+	-	1
200	+	+	+	-	+	+	-	+	-	-	+	+	+	+	7
201	-	+	+	-	-	-	+	-	-	-	+	-	-	+	10
202	-	+	nd	-	-	-	-	-	+	-	+	+	+	+	18
203	-	+	+	-	+	-	+	-	-	-	+	+	+	+	6
204	-	+	+	-	+	-	-	-	+	-	-	-	+	-	13
205	-	+	+	-	+	-	+	+	+	+	+	+	-	+	20
206	+	+	+	-	+	+	-	-	+	-	+	+	+	+	2
207	+	+	+	+	+	+	+	-	+	-	+	+	+	+	2
208	+	+	+	+	+	+	-	-	+	-	+	+	+	+	2
209	+	+	+	-	+	+	-	-	+	-	+	+	+	+	2
210	+	+	+	-	+	+	-	-	+	-	+	+	+	+	2
211	-	+	-	+	-	-	-	-	+	-	+	+	+	+	2
212	+	+	+	-	+	+	-	-	+	-	+	+	+	+	2
213	-	+	+	-	+	-	-	-	+	-	+	+	+	-	1
214	+	+	+	-	+	-	-	-	+	-	+	+	+	+	nd
215	-	+	+	-	-	-	+	-	-	+	+	-	-	-	11
216	+	+	-	+	+	-	-	-	+	-	+	+	+	-	1
217	+	+	-	-	+	-	-	-	+	-	+	+	+	+	2
218	+	+	-	-	+	-	-	-	+	-	+	+	+	+	2
219	+	+	-	-	-	-	-	-	+	-	+	+	+	+	2
220	+	+	-	-	+	-	-	-	+	-	+	+	+	+	2
221	+	+	-	-	+	-	-	-	+	-	+	+	+	+	2
222	+	+	-	-	+	-	-	-	+	-	+	+	+	+	2
223	+	+	-	-	+	-	-	-	+	-	+	+	+	+	2
224	+	+	-	-	+	-	-	-	+	-	+	+	+	+	2
225	+	+	+	+	+	-	-	-	+	-	+	+	+	+	2
226	+	+	+	+	+	-	+	-	+	-	+	+	+	+	2
227	+	+	+	+	+	-	-	+	+	-	+	+	+	+	25
228	+	+	+	+	+	-	-	-	-	-	+	+	+	+	6
229	+	+	+	+	+	-	-	+	+	-	+	+	+	+	25
230	+	+	+	+	+	-	-	+	+	-	+	+	+	+	25
231	+	+	+	+	+	-	-	-	+	-	+	+	+	+	2
232	+	+	+	-	+	-	-	+	+	-	+	+	+	+	26
233	+	+	+	+	+	-	-	+	-	-	+	+	+	+	7
234	+	+	+	+	+	-	-	+	+	-	+	+	+	+	25
235	+	+	+	+	+	-	-	+	+	-	+	+	+	+	25
236	+	+	+	+	+	-	-	+	+	-	+	+	+	+	25
237	+	+	+	+	+	-	-	+	+	-	+	+	+	+	25
238	+	+	+	+	+	-	-	-	+	-	+	+	+	+	2
239	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
240	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
241	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
242	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20

SFC	Biochemical Test Results														Cluster
	Pigment	N_2 reduction	Arginine dihydrolase	Indole	Methyl red	Voges-Proskauer	Aesculin hydrolysis	Acid from arabinose	Acid from galactose	Gas from glucose	Acid from maltose	Acid from mannitol	Acid from sucrose	Acid from trehalose	
243	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
244	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
245	+	+	+	+	+	-	+	-	+	-	+	+	+	+	2
246	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
247	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
248	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
249	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
250	+	+	+	-	+	-	+	+	+	+	+	+	-	-	19
251	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
252	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
253	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
254	+	+	+	-	+	-	+	+	+	+	+	+	+	+	24
255	+	+	+	-	+	-	+	+	+	+	+	+	+	+	24
256	+	+	+	+	+	-	+	-	+	-	+	+	+	+	2
258	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
260	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
261	+	+	+	-	+	-	+	+	+	+	+	+	+	+	24
262	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
263	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
264	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
265	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
266	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
267	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
268	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
269	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
270	+	+	+	-	+	-	+	+	+	+	+	+	-	-	19
271	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
272	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
273	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
274	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
275	+	+	+	-	+	-	+	+	+	+	+	+	-	-	19
276	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
277	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
278	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
279	+	+	+	-	+	-	+	+	+	+	+	-	-	+	20
280	+	+	+	-	+	-	+	+	+	+	+	-	-	-	22
281	+	+	+	-	+	-	+	+	+	+	+	+	-	-	19
282	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
283	+	+	+	-	+	-	+	+	+	+	+	-	-	+	22
284	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
285	+	+	+	-	+	-	+	+	+	+	+	+	-	-	19
286	+	+	+	-	-	-	+	+	+	+	+	+	-	+	20
287	+	+	+	-	+	-	+	+	+	+	+	+	-	-	19
288	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20

SFC	Biochemical Test Results														Cluster
	Pigment	N_2 reduction	Arginine dihydrolase	Indole	Methyl red	Voges-Proskauer	Aesculin hydrolysis	Acid from arabinose	Acid from galactose	Gas from glucose	Acid from maltose	Acid from mannitol	Acid from sucrose	Acid from trehalose	
289	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
290	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
291	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
292	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
293	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
294	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
296	-	+	+	-	+	-	-	-	-	-	-	-	-	-	14
298	-	+	+	-	+	-	+	-	+	-	+	-	+	-	4
299	-	+	-	-	+	+	+	-	-	-	+	-	-	-	9
300	-	+	+	-	+	-	-	-	-	-	+	-	+	+	15
301	-	+	+	-	+	-	+	-	+	+	+	+	-	+	28
302	-	+	+	-	+	-	+	-	+	+	+	+	-	+	28
303	-	+	+	-	+	-	-	-	-	-	+	+	+	+	6
305	+	+	+	+	+	-	-	-	+	+	+	-	+	+	30
306	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
307	+	+	+	+	+	-	-	-	+	-	+	+	+	+	2
326	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
327	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
328	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
362	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
363	+	+	+	+	+	-	-	-	-	-	+	+	+	+	6
364	-	+	+	-	+	-	-	-	-	-	+	+	+	-	5
365	-	+	+	+	+	+	+	+	+	+	+	+	+	+	24
366	-	-	+	-	-	-	-	-	-	-	+	-	+	-	16
469	-	+	-	-	nd	nd	+	-	-	-	+	-	-	+	10
470	-	-	-	-	+	-	-	-	-	-	+	-	+	+	15
471	-	-	+	nd	nd	nd	+	-	-	-	-	-	+	+	17
472	-	+	-	nd	nd	nd	-	-	-	-	-	-	+	+	17
473	-	+	-	-	nd	nd	+	-	+	-	-	-	+	-	13
474	-	+	-	nd	nd	nd	-	-	+	-	-	-	+	-	13
475	+	+	+	+	+	-	-	-	+	-	+	+	+	+	2
476	+	+	+	+	+	-	-	-	+	-	+	+	+	+	2
477	+	+	+	+	+	-	-	-	+	-	+	+	+	+	2
478	+	+	+	+	+	-	-	-	+	-	+	+	+	+	2
479	+	+	+	+	+	-	-	-	+	-	+	+	+	-	1
480	-	+	+	-	+	-	-	-	+	-	+	-	+	-	4
481	-	+	+	+	+	-	-	-	+	-	+	+	+	-	1
483	-	+	nd	-	nd	nd	-	-	-	-	-	-	+	-	12
484	+	+	+	-	+	-	-	-	+	-	+	+	+	+	2
485	+	+	+	+	+	-	-	-	+	-	+	+	+	+	2
486	-	+	+	-	+	-	-	-	+	-	+	+	-	+	29
487	+	nd	+	-	+	-	+	+	+	+	+	+	-	+	20
488	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
489	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20

SFC	Biochemical Test Results														Cluster
	Pigment	N_2 reduction	Arginine dihydrolase	Indole	Methyl red	Voges-Proskauer	Aesculin hydrolysis	Acid from arabinose	Acid from galactose	Gas from glucose	Acid from maltose	Acid from mannitol	Acid from sucrose	Acid from trehalose	
490	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
491	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
492	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
493	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
494	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
495	-	+	-	-	+	-	-	-	-	-	+	-	-	-	9
496	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
497	+	+	+	-	+	-	+	+	+	+	+	+	-	-	19
498	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
499	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
500	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
501	+	+	+	-	+	-	+	+	+	+	+	+	+	+	24
502	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
503	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
504	-	+	+	+	+	-	-	-	+	-	+	+	+	-	1
505	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
506	+	+	+	-	+	-	-	-	+	-	+	+	+	+	2
507	+	nd	+	+	+	-	-	-	+	-	+	+	+	-	1
508	+	+	+	-	+	-	-	-	+	-	+	+	+	-	1
509	+	+	+	-	+	-	+	+	+	+	+	+	-	-	19
510	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
511	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
512	+	+	+	-	+	-	+	+	+	+	+	+	-	+	20
513	+	+	+	-	+	-	+	+	+	-	+	+	+	+	25
702	-	+	nd	+	+	+	+	+	+	+	+	+	+	+	24

Appendix 2 Results of PCR *in vitro* testing

SFC	AP	PAAS	MIY	Comments	Cluster
1	+	+	-	GUD, Australia	1
2	+	+	-	GUD, Australia	1
3	+	+	-	GUD, Australia	1
4	+	+	-	GUD, Australia	1
5	+	+	-	GUD, Australia	1
6	+	+	-	GUD, Australia	1
7	-	+	-	GUD, Australia	1
8	-	+	-	GUD, Australia	5
9	+	+	-	GUD, Australia	1
10	+	+	-	GUD, USA	12
11	+	+	-	GUD, Italy	1
12	+	+	-	GUD, USA	1
13	+	+	-	GUD, Japan	1
14	+	+	-	GUD, USA	1
15	+	+	-	GUD, USA	1
16	-	+	-	GUD, USA	1
17	+	+	-	GUD, Australia	1
18	+	+	-	GUD, Italy	5
19	+	+	-	GUD, England	5
20	+	-	-	GUD, Sweden	1
21	+	-	-	GUD, England	1
22	+	-	-	GUD, England	5
23	+	+	-	GUD, Australia	1
24	+	+	-	GUD, Australia	1
25	+	+	-	GUD, Australia	1
26	+	+	-	GUD, Australia	1
27	+	+	-	GUD, Australia	1
28	+	+	-	GUD, Australia	1
29	+	+	-	GUD, Australia	1
30	+	+	-	GUD, Australia	1
31	+	+	-	GUD, Australia	1
32	+	+	-	GUD, Australia	1
33	+	+	-	GUD, Australia	1
34	+	+	-	GUD, Australia	1
35	+	+	-	GUD, Australia	1
36	-	+	+	*A. salmonicida* subsp. *salmonicida*	21
97	+	+	-	*A. salmonicida* subsp. *achromogenes* (formerly *H. piscium*)	3
105	+	+	-	GUD, Australia	1
106	+	+	-	GUD, Australia	1
120	+	+	-	GUD, Australia	1
121	+	+	-	*A. salmonicida* subsp. *achromogenes*	2
122	+	+	+	*A. salmonicida* subsp. *salmonicida*	20
123	+	-	-	*A. salmonicida* subsp. *masoucida*	25
153	+	+	+	typical isolate	24
154	+	+	+	typical isolate	20
155	+	+	+	typical isolate	20
156	+	+	+	typical isolate	20
157	+	+	+	typical isolate	19
158	+	+	+	typical isolate	20
159	+	+	+	typical isolate	20
160	+	+	+	typical isolate	20
161	+	+	-	atypical isolate	2
162	+	+	-	atypical isolate	2
163	+	+	-	atypical isolate	2
164	+	-	-	atypical isolate	2
165	+	+	-		24
166	+	+	+		24
167	+	+	+		20
168	+	-	+	typical isolate	20

SFC	AP	PAAS	MIY	Comments	Cluster
169	+	+	+	typical isolate	20
170	+	-	+	typical isolate	20
171	-	+	+		20
172	+	+	+		20
173	+	+	+		20
174	+	+	+		20
175	+	+	+		19
176	-	+	+		20
177	+	+	+		20
178	+	+	+		20
179	-	+	+		20
180	+	+	+		20
186	+	+	-	atypical isolate	1
187	-	-	-	atypical isolate	2
188	+	-	-	atypical isolate	2
189	+	+	-	atypical isolate	2
190	+	+	+	*A. salmonicida* subsp. *salmonicida*	20
191	-	-	-	atypical isolate	2
192	+	-	-	atypical isolate	2
193	+	+	-	atypical isolate	27
194	+	+	-	indole +	1
195	+	+	-	indole+	1
196	+	+	-	indole+	1
197	+	-	-	indole+	1
198	+	-	-	indole+	1
199	+	+	-	indole+	1
200	+	+	-		7
201	+	+	-	oxidase -ve	10
203	+	+	-	oxidase -ve	6
204	+	+	-		13
205	+	+	+	*A. salmonicida* subsp. *salmonicida* (non-pigmented)	20
206	+	+	-		2
207	+	+	-	indole +	2
208	+	+	-	indole +	2
209	+	+	-		2
210	+	+	-		2
211	-	+	-	indole +	2
212	+	+	-		2
213	+	-	-		1
214	+	+	-		1
215	+	+	-		11
216	+	+	-	indole +	1
217	+	+	-		2
218	+	+	-		2
219	+	+	-		2
220	+	+	-		2
221	+	+	-		2
222	+	-	-		2
223	+	+	-		2
224	+	+	-		2
225	+	+	-	indole w+	2
226	+	-	-	atypical isolate (indole w+)	2
227	+	+	-	indole w+	25
228	+	+	-	indole w+	6
229	+	-	-	indole w+	25
230	+	+	-	indole w+	25
231	+	+	-	indole w+	2
232	+	+	-		26
233	+	+	-	indole w+	7
234	+	+	-	indole w+	25
235	+	+	-	indole w+	25

SFC	AP	PAAS	MIY	Comments	Cluster
236	+	+	-	indole w+	25
237	+	+	-	indole w+	25
238	+	+	-	indole w+	2
239	+	+	+		20
240	+	+	+		20
241	+	+	+		20
242	+	+	+		20
243	+	+	+		20
244	+	+	+		20
245	+	+	-	indole +	2
246	+	+	+		20
247	+	+	+		20
248	-	+	+		20
249	-	+	+		20
250	+	+	+		19
251	+	+	+		20
252	+	+	+		20
253	+	+	+		20
254	+	+	+		24
255	+	+	+		24
256	+	+	-	indole +	2
258	+	+	+		20
260	+	+	+		20
261	+	+	+		24
262	+	+	+		20
263	+	+	+		20
264	+	+	+		20
265	+	+	+		20
266	+	+	+		20
267	+	+	+		20
268	+	+	-		20
269	+	+	+		20
270	+	+	+		19
271	+	+	+		20
272	+	+	+		20
273	+	+	+		20
274	+	+	+		20
275	+	+	+		19
276	+	+	+		20
277	+	+	+		20
278	+	+	+		20
279	+	+	+		20
280	+	+	+		22
281	+	+	+		19
282	+	+	+		20
283	+	+	+		22
284	+	+	+		20
285	+	+	+		19
286	+	+	+		20
287	+	+	+		19
288	+	+	+		20
289	+	+	+		20
290	+	+	+	*A. salmonicida* subsp. *salmonicida*	20
291	+	+	+	*A. salmonicida* subsp. *salmonicida*	20
292	+	+	+	*A. salmonicida* subsp. *salmonicida*	20
293	+	+	+	*A. salmonicida* subsp. *salmonicida*	20
294	+	+	+	*A. salmonicida* subsp. *salmonicida*	20
296	+	+	-	*A. salmonicida* subsp. *nova*	14
298	+	+	-	*A. salmonicida* subsp. *nova*	4
299	-	+	-	atypical isolate	9
300	+	+	-	atypical isolate	15
301	-	+	-	atypical isolate (motile)	28

SFC	AP	PAAS	MIY	Comments	Cluster
302	+	+	-	atypical isolate (motile)	28
303	+	+	-		6
305	+	+	-	atypical isolate (indole +)	30
306	+	+	+		20
307	+	+	-	atypical isolate (indole +)	2
308	-	+	-	greenback flounder isolate	nd
309	+	+	-	greenback flounder isolate	nd
310	+	+	-	greenback flounder isolate	nd
311	+	+	-	greenback flounder isolate	nd
312	+	+	-	greenback flounder isolate	nd
313	+	+	-	greenback flounder isolate	nd
314	+	+	-	greenback flounder isolate	nd
315	+	+	-	greenback flounder isolate	nd
316	+	+	-	greenback flounder isolate	nd
317	-	+	-	greenback flounder isolate	nd
318	+	+	-	greenback flounder isolate	nd
319	+	+	-	greenback flounder isolate	nd
320	+	+	-	greenback flounder isolate	nd
321	+	+	-	greenback flounder isolate	nd
322	+	+	-	stripey trumpeter isolate	nd
323	+	+	-	stripey trumpeter isolate	nd
324	+	+	-	greenback flounder isolate	nd
325	+	+	-	stripey trumpeter isolate	nd
326	+	+	-	GUD, Australia	1
327	+	+	-	GUD, Australia	1
328	+	+	-	GUD, Australia	1
345	+	+	+	large colony variant SFC 246	nd
346	-	+	+	large colony variant SFC 250	nd
347	+	+	+	large colony variant SFC 254	nd
349	+	+	+	large colony variant SFC 239	nd
362	-	+	+	*A. salmonicida* subsp. *salmonicida*	20
363	+	+	-	*A. salmonicida subsp.achromogenes*	6
364	+	+	-	*A. salmonicida subsp.achromogenes*	5
365	+	+	-	*A. salmonicida subsp.masoucida*	24
366	+	+	-	*A. salmonicida subsp.smithia*	16
469	+	+	-	atypical isolate (oxidase -ve)	10
470	+	+	-		15
471	+	+	-		17
472	+	+	-		17
473	-	+	-		13
474	+	+	-		13
475	+	+	-	indole +	2
476	+	+	-	indole +	2
477	+	+	-	indole +	2
478	+	+	-	indole +	2
479	+	+	-	indole +	1
480	+	-	-		4
481	+	+	-	indole +	1
483	-	+	-		12
484	+	-	-		2
485	+	+	-	indole +	2
486	+	+	-		29
487	+	+	+		20
488	+	+	+		20
489	+	+	+		20
490	+	+	+		20
491	+	-	+		20
492	+	+	+		20
493	+	+	+	typical isolate	20
494	+	+	+		20
496	+	+	+		20
497	+	+	-		19

SFC	AP	PAAS	MIY	Comments	Cluster
498	-	+	-		20
499	+	+	+		20
500	+	+	+		20
501	+	+	+		24
502	+	+	+		20
503	+	+	+		20
504	+	+	-	atypical isolate (indole +)	1
505	-	+	+		20
506	+	+	-	atypical isolate	2
507	+	+	-	atypical isolate (indole +)	1
508	+	+	-	atypical isolate	1
509	+	+	+		19
510	+	+	+		20
511	+	+	+		20
512	+	+	+		25
513	+	+	+		nd
724	+	+	-	subm as *salmonicida*, pigmented; MIY -ve	nd
725	+	-	-	subm as *salmonicida*, + by Qiaprep, pigm; MIY -ve	nd
726	+	+	+	*A. salmonicida* subsp. *salmonicida*	nd
727	+	+	+	*A. salmonicida* subsp. *salmonicida*	nd
728	+	-	-	*A.salmonicida* subsp. *masoucida*	nd
729	+	+	-	*A.salmonicida* subsp. *achromogenes*	nd
730	+	+	-	*A.salmonicida* subsp. *achromogenes*	nd
731	+	+	-	*A.salmonicida* subsp. *achromogenes*	nd
732	+	+	-	*A.salmonicida* subsp. *achromogenes*	nd
733	+	+	-	*A.salmonicida* subsp. *smithia*	nd
734	+	+	-	*A.salmonicida* subsp. *smithia*	nd
735	+	+	-	atypical isolate	nd
736	+	+	-	atypical isolate	nd
737	+	+	-	atypical isolate	nd
738	+	+	-	atypical isolate	nd
739	+	+	-	atypical isolate	nd
740	+	+	-	atypical isolate	nd
741	+	+	-	atypical isolate	nd
742	+	+	-	atypical isolate	nd
743	+	+	-	typical isolate	nd
744	+	+	-	typical isolate	nd
745	+	+	-	atypical isolate	nd
746	+	+	-	atypical isolate	nd
747	+	+	-	typical isolate	nd
748	+	+	-	atypical isolate	nd
749	+	+	-	atypical isolate	nd
750	+	+	-	typical isolate	nd
751	+	+	-	atypical isolate	nd
752	+	-	-	*A.salmonicida* subsp. *masoucida*	nd
753	+	-	-		nd
754	+	+	-		nd
758	+	+	+		nd
759	+	+	+		nd
760	+	+	+		nd
761	+	+	+		nd
763	+	+	+		nd
764	+	+	+		nd
765	+	+	+		nd
766	+	+	+		nd
767	+	+	+		nd
768	+	+	+		nd
769	+	+	+		nd
770	+	+	+	oxidase -ve	nd
771	+	+	+		nd
772	+	+	+		nd

SFC	AP	PAAS	MIY	Comments	Cluster
773	+	-	+		nd
774	+	+	+		nd
775	+	+	+		nd

Appendix 3

Table 1 Enumeration and identification of bacterial flora from the mucus of 100 covertly infected Atlantic salmon held in Raceway 3 at the RCNSS, Massachussets, USA.

Fish #	Total cfu/g	A.sal cfu/g	eg of species
1	3.9E+03	0	*Aeromonas hydrophila*
2	5.3E+02	0	*Aeromonas hydrophila*
3	8.3E+03	0	*Aeromonas hydrophila*
4	9.0E+04	0	*Aeromonas hydrophila*
5	0	0	
6	8.2E+03	0	*Aeromonas hydrophila*
7	7.2E+03	0	*Aeromonas hydrophila*
8	1.0E+04	0	*Aeromonas hydrophila*
9	0.0E+00	0	
10	1.1E+04	0	*Aeromonas hydrophila*
11	3.1E+03	0	*Aeromonas hydrophila, Staphylococcus* sp.
12	2.5E+03	0	*Shewanella putrefasciens, Moraxella* sp.
13	0	0	
14	1.5E+03	0	*Pseudomonas fluorescens, Aeromonas hydrophila*
15	2.5E+03	0	*Aeromonas hydrophila*
16	3.3E+03	0	*Aeromonas hydrophila*
17	0	0	
18	2.0E+04	2.0E+03	*Aeromonas hydrophila, Aeromonas salmonicida*
19	2.1E+03	0	*Aeromonas hydrophila*
20	1.3E+03	0	*Pseudomonas fluorescens, Aeromonas hydrophila*
21	1.7E+05	0	*Aeromonas hydrophila*
22	2.5E+04	0	*Aeromonas hydrophila, Shewanella putrefasciens*
23	7.7E+03	7.7E+03	*Aeromonas salmonicida*
24	8.8E+03	0	*Aeromonas hydrophila*
25	3.8E+04	0	*Comamonas terrigenia, Aeromonas hydrophila*
26	2.9E+04	0	*Aeromonas hydrophila*
27	3.8E+04	0	*Aeromonas hydrophila, Pseudomonas fluorescens*
28	2.0E+04	0	*Aeromonas hydrophila*
29	7.1E+03	0	*Aeromonas hydrophila*
30	4.0E+04	0	*Aeromonas hydrophila, Shewanella putrefasciens*
31	4.0E+03	0	*Aeromonas hydrophila*
32	1.2E+03	0	*Aeromonas hydrophila*
33	2.0E+04	0	*Aeromonas hydrophila*
34	5.7E+03	0	*Aeromonas hydrophila*
35	3.0E+03	0	*Aeromonas hydrophila, Pseudomonas fluorescens*
36	8.8E+03	0	*Aeromonas hydrophila*
37	1.4E+04	0	*Aeromonas hydrophila*
38	5.7E+04	0	*Aeromonas hydrophila*
39	1.1E+03	0	*Aeromonas hydrophila*
40	6.0E+03	0	*Aeromonas hydrophila*
41	3.6E+03	0	*Aeromonas hydrophila*
42	3.0E+04	0	*Aeromonas hydrophila*
43	5.0E+03	0	**Aeromonas hydrophila**
44	2.5E+03	0	*Aeromonas hydrophila*
45	5.0E+03	0	*Aeromonas hydrophila*
46	1.4E+04	0	*Aeromonas hydrophila*
47	1.7E+03	0	*Pseudomonas fluorescens*
48	0	0	
49	4.0E+03	0	*Aeromonas hydrophila*
50	1.0E+04	0	*Aeromonas hydrophila*
51	1.7E+03	0	*Aeromonas hydrophila*
52	0	0	
53	3.0E+03	0	*Moraxella* sp., *Pseudomonas fluorescens*
54	2.3E+03	0	*Aeromonas hydrophila*

Fish #	Total cfu/g	A.sal cfu/g	eg of species
55	0	0	
56	7.5E+03	8.3E+02	*Aeromonas salmonicida, Aeromonas hydrophila*
57	2.0E+04	0	*Aeromonas hydrophila*
58	5.9E+02	0	*Aeromonas hydrophila*
59	0	0	
60	3.8E+03	0	*Shewanella putrefasciens*
61	0	0	
62	4.0E+03	0	*Pseudomonas fluorescens*
63	5.0E+03	5.0E+03	*Aeromonas salmonicida*
64	1.2E+03	0	*Aeromonas hydrophila*
65	3.3E+03	0	*Aeromonas hydrophila*
66	2.2E+03	1.1E+03	*Aeromonas hydrophila, Aeromonas salmonicida*
67	1.0E+04	0	*Aeromonas hydrophila*
68	2.2E+03	0	*Aeromonas hydrophila*
69	5.0E+03	0	*Aeromonas hydrophila*
70	2.5E+03	0	*Aeromonas hydrophila*
71	7.5E+03	0	*Aeromonas hydrophila*
72	4.0E+06	0	*Moraxella* sp.
73	0	0	
74	5.0E+03	0	*Shewanella putrefasciens*
75	5.0E+03	0	*Aeromonas hydrophila*
76	0	0	
77	8.3E+03	0	*Pseudomonas fluorescens*
78	4.0E+03	1.0E+03	*Aeromonas hydrophila, Aeromonas salmonicida*
79	3.3E+03	0	*Pseudomonas diminuta*
80	0	0	
81	5.0E+03	0	*Aeromonas hydrophila*
82	3.1E+03	0	*Pseudomonas fluorescens, Aeromonas hydrophila*
83	5.9E+02	0	*Aeromonas hydrophila*
84	3.3E+03	0	*Aeromonas hydrophila*
85	1.7E+03	0	*Pseudomonas pseudoalcaligenes*
86	2.9E+03	0	*Staphylococcus* sp.
87	1.0E+04	0	*Shewanella putrefasciens, Aeromonas hydrophila*
88	0	0	
89	4.4E+03	0	*Aeromonas hydrophila*
90	6.7E+03	0	*Pseudomonas fluorescens*
91	8.3E+03	0	*Aeromonas hydrophila*
92	7.5E+03	0	*Aeromonas hydrophila*
93	1.6E+04	0	*Pseudomonas fluorescens*
94	2.0E+03	0	*Aeromonas hydrophila*
95	3.3E+03	0	*Aeromonas hydrophila*
96	7.7E+03	0	*Aeromonas hydrophila*
97	2.8E+04	2.5E+03	*Pseudomonas fluorescens, Aeromonas salmonicida*
98	0	0	
99	1.1E+03	0	**Aeromonas hydrophila**
100	2.9E+04	1.4E+04	*Aeromonas salmonicida, Aeromonas hydrophila*

Table 2 Range of bacteria isolated from lethally-sampled covertly infected salmonids

Location	Salmonid species	Tissue	Fish #	Bacterial spp.
RCNSS, Raceway 5	Atlantic salmon	Gill	7	*Pseudomonas fluorescens*
			8	*Pseudomonas fluorescens*
			9	*Pseudomonas fluorescens*
			12	*Moraxella* sp.
			15	*Pseudomonas fluorescens*
			16	*Pseudomonas fluorescens*
			17	*Pseudomonas fluorescens*
			19	*Aeromonas hydrophila*
		Intestine	1	*Acinetobacter* sp.
			3	*Shewanella putrefasciens*
			4	*Shewanella putrefasciens*
			7	*Shewanella putrefasciens*
			9	*Shewanella putrefasciens*
			10	*Shewanella putrefasciens*
			11	*Aeromonas hydrophila*
			12	*Enterobacter* sp.
			13	*Pseudomonas fluorescens*
			15	*Shewanella putrefasciens*
			16	*Shewanella putrefasciens*
			17	*Shewanella putrefasciens*
			18	*Shewanella putrefasciens*
			19	*Aeromonas hydrophila*
			20	*Shewanella putrefasciens*
RCNSS, Raceway 6	Atlantic salmon	Gill	5	*Aeromonas hydrophila, Pseudomonas fluorescens*
			7	*Pseudomonas fluorescens*
			16	*Staphylococcus* sp.
			18	*Pseudomonas fluorescens*
		Intestine	1	*Aeromonas hydrophila*
			2	*Shewanella putrefasciens*
			5	*Shewanella putrefasciens*
			6	*Staphylococcus* sp.
			7	*Shewanella putrefasciens*
			8	*Shewanella putrefasciens*
			10	*Aeromonas hydrophila*
			11	*Shewanella putrefasciens*
			12	*Shewanella putrefasciens*
			13	*Aeromonas hydrophila*
			14	*Shewanella putrefasciens*
			15	*Pseudomonas fluorescens*
			17	*Shewanella putrefasciens*
			18	*Pseudomonas fluorescens*
			19	*Shewanella putrefasciens*
Bennington	Brown trout	Mucus	1	*Pseudomonas fluorescens, Aeromonas hydrophila*
			2	*Pseudomonas fluorescens*
			3	*Moraxella* sp.
			4	*P. fluorescens, A. hydrophila, Moraxella* sp.
			5	*Moraxella* sp.
			6	*Staphylococcus sp., Comamonas terrigenia*
			7	*Pseudomonas fluorescens, Moraxella* sp.
			8	*Moraxella* sp.
			9	*Pseudomonas fluorescens, Moraxella* sp.
			10	*Pseudomonas fluorescens*
			12	*Pseudomonas fluorescens, Moraxella* sp.
			13	*Pseudomonas fluorescens*
			14	*Pseudomonas diminuta, Staphylococcus* sp.
			15	*Staphylococcus* sp., *Moraxella* sp.
			16	*Acinetobacter* sp., *Moraxella* sp.

Location	Salmonid species	Tissue	Fish #	Bacterial spp.
			17	*Pseudomonas fluorescens*
			19	*Staphylococcus* sp., *Moraxella* sp.
			20	*Pseudomonas diminuta, Staphylococcus* sp.
		Gill	8	*Pseudomonas fluorescens, Moraxella* sp.
			10	*Pseudomonas fluorescens*
			11	*Pseudomonas fluorescens*
			14	*Pseudomonas fluorescens*
			17	*Moraxella* sp.
			20	*Pseudomonas fluorescens, Moraxella* sp.
		Spleen	17	*Moraxella* sp., *Pseudomonas pseudoalcaligines*
			20	*Moraxella* sp.
		Intestine	1	*Enterobacter* sp.
			7	*Shewanella putrefasciens*
			8	*Pseudomonas fluorescens*
			10	*Pseudomonas diminuta*
			12	*enteric bacterium*
			19	*Aeromonas hydrophila*
			20	*Aeromonas hydrophila*
Leetown (pre SIF)	Rainbow trout	Mucus	1	*Pseudomonas fluorescens*
			2	*Pseudomonas fluorescens*
			3	*Comamonas terrigenia*
			4	*Moraxella* sp., *Aeromonas hydrophila*
			5	*Aeromonas salmonicida, Acinetobacter* sp.
			6	*Comamonas terrigenia, Aeromonas hydrophila*
			7	*Comamonas terrigenia*
			8	*Moraxella* sp.
			9	*Moraxella* sp.
			10	*Aeromonas hydrophila, Staphylococcus* sp.
			11	*Pseudomonas alcaligenes*
			12	*Comamonas terrigenia, Staphylococcus* sp.
			13	*Comamonas terrigenia*
			14	*Comamonas terrigenia, Moraxella* sp.
			15	*Comamonas terrigenia, Moraxella* sp.
			16	*Comamonas terrigenia, Moraxella* sp.
			17	*Moraxella* sp., *Pseudomonas alcaligenes*
			18	*Pseudomonas fluorescens, Moraxella* sp.
			19	*Aeromonas salmonicida*
			20	*Aeromonas salmonicida, P. diminuta*
		Gill	1	*Aeromonas hydrophila*
			2	*Aeromonas hydrophila*
			4	*Aeromonas hydrophila*
			5	*Aeromonas salmonicida*
			6	*Staphylococcus* sp.
			7	*Aeromonas hydrophila*
			8	*Staphylococcus* sp.
			9	*Aeromonas hydrophila*
			10	*Staphylococcus* sp.
			11	*Pseudomonas fluorescens*
			12	*Staphylococcus* sp.
			13	*Acinetobacter* sp.
			14	*Staphylococcus* sp.
			15	*Staphylococcus* sp., *Acinetobacter* sp.
			16	*Staphylococcus* sp., *Aeromonas hydrophila*
			17	*Staphylococcus* sp., *Aeromonas hydrophila*
			18	*Aeromonas hydrophila*
			19	*Aeromonas salmonicida*
			20	*Comamonas terrigenia*
		Spleen	5	*Aeromonas salmonicida*
			18	*Aeromonas salmonicida*
		Kidney	5	*Aeromonas salmonicida*

Location	Salmonid species	Tissue	Fish #	Bacterial spp.
			18	*Aeromonas salmonicida*
		Intestine	1	*Aeromonas hydrophila*
			2	*Aeromonas hydrophila*
			3	*Aeromonas hydrophila*
			4	*Enterobacter* sp.
			5	*Aeromonas salmonicida*
			18	*Aeromonas salmonicida*
			20	*Enterobacter* sp.
Leetown (pre SIF)	Atlantic salmon (non vaccinated)	Mucus	1	*A. hydrophila, C. terrigenia, Acinetobacter denitrificans*
			2	*Aeromonas hydrophila*
			3	*Aeromonas hydrophila*
			4	*Acinetobacter denitrificans*
			5	*Aeromonas salmonicida, Aeromonas hydrophila*
			6	*Moraxella* sp., *Aeromonas hydrophila*
			7	*Aeromonas salmonicida, Aeromonas hydrophila*
			8	*Aeromonas hydrophila*
			9	*Aeromonas hydrophila*
			10	*Moraxella* sp.
			11	*Staphylococcus* sp., *Moraxella* sp.
			12	*Aeromonas hydrophila, Acinetobacter denitrificans*
			13	*Comomonas terrigenia, Moraxella* sp.
			14	*Moraxella* sp.
			15	*Comomonas terrigenia, Moraxella* sp.
			16	*Aeromonas hydrophila, Moraxella* sp.
			17	*Staphylococcus* sp.
			18	*Staphylococcus* sp.
			19	*Aeromonas salmonicida, Aeromonas hydrophila*
			20	*Aeromonas salmonicida, Aeromonas hydrophila*
		Gill	1	*Aeromonas salmonicida*
			2	*Moraxella* sp.
			3	*Comamonas terrigenia*
			4	*Moraxella* sp.
			6	*Moraxella* sp., *Aeromonas hydrophila*
			7	*Comamonas terrigenia*
			8	*Aeromonas salmonicida, Aeromonas hydrophila*
			9	*Aeromonas salmonicida, Aeromonas hydrophila*
			10	*Aeromonas hydrophila*
			11	*Aeromonas salmonicida, Moraxella* sp.
			13	*Aeromonas hydrophila*
			14	*Comamonas terrigenia, Moraxella* sp.
			15	*Aeromonas salmonicida, Aeromonas hydrophila*
			16	*Comamonas terrigenia*
			17	*Aeromonas salmonicida, Aeromonas hydrophila*
			18	*Aeromonas salmonicida, Aeromonas hydrophila*
			19	*Pseudomonas fluorescens, Comamonas terrigenia*
			20	*Aeromonas salmonicida, Aeromonas hydrophila*
		Spleen	4	*Aeromonas hydrophila*
			8	*Aeromonas hydrophila*
			9	*Staphylococcus* sp., *Pseudomonas diminuta*
			13	*Comamonas terrigenia*
		Kidney	5	*Aeromonas salmonicida*
			6	*Aeromonas hydrophila*
			7	*Aeromonas hydrophila*
			12	*Comamonas terrigenia*
			13	*Aeromonas hydrophila*
			14	*Aeromonas hydrophila*
			15	*Pseudomonas diminuta*
			16	*Comamonas terrigenia*
			17	*Aeromonas hydrophila*
			18	*Aeromonas hydrophila*

Location	Salmonid species	Tissue	Fish #	Bacterial spp.
			20	*Aeromonas hydrophila*
		Intestine	1	*Aeromonas hydrophila*
			2	*Aeromonas hydrophila*
			3	*Aeromonas hydrophila*
			4	*Aeromonas hydrophila*
			5	*Aeromonas hydrophila*
			6	*Aeromonas hydrophila*
			7	*Aeromonas hydrophila*
			8	*Aeromonas hydrophila*
			10	*Aeromonas hydrophila*
			11	*Aeromonas hydrophila*
			12	*Staphylococcus* sp.
			13	*Aeromonas hydrophila*
			14	*Aeromonas hydrophila, Staphylococcus* sp.
			15	*Aeromonas hydrophila*
			16	*Aeromonas hydrophila*
			17	*Aeromonas hydrophila, Acinetobacter* sp.
			18	*Staphylococcus* sp.
			19	*Aeromonas hydrophila, Enterobacter* sp.
			20	*Acinetobacter* sp.

Appendix 4

Table 1 Enumeration of bacterial flora from the mucus of covertly infected Atlantic salmon (2+ surplus domestic stock) held in Raceway 5 at the RCNSS, Massachussets, USA.

Fish #	Mucus		Gill		Spleen		Kidney		Intestine	
	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g
1	1.0E+05	0	0	0	0	0	0	0	0	0
2	1.7E+04	0	0	0	0	0	0	0	5.3E+05	0
3	1.8E+05	0	0	0	0	0	0	0	1.1E+08	0
4	1.0E+05	0	0	0	0	0	0	0	2.9E+03	0
5	2.5E+05	0	0	0	0	0	0	0	0	0
6	8.0E+04	0	0	0	0	0	0	0	0	0
7	1.7E+05	0	2.0E+03	0	0	0	0	0	9.2E+03	0
8	3.3E+05	0	1.4E+03	0	0	0	0	0	0	0
9	5.0E+05	0	5.0E+03	0	0	0	0	0	1.0E+06	0
10	8.0E+05	0	0	0	0	0	0	0	1.0E+07	0
11	4.3E+04	0	0	0	0	0	0	0	3.3E+03	0
12	2.5E+04	0	1.0E+04	0	0	0	0	0	7.1E+07	0
13	8.8E+04	0	2.4E+02	0	0	0	0	0	1.1E+07	0
14	1.2E+05	0	5.6E+02	0	0	0	0	0	6.3E+04	0
15	1.1E+05	0	2.0E+03	0	0	0	0	0	1.0E+05	0
16	7.5E+04	0	0	0	0	0	0	0	4.2E+07	0
17	0	0	1.3E+03	0	0	0	0	0	3.7E+04	0
18	5.0E+04	0	0	0	0	0	0	0	4.2E+07	0
19	3.0E+04	0	3.7E+02	0	0	0	0	0	1.2E+07	0
20	2.7E+04	0	0	0	0	0	0	0	5.7E+02	0

Table 2 Enumeration of bacterial flora from the mucus of covertly infected Atlantic salmon (2+ surplus domestic stock) held in Raceway 6 at the RCNSS, Massachussets, USA.

Fish #	Mucus		Gill		Spleen		Kidney		Intestine	
	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g
1	2.2E+03	0	0	0	0	0	0	0	1.2E+04	0
2	3.6E+04	0	0	0	0	0	0	0	1.3E+07	0
3	1.1E+04	0	0	0	0	0	0	0	0	0
4	1.3E+03	0	0	0	0	0	0	0	0	0
5	4.3E+03	0	5.0E+03	0	0	0	0	0	8.8E+03	0
6	1.5E+05	0	0	0	0	0	0	0	5.0E+05	0
7	1.4E+04	0	1.7E+03	0	0	0	0	0	5.4E+03	0
8	4.5E+03	0	0	0	0	0	0	0	2.6E+03	0
9	4.0E+04	0	0	0	0	0	0	0	0	0
10	1.0E+04	0	0	0	0	0	0	0	9.1E+06	0
11	4.4E+04	0	0	0	0	0	0	0	3.1E+04	0
12	1.1E+04	0	0	0	0	0	0	0	1.3E+04	0
13	1.3E+04	0	0	0	0	0	0	0	3.3E+07	0
14	6.2E+04	0	0	0	0	0	0	0	3.3E+04	0
15	8.3E+03	0	0	0	0	0	0	0	7.5E+05	0
16	3.8E+04	0	1.4E+03	0	0	0	0	0	0	0
17	7.1E+03	0	0	0	0	0	0	0	1.0E+07	0
18	1.3E+04	0	8.3E+02	0	0	0	0	0	6.0E+03	0
19	2.3E+04	0	0	0	0	0	0	0	1.3E+05	0
20	1.3E+04	0	0	0	0	0	0	0	0	0

Table 3 Enumeration of bacterial flora from the mucus of covertly infected brown trout from the Bennington State Fish Hatchery, Vermont USA

Fish #	Mucus		Gill		Spleen		Kidney		Intestine	
	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g
1	3.8E+03	0	0	0	0	0	0	0	1.1E+03	0
2	2.5E+03	0	0	0	0	0	0	0	0	0
3	1.7E+03	0	0	0	0	0	0	0	0	0
4	7.1E+03	0	0	0	0	0	0	0	0	0
5	1.7E+03	0	0	0	0	0	0	0	0	0
6	2.9E+03	0	0	0	0	0	0	0	0	0
7	3.8E+03	0	0	0	0	0	0	0	7.1E+03	0
8	2.0E+03	0	2.9E+03	0	0	0	0	0	1.0E+03	0
9	1.2E+04	0	0	0	0	0	0	0	0	0
10	2.9E+03	0	1.7E+03	0	0	0	0	0	5.0E+03	0
11	0	0	1.3E+03	0	0	0	0	0	0	0
12	1.3E+04	0	0	0	0	0	0	0	1.5E+03	0
13	5.0E+03	0	0	0	0	0	0	0	0	0
14	4.0E+03	0	5.0E+03	0	0	0	0	0	0	0
15	5.5E+03	0	0	0	0	0	0	0	0	0
16	1.3E+04	0	0	0	0	0	0	0	0	0
17	1.7E+03	0	5.0E+03	0	2.0E+04	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0
19	2.0E+04	0	0	0	0	0	0	0	0	0
20	1.2E+04	0	2.0E+04	0	3.3E+03	0	0	0	0	0

Table 4 Enumeration of bacterial flora from the mucus of covertly infected rainbow trout (average weight 1.08 kg) from the NFHRL, Leetown, W.V., USA

Fish #	Mucus		Gill		Spleen		Kidney		Intestine	
	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g
1	1.1E+05	0	2.0E+03	0	0	0	0	0	1.5E+05	0
2	9.4E+04	0	3.3E+04	0	0	0	0	0	3.5E+06	0
3	6.0E+04	0	1.0E+07	0	0	0	1.3E+03	0	1.7E+05	0
4	1.1E+05	0	5.0E+04	0	0	0	0	0	2.0E+06	0
5	2.7E+04	1.3E+04	2.0E+05	6.7E+04	1.7E+05	1.7E+05	4.0E+05	4.0E+05	3.9E+04	3.9E+04
6	8.5E+04	0	5.0E+05	0	0	0	0	0	1.3E+07	0
7	3.3E+04	0	1.8E+04	0	0	0	3.8E+05	0	7.7E+06	0
8	1.4E+05	0	1.5E+07	0	0	0	0	0	1.9E+08	0
9	1.7E+04	0	1.0E+05	0	0	0	0	0	9.1E+06	0
10	1.2E+05	0	2.5E+04	0	0	0	0	0	4.0E+05	0
11	9.1E+03	0	1.0E+05	0	0	0	7.1E+02	0	1.3E+07	0
12	1.2E+05	0	0	0	0	0	0	0	9.1E+06	0
13	4.0E+04	0	4.3E+08	0	0	0	0	0	4.0E+03	0
14	7.3E+04	0	1.0E+06	0	0	0	1.5E+03	0	4.7E+06	0
15	2.9E+05	0	8.8E+04	0	0	0	0	0	8.7E+04	0
16	5.7E+04	0	6.7E+05	0	0	0	0	0	1.2E+05	0
17	8.6E+04	0	2.0E+04	0	0	0	0	0	0	0
18	7.7E+04	0	3.0E+04	0	2.0E+06	2.0E+06	1.9E+05	1.9E+05	1.8E+05	1.8E+05
19	2.7E+05	1.3E+05	3.8E+04	1.3E+04	0	0	0	0	8.2E+04	0
20	2.5E+04	1.9E+04	1.2E+05	0	0	0	0	0	8.3E+03	0

Table 5 Enumeration of bacterial flora from the mucus of unvaccinated covertly infected Atlantic salmon (average weight 58.04 g) from the NFHRL, Leetown, W.V., USA

Fish #	Mucus		Gill		Spleen		Kidney		Intestine	
	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g	Total cfu/g	A.sal cfu/g
1	3.0E+05	0	2.0E+03	2.0E+03	0	0	0	0	3.0E+06	0
2	7.1E+04	7.1E+04	3.3E+04	0	0	0	0	0	2.9E+07	0
3	2.5E+05	0	1.0E+07	0	0	0	0	0	2.5E+05	0
4	1.0E+05	0	5.0E+04	0	2.5E+03	0	0	0	7.0E+07	0
5	1.0E+05	5.0E+04	2.0E+04	0	0	0	6.3E+03	6.3E+03	2.0E+08	0
6	1.3E+05	0	5.0E+05	0	0	0	2.2E+06	0	1.0E+07	0
7	2.0E+05	2.0E+05	1.8E+04	0	0	0	5.0E+03	0	1.4E+03	0
8	6.7E+04	0	1.5E+07	0	1.0E+04	0	0	0	1.7E+03	0
9	1.2E+05	0	1.0E+05	2.9E+04	0	0	0	0	0	0
10	4.3E+04	0	2.5E+04	0	0	0	0	0	1.4E+08	0
11	2.2E+04	0	1.0E+05	5.0E+04	0	0	0	0	4.6E+07	0
12	1.5E+04	0	0	0	0	0	1.4E+03	0	2.2E+05	0
13	1.0E+05	0	4.3E+08	0	2.0E+03	0	8.8E+04	0	1.1E+08	0
14	1.4E+07	0	1.0E+06	0	0	0	4.0E+04	0	6.7E+06	0
15	5.0E+05	0	8.8E+04	2.5E+04	0	0	6.7E+02	0	1.4E+08	0
16	7.7E+03	0	6.7E+05	0	0	0	8.8E+03	0	1.3E+05	0
17	1.0E+04	0	2.0E+05	1.0E+05	0	0	5.0E+03	0	2.0E+07	0
18	9.1E+04	0	3.0E+04	1.0E+04	0	0	1.7E+03	0	1.4E+08	0
19	5.7E+05	2.9E+05	3.8E+04	0	0	0	0	0	1.3E+08	0
20	6.2E+04	2.3E+04	1.2E+05	6.0E+04	0	0	4.3E+04	0	2.0E+04	0

Appendix 5

Table 1 Comparison of PCR testing of *A. salmonicida* isolates and tissue extracts taken from overtly infected Arctic char held at the NFHRL, WV.

Fish #	*Tissue*	*PCR of Isolates*		*PCR of Tissues*	
		AP	PAAS	AP	PAAS
1	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
2	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
3	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
4	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
5	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
6	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	-	+
	kidney	+	+	+	+
	intestine	+	+	-	-
7	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
8	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
9	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
10	mucus	+	+	+	+
	gill	+	+	-	(+)
	spleen	+	+	-	-
	kidney	+	+	+	+
	intestine	+	+	+	+
11	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
12	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	+	+
	intestine	+	+	-	-
13	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	-	-

Fish #	*Tissue*	*PCR of Isolates*		*PCR of Tissues*	
		AP	**PAAS**	**AP**	**PAAS**
14	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
15	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	-	-
	kidney	+	+	+	+
	intestine	+	+	-	-
16	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	-	-
17	mucus	+	+	+	+
	gill	+	+	+	+
	lesion	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	-	-
18	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+

Table 2 Comparison of PCR testing of *A. salmonicida* isolates and tissue extracts taken from overtly infected rainbow trout held at the NFHRL, WV.

Fish #	*Tissue*	*PCR of Isolates*		*PCR of Tissues*	
		AP	PAAS	AP	PAAS
1	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
2	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
3	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
4	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
5	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	-	-
	lesion	+	+	+	+
6	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
7	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
8	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	+	+
	intestine	+	+	+	+
9	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	-	-
	kidney	+	+	+	+
	intestine	+	+	-	-
	lesion	+	+	+	+
10	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	-	-
	intestine	+	+	-	-
11	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
12	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
	lesion	+	+	+	+
13	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	+	+
	kidney	+	+	-	-
	intestine	+	+	+	+

Fish #	*Tissue*	*PCR of Isolates*		*PCR of Tissues*	
		AP	PAAS	AP	PAAS
14	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
15	mucus	+	+	-	-
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
16	mucus	+	+	-	-
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	+	+
	intestine	+	+	+	+
	lesion	+	+	+	+
17	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
18	mucus	+	+	+	+
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
19	mucus	+	+	-	-
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-
20	mucus	+	+	-	-
	gill	+	+	-	-
	spleen	+	+	-	-
	kidney	+	+	-	-
	intestine	+	+	-	-

Table 3 Comparison of PCR testing of *A. salmonicida* isolates and tissue extracts taken from overtly infected Atlantic salmon (vaccinated) held at the NFHRL, WV.

Fish #	*Tissue*	*PCR of Isolates*		*PCR of Tissues*	
		AP	PAAS	AP	PAAS
1	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
2	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
3	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
4	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
5	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
6	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
7	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
8	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
9	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
10	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
11	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
12	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
13	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
14	mucus	+	+	+	+

Fish #	*Tissue*	*PCR of Isolates*		*PCR of Tissues*	
		AP	PAAS	AP	PAAS
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
15	mucus	+	+	+	+
	gill	+	+	+	+
	lesion	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
16	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
17	mucus	nd*	nd	+	+
	gill			-	-
	spleen			-	-
	kidney			-	-
	intestine			-	-
18	mucus			-	-
	gill			+	+
	spleen			-	-
	kidney			-	-
	intestine			-	-
19	mucus			-	-
	gill			-	-
	spleen			+	+
	kidney			-	-
	intestine			+	+
20	mucus			-	-
	gill			-	-
	spleen			-	-
	kidney			-	-
	intestine			-	-
21	mucus			-	-
	gill			-	-
	spleen			+	+
	kidney			+	+
	intestine			-	-
22	mucus			-	-
	gill			-	-
	spleen			-	-
	kidney			+	+
	intestine			-	-
23	mucus			+	+
	gill			-	-
	spleen			-	-
	kidney			-	-
	intestine			-	-
24	mucus			-	-
	gill			-	-
	spleen			-	-
	kidney			+	+
	intestine			-	-

*The tissues from fish #17-24 were not cultured.

Table 4 Comparison of PCR testing of *A. salmonicida* isolates and tissue extracts taken from overtly infected Atlantic salmon (unvaccinated) held at the NFHRL, WV.

Fish #	*Tissue*	*PCR of Isolates*		*PCR of Tissues*	
		AP	PAAS	AP	PAAS
1	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
2	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
3	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
4	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
5	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
6	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
7	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
8	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
9	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
10	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
11	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
12	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
13	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
14	mucus	+	+	+	+
	gill	+	+	+	+

Fish #	*Tissue*	*PCR of Isolates*		*PCR of Tissues*	
		AP	PAAS	AP	PAAS
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
15	mucus	+	+	+	+
	gill	+	+	+	+
	lesion	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+
16	mucus	+	+	+	+
	gill	+	+	+	+
	spleen	+	+	+	+
	kidney	+	+	+	+
	intestine	+	+	+	+

Appendix 6: HC-PCR Probe and AP PCR Target Sequences on the *A.salmonicida vapA* gene

A. salmonicida *vap*A gene (1506bp) contains a 21 base repeat (underlined) separated by 795 bases beginning at nucleotides 219 and 1035 of the 1506 base gene sequence. **ATG** start codon at nucleotide 121 and end of *vap*A is at nucleotide 1626. *vap*A gene has 2 BstEII cleavage sites (lower case), G/GTNACC (N=A,C,G or T), at nucleotides 307/8 and 1395/6 (vapA nucleotides 186/7 and 1274/5).

AS1 capture probe primer binding sites (nucleotides numbers are from the whole 1800bp fragment, not *vap*A nucleotide sequence)

AS 1f808 5'-**GTTTCCGAAGGTTTCTTG**-3' nucleotides 808-825 (*vap*A nucleotides 687-704 +ve)
AS 1r1203 5'-**CTCGTCCTTGAAGTAGTTATAGG**-3' (primer is rev. comp.) nucleotides 1181-1203 (*vap*A nucleotides 1082-1060 -ve)
Product size: 396bp

G[808]**TTTCCGAAGGTTTCTTG**[825]AAACTTAATATCGGTGATGCGAATATTTCTGCTACTGATTTCGCGATTACCAACGTTACTACTAACC
AGACCATCCAACGTGACAAGGTTAACCTGACCCTGACTGGTGATGTTTCTGCCTTCAAGAAAGATGCCAACGGTAACTTGGTAAACA
AAGCTGGTGCTAGCATCGGTTGGAAAGCTGCTGCTGATGGTCAATCTGCTACAGCTGTCTTGGGTGCTGGCAACATGGCCGGTGG
GGTTCAAAATGCTCTGGCTGCTTTTGGTACACTGTACGTTGCTGCAGATAACACTGTTCCGGTTCCTGCTGTTAACTTCAATGTTAA
GGCTGAAATCCAAGGTGATAGCCAAGCTA**C**[1181]**CTATAACTACTTCAAGGACGAG**[1203]

AP1f / AP2r primer binding sites (nucleotides numbers are from the whole 1800bp fragment, not *vap*A nucleotide sequence)

AP1f 5'-**GGCTGATCTCTTCATCCTCACCC**-3' nucleotides 1206-1228 (*vap*A nucleotides 1085 to 1108, + strand)
AP2r 5'-**GCACCGCTGGTAGATTTCACTCTG**-3' (primer is rev. comp.) nucleotides 1603-1626 (*vap*A nucleotides 1505 to 1482, - strand)
Product size: 421bp

G[1206]**GCTGATCTCTTCATCCTCACCC**[1228]GTGATGGTATGAAGTTTGACACAATTACTACTGGTACCACTTCTGCCAACCTCATCCACA
TTCGTGATGTATCTAACATCCTGCCTACTGAAGGTGGCAAGATCTTCGTAACTATCACTGAATATGCAGATCATGCTGCCAATGGTC
GTGGTGAAGGTACTGTATTg/gttaccCGTAAAGCACTGTCTGTTACCCTGCCAAGCGGTGGTGCAGTGACTCTGAAGCCTGCTGATGT
TGCTGCTGACGTTGGTGCTTCTATCACTGCTGGCCGTCAGGCTCGCTTGGTGTTTGAAGTTGAAACCAATCAGGGTGAAGTAGCTG
TTAAGAAATCCAATGCTGAAGGCGTGGATATTCAGAATGGTACCCGCGGCACA**G**[1603]**CACCGCTGGTAGATTTCACTCTG**[1626]

BstEII digestion of AP1f / AP2r product should yield 2 fragments of 190 and 231bp respectively.

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   1  GCCTTGCAAT CCATCTTGTT CAATCATGCG AAACGAACCT CATCCTGTCT
  51  CTTGATCAGA TCAACGGATA GGTTCAACCC TATTTGTATA TAATCAATAA
 101  ACTTCCTTAA GGAAAAAAGT ATGTTTAAGA AGACTTTGAT TGCAGCTGCC
 151  ATTGTGGTCG GTTCCGCTGC ACCTGCGTTT GCTGATGTCG TGATTAGCCC
 201  GAACGACAAC ACTTTCGTTA CTACCTCCCT CGCATCTGTA ACTAAGCAGC
 251  CGGTACTGGA CTTCTCCACT GCTCAACAAA ACCTGACCCT TAACTTCAGT
 301  GAAGTTggtg accTTAAGAA CAACGGTTTC ATTGTGTTGG AAATCCAAGG
 351  TGAAGGCCAA TTCAACGACG CGGAAATCCG TCAGTGGCTG TCCAACGGTT
 401  TCTGGCGTAG GCCGTTTACC GGTCTGCTGG TTAACCCGAA TGATCATGGT
 451  AATTTTGCCA ATAGCGGTGA AGTTAATGAC GTTCGGAAGT TCTTTAAGAT
 501  TATTTCCGAC GGTACCCAGC TGACCATCGT TCACACTATC GACAGCAATG
 551  GCAAGCGTCT GCGTCTTGCT CTTGCTTCTG ATGTAGAAGA GACAATCAAT
 601  TTTGCTGATG CAGAGGTTGA GCTGAAGCTG AACTTAGCTA ACCAAGCCTT
 651  TAAGCTGACC TCCGGTTCTC AAGGTACAGT AGCTCTGACC GCAGGAGCTC
 701  TGTGGAACGC TTCTTACACT GCTGATCCGG TTGCTACCAA GCCGCTGTTC
 751  AAACTGGGTA AGCTGTTCCA GTTGAGTTTG ACTAACGCTG GTAAAGCTAC
 801  CGCTCTGGTT TCCGAAGGTT TCTTGAAACT TAATATCGGT GATGCGAATA
 851  TTTCTGCTAC TGATTTCGCG ATTACCAACG TTACTACTAA CCAGACCATC
 852  CAACGTGACA AGGTTAACCT GACCCTGACT GGTGATGTTT CTGCCTTCAA
 853  GAAAGATGCC AACGGTAACT TGGTAAACAA AGCTGGTGCT AGCATCGGTT
 854  GGAAAGCTGC TGCTGATGGT CAATCTGCTA CAGCTGTCTT GGGTGCTGGC
 855  AACATGGCCG GTGGGGTTCA AAATGCTCTG GCTGCTTTTG GTACACTGTA
 856  CGTTGCTGCA GATAACACTG TTCCGGTTCC TGCTGTTAAC TTCAATGTTA
 857  AGGCTGAAAT CCAAGGTGAT AGCCAAGCTA CCTATAACTA CTTCAAGGAC
1201  GAGCTGGCTG ATCTCTTCAT CCTCACCCGT GATGGTATGA AGTTTGACAC
1251  AATTACTACT GGTACCACTT CTGCCAACCT CATCCACATT CGTGATGTAT
1301  CTAACATCCT GCCTACTGAA GGTGGCAAGA TCTTCGTAAC TATCACTGAA
1351  TATGCAGATC ATGCTGCCAA TGGTCGTGGT GAAGGTACTG TATTggttac
1401  cCGTAAAGCA CTGTCTGTTA CCCTGCCAAG CGGTGGTGCA GTGACTCTGA
1451  AGCCTGCTGA TGTTGCTGCT GACGTTGGTG CTTCTATCAC TGCTGGCCGT
1501  CAGGCTCGCT TGGTGTTTGA AGTTGAAACC AATCAGGGTG AAGTAGCTGT
1551  TAAGAAATCC AATGCTGAAG GCGTGGATAT TCAGAATGGT ACCCGCGGCA
1601  CAGCACCGCT GGTAGATTTC ACTCTGTAAG AGCGAATCTA GTCGATAAAG
1651  GGCGCCGAGG CGCCCTTTTT AATTAATGTA AAACTAAGCA GTTTCATTTG
1701  TTCCCTCACT CATCAATCTC TTCATTTAGC GAGGTAGACA AGCTCTGCCG
1751  TTGCTCGTGA CCACAGCCCT TATTTCAGTT GTCATTTATT GTTATACTCT
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AS1 capture probe

AP detection PCR