Development of molecular probes for use in bacterial disease diagnosis and health monitoring of farmed and wild finfish in Australia

Final Report on FRDC project 93/128

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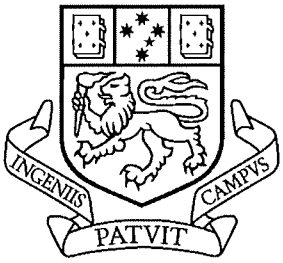
Department of Primary Industry & Fisheries



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Development of molecular probes for use in bacterial disease diagnosis and health monitoring of farmed and wild finfish in Australia

Fisheries Research & Development Corporation
Project 93/128

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OBJECTIVES:

1. Develop procedures for testing fish by molecular probes for the presence of bacterial fish pathogens.
2. Develop methodologies using molecular probes for the rapid identification of bacterial fish pathogens recovered by conventional culture techniques.
3. Develop molecular probes for enzootic strains of *Aeromonas salmonicida*, *Enterococcus seriolicida*, *Flexibacter maritimus* and *Yersinia ruckeri*.
4. Develop a secondary confirmation system for PCR assays using internal probes and hybridization
5. Formulate the developed test procedures for transfer to, and adoption by, veterinary diagnostic and research laboratories.

NON TECHNICAL SUMMARY:

Farming of Atlantic salmon and rainbow trout in Australia is a multi-million dollar industry estimated to be worth over $100 million dollars per annum. A key factor in the success of the industry is Australia's relative freedom from major salmonid diseases caused by viruses and bacteria. Although free of the more devastating diseases that can seriously limit production in Northern Hemisphere countries, Australia nevertheless has several bacterial diseases that can cause significant disease outbreaks.

Current methods of disease diagnosis and detection are aimed at confirming the cause of disease outbreaks and for a relatively limited range of bacterial species. There is a growing need to acquire diagnostic tools that can provide a means of rapid identification of infectious bacteria during disease outbreaks. More importantly however, there is an urgent need to develop techniques that can be used in surveillance and monitoring programmes to detect significant bacterial pathogens before major disease outbreaks occur.

In Australia, significant disease outbreaks caused by *Enterococcus seriolicida, Flexibacter maritimus* and *Yersinia ruckeri* have occurred in rainbow trout and Atlantic salmon. Although there have been no reports of disease in Atlantic salmon caused by atypical strains of *Aeromonas salmonicida*, this bacterium remains a potent threat to salmon farming. Despite the obvious impact of these pathogens on fish health there are few if any versatile or rapid techniques available to diagnose disease caused by these bacteria.

The major goal of this project was to develop rapid, specific and sensitive techniques to identify bacterial fish pathogens. A set of diagnostic tools based on gene probe technology has been developed to detect these four major bacterial pathogens. Probes were designed as DNA primer pairs for use in the polymerase chain reaction (PCR), a highly sensitive DNA amplification test. The primers target the 16S ribosomal RNA gene a conserved region of the bacterial genome that contains information unique to each species of bacterium. In addition, a system based on an internal gene probe was developed to verify the accuracy of the DNA sequences amplified by PCR in respect of the bacterial fish pathogens.

The PCR system was adapted for each bacterium and the specificity of the test extensively evaluated. A wide range of target bacteria were used comprising enzootic and exotic strains, near related bacteria with some genetic similarity to the target bacteria and a wide range of normal flora commonly associated with fish. The gene probes in the PCR system were found to be highly specific for *E. seriolicida, Fx. maritimus* and *Y. ruckeri.* No cross reactions were detected and the PCR test identified all known strains and isolates correctly. The PCR test for *A. salmonicida* was less specific than desirable. Cross reactions occurred with some strains of *A. hydrophila* as well as *A. bestiarum* and as a consequence, application of the probe is limited to specific situations.

Development of the PCR systems has enabled the development of a rapid identification test for these important fish pathogens. The test has been configured so that a single bacterial colony can be identified within four hours, a task that for some of the fish pathogens may take up to two weeks using conventional culture methods. It is intended that the PCR test will be used for the rapid identification of bacterial cultures, in particular the hard to identify species such as *A. salmonicida* and *Fx. maritimus*.

The PCR test has also been adapted for use as a direct detection method in fish tissue. Fish which are known to be diseased can be tested by the PCR method and a presumptive identification made within six hours. The ability to detect very small numbers of bacteria in fish tissue is not currently achievable with existing formats. The smallest number of bacteria which could be detected by direct detection PCR was $1x10^4$ bacteria per gram of tissue. At this level of detection it may not be possible to identify with certainty all fish which might be symptomless carriers of a bacterial pathogen. It is anticipated that the direct detection PCR test will be used as a means of rapid detection of bacterial pathogens in overtly diseased fish and be used with caution for monitoring normal fish as a means of identifying carriers.

Significant advances have been made in this project to developing an important diagnostic technology based on PCR. It is evident however that further work is required to improve the sensitivity of direct detection PCR if it is to be used as a tool for the early detection of bacterial pathogens in farmed and wild fish. Further work is also required to package the PCR technology in a format that can be used reliably and simply when testing large numbers of fish.

Access to the PCR technology has been facilitated by conducting a training workshop at the end of the project. Participants at the workshop came from the principal diagnostic veterinary laboratory responsible for fish disease diagnosis in each state. As a result of the workshop, the techniques are now being used for disease diagnosis and research for the salmonid aquaculture industry.

Acquisition of these tools must be seen as a significant advance in disease diagnostic capability and a tangible means to maintaining and protecting Australia's disease free status in aquaculture and wild fisheries.

KEYWORDS: Bacteria, fish pathogens, aquaculture, gene probes, PCR, detection, salmonids

Contents

Project Team Members

Name	Dates	Role	Institution
Jeremy Carson	1993-96	Team Leader	Dept. Primary Industry & Fisheries, Tasmania
Peter Franzmann	1993-94	Supervising Scientist	University of Tasmania
Sue Dobson	1994-96	Supervising Scientist	University of Tasmania
Janine Miller	1993-94	Research Scientist	University of Tasmania
Sharee McCammon	1994-95	Research Scientist	University of Tasmania
Michelle Williams	1995-96	Research Scientist	University of Tasmania

Jeremy Carson managed the project and undertook the intra-species and inter-species testing of the PCR probes.

Peter Franzmann and *Janine Miller* sequenced the bacteria, undertook the alignments and nominated the probe sequences.

Sue Dobson and *Sharee McCammon* verified the probe sequences and identified the range of bacteria for specificity testing. They developed the basic PCR process for the pathogens and investigated the rRNA hybridization method.

Michelle Williams refined and optimised the PCR assays, undertook key specificity testing and determined detection levels of the tests. She also developed and tested the protocol for direct detection PCR in fish as well as the PCR confirmation probes. Michelle developed the technology transfer package for the assays.

Acknowledgments

The author wishes to acknowledge the support, guidance and assistance that was given with unfailing enthusiasm, courtesy and co-operation by members of the project team. Particular thanks to Michelle Williams and Sharee McCammon for their patience during the technology transfer process.

Nick Gudkovs, Helen Byers and Eva-Marie Bernoth from the Fish Disease Laboratory, Australian Animal Health Laboratory, CSIRO entered into many stimulating and incisive discussions that have helped shape the outcomes of the project. The provision of reference cultures and DNA, materially helped the project and is gratefully acknowledged.

During the project, Steinar Høie from the Central Veterinary Laboratory, Oslo, Norway freely provided data and insights regarding his experiences with 16s rRNA *Aeromonas salmonicida* probes and his contribution is acknowledged.

List of Abbreviations

ANGIS	Australian National Genomic Information Service
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
cfu	colony forming units
CIP	Collection de l'Institut Pasteur
ddNTP	di-deoxynucleotide triphosphate
dITP	deoxyinosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DPIF	Department of Primary Industry & Fisheries
EDTA	ethylene-diamine-tetra acetic acid
ELISA	enzyme linked immuno sorbent assay
EMBL	European Molecular Biology Laboratory
FAT	fluorescent antibody test
FCLB	*Flexibacter-Cytophaga*-like bacteria
fg	femtogram
GE	genome equivalent
GSDB	Genome Sequence Data Base
HG	Hybridization Group
kDa	kilo Daltons
MΩ	mega ohm
mM	millimole
NCDO	National Collection of Dairy Organisms
NCFB	National Collection of Food Bacteria
ng	nanogram
OIE	Office International des Épizooties
PAAS	probe assay *A. salmonicida* (plasmid)
PCR	polymerase chain reaction
pg	picogram
RAPD	random amplification of polymorphic DNA
RDP	Ribosomal Database Project
RNA	ribonucleic acid
rRNA	ribosomal RNA
SALTAS	Salmon Enterprises of Tasmania
SDS	sodium dodecyl sulphate
sp.	species
SSC	salt sodium citrate
ssp.	subspecies
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBE	Tris-boric acid-EDTA
T_m	melting temperature (of DNA)
T_{HYB}	oligonucleotide probe hybridization temperature
T_W	wash temperature during oligonucleotide probe hybridization

"Knowledge is of two kinds. We know a subject ourselves or we know where we can find information upon it."
- Samuel Johnson (1709-1784) *Boswell's Life of Johnson*

"Truth lies within a little and certain compass, but error is immense"
- Henry St John, Viscount Bolingbroke 1678-1751. *Reflections upon Exile*

"It is of the highest importance in the art of detection to be able to recognise out of a number of facts which are incidental and which are vital."
- Arthur Conan Doyle 1859-1930. *The Adventure of the Reigate Squire*

"I had," said he, "come to an entirely erroneous conclusion which shows, my dear Watson, how dangerous it always is to reason from insufficient data."
- Arthur Conan Doyle 1859-1930. *The Speckled Band*

"The nice thing about standards is that you have so many to choose from.
- Andrew S. Tanenbaum 1988. *Computer Networks*

Section 1

INTRODUCTION

1.1 Background

Unlike traditional forms of farming in which animal species have been domesticated and bred for resistance to disease over hundreds of years, aquaculture does not have this advantage of time in selecting strains of fish tolerant to the stress of intensive production methods. Caging of fish inevitably leads to a compromise in the optimum micro-environment of the animal in domains such as water quality, nutrition, stocking density and social

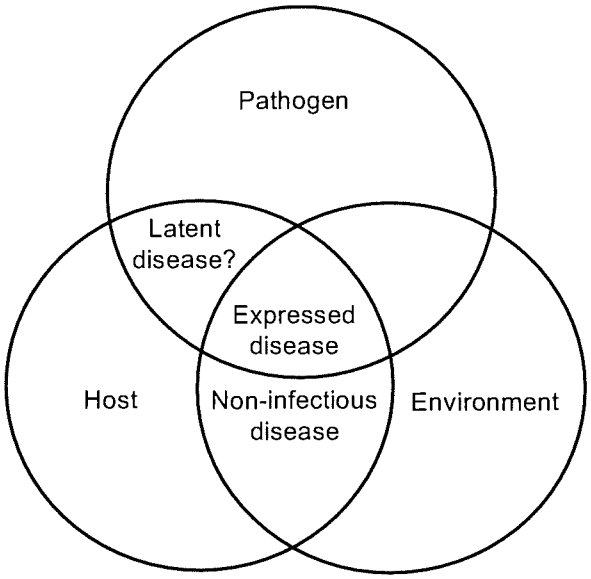

Figure 1.1 Interactions of host, pathogen and environment and expression of disease (after Snieszko *op. cit.* Langdon, 1988)

hierarchy pressures (Schreck 1981). The interaction of these factors with the host can be complex (Figure 1.1) and are central to the expression of disease. These interactions contribute to the development of physiological stress in the fish leading to a reduction of immune competence (Ellis 1981; Langdon 1988). In many situations this may not be critical, but it does increase the vulnerability of fish to infection either by primary microbial pathogens or endogenous opportunistic pathogens.

1.2 Disease agent inventory

Currently Australia is free of the following major bacterial pathogens: *A. salmonicida* subspecies *salmonicida*, *achromogenes*, *masoucida*, *smithia*, *Piscirickettsia salmonis*, *Renibacterium salmoninarum*, *Vibrio damsela* ssp.

piscicida (syn. *Pasteurella piscicida*), *Vibrio ordalii*, *Vibrio salmonicida* and the rainbow trout pathovar of *Yersinia ruckeri*, serotype O1a (Romalde *et al.* 1993).

Although these primary pathogens are exotic, a number of other significant bacterial pathogens of fish have been detected (Table 1.1). At least two atypical biovars of *A. salmonicida* are known to exist in the Australian temperate regions of Victoria, New South Wales and Tasmania. One of the biovars, introduced form Japan in 1974 (Humphrey & Ashburner 1993), is a natural host of goldfish (*Carassius auratus*) and is enzootic in Victoria and New South Wales but not Tasmania. This biovar, under appropriate conditions, is a potent pathogen of Atlantic salmon (Carson & Handlinger 1988) as well as silver perch (*Bidyanus bidyanus*) (Whittington *et al.* 1995), a farmed native fish. The second atypical biovar of *A. salmonicida* has only been detected in wild and farmed Tasmanian greenback flounder (*Rhombosolea tapirina*). On the basis of phenotype, the biovar is unlike any of the named subspecies of *A. salmonicida* and there is sufficient taxonomic evidence to consider classification of the biovar as a new subspecies, *A. salmonicida* subspecies "*lerunnica*" (Carson, Pinkard & Schmidtke unpublished findings). Its low pathogenicity in flounder and unique characteristics suggest that the pathogen may form part of the natural bacterial flora of the region. Experimental pathogenicity trials with this flounder biovar have established that it is a significant pathogen in Atlantic salmon as well as striped trumpeter (*Latris lineata*).

Pathogen	Disease	Host range
A. salmonicida atypical	Goldfish ulcer disease	goldfish silver perch salmonids
A. salmonicida ssp "*lerunnica*"	Flounder ulcer disease	greenback flounder striped trumpeter salmonids
Carnobacterium piscicola	Egg peritonitis	salmonids
Edwardsiella tarda	Edwardsiellosis	rainbow trout
Flavobacterium psychrophilum	Fin erosion	Atlantic salmon
Flexibacter maritimus	Cutaneous erosion disease	salmonids marine fish
Lactococcus garvieae	Streptococcaceosis	rainbow trout
Vagococcus salmoninarum	Egg peritonitis	salmonids
Vibrio anguillarum	Vibriosis	salmonids marine fish
Yersinia ruckeri	Yersiniosis	salmonids

Table 1.1 Significant bacterial pathogens of temperate farmed Australian fin fish

Carnobacterium piscicola and *Vagococcus salmoninarum* (Schmidtke & Carson, 1994) cause chronic peritonitis in sexually mature rainbow trout and Atlantic salmon. The disease is associated with poor husbandry techniques and occurs only sporadically. A significantly more virulent pathogen is *Lactococcus garvieae* (formerly *Enterococcus seriolicida*), the cause of the septicaemic disease in rainbow trout called streptococcaceosis (Carson, Gudkovs & Austin, 1993). The disease is associated with high stocking densities, poor water quality and raised water temperatures. Through improved management practices the disease is in rapid decline in Australia although in Japan, streptococcaceosis is still the cause of major loss in farmed yellowtail (*Seriola quinqueradiata*).

Edwardsiella tarda is an economically important pathogen of farmed eels in Japan, China and Taiwan. A pathovar of the bacterium has also been isolated from rainbow trout in Australia (Reddacliff *et al.*1994). The disease has caused only low levels of mortality in a limited geographic location and was associated with poor water quality. Of greater significance is the disease caused by *Y. ruckeri* serotype O1b (syn. I′, III) the so-called Australian strain. Like infections caused by *E. tarda*, yersiniosis is associated with poor water quality and elevated water temperatures. Under intensive culture conditions, moderate and persistent outbreaks of yersiniosis have occurred in Atlantic salmon hatcheries in Tasmania. These disease episodes result in low levels of mortality and an increase in the number of asymptomatic carriers in the population. Further disease episodes occur when fish move from the hatchery to sea-cages where acclimatization stress can precipitate outbreaks of yersiniosis in carriers.

Infections caused by gliding, filamentous Gram-negative bacteria occur in both freshwater and marine environments. *Flavobacterium psychrophilum* (Schmidtke & Carson, 1995) has been isolated from only one location in Tasmania and is associated with fin erosion of cold stressed Atlantic salmon. Although the disease is not associated with mortalities, the number of fish affected can be high, exceeding 90% in some groups of fish. This contrasts with the European pathovar of *Fl. psychrophilum*, which causes the disease

rainbow trout fry syndrome, an acute disease associated with high levels of mortality. This disease has not been seen in Australia. The marine filamentous bacterium, *Flexibacter maritimus* (Schmidtke *et al.* 1991), appears to have a worldwide distribution and is the cause of acute to chronic skin erosions in temperate water fish. The disease is associated with handling stress and can, if untreated, lead to significant losses. The pathogen has a wide host range and has been identified as a cause of skin erosions in many commercially and experimentally farmed fish species including Atlantic salmon, rainbow trout, greenback flounder, and striped trumpeter.

Vibrio anguillarum is a widely distributed pathogen of many teleost species of fish. This pathogen can cause a severe septicaemia in salmonids and under farm conditions losses can be severe. In Tasmanian coastal waters only serotype O1, the commonest salmonid serotype, has been found in diseased Atlantic salmon and rainbow trout (Carson, 1990). Disease prevention in Tasmania has been effected through the use of a commercial vaccine *Anguillvac-C* (DPIF, Launceston) which can achieve protection levels in excess of 90% (Munday *et al.* 1992).

1.3 Value of salmonid production

Finfish aquaculture production in Australia for 1989-90 has been estimated to be worth $47.4 million of which salmonid production accounted for $41.5 million (O'Sullivan 1991). By 1994-95, production had jumped to $135 million of which $105.5 million was due to salmonid production (ABARE 1995). The rapid development of the industry and its evident economic sustainability is partially attributable to the absence of major salmonid pathogens. The importance of freedom from disease for sustainable production has been demonstrated through economic modelling. The impact of the exotic disease agents, *Aeromonas salmonicida* subspecies *salmonicida* and infectious haematopoietic necrosis virus if they became established in Australia was forecast to lead to a significant economic down turn in the industry. Such a decline was forecast to be so severe that salmonid farming in Australia would be abandoned (ABARE 1994).

1.4 Economic loss associated with Australian salmonid diseases

The bacterial diseases listed in Table 1.1 are known to occur in some or all of the south eastern states of Australia and cause considerable loss to the aquaculture industry. Streptococcaceosis has been reported in Tasmania, New South Wales, Victoria. In Tasmania alone, this disease has been calculated to cost $6 million between 1983 and 1991. Cutaneous erosion disease which affects striped trumpeter as well as rainbow trout and Atlantic salmon has been estimated to have cost the salmonid industry $400,000 pa. (SALTAS, Tasmanian industry estimate, 1992) between 1991 and 1993.

Although furunculosis has not been reported in Australia, this disease, if established, will have severe biological, economic and social consequences for aquaculture and recreational fisheries (Anon. 1997). In Scotland (1990/91), 35% of salmonid stocks were lost as a result of furunculosis and the cost to industry determined at £18 million (G. Rae pers. comm. Scottish Salmon Growers Association, 1993). For several years mortalities attributable to Yersiniosis in Australia have not been significant but recently, as production of Atlantic salmon has intensified, there have been significant disease episodes in Tasmania. The direct and indirect costs of the disease in Tasmania has been estimated to be $110,000 pa. (Schmidtke 1995). In Europe the disease occurs widely and has resulted in significant levels of mortality. In a UK survey (Rodgers 1991), 22% of farms had experienced the disease at least once and half the sites had repeat outbreaks. The cost of treatment by vaccination and chemotherapy per farm was put at just under £3000 pa.

1.5 Trade implications

Active surveillance of animal populations is an important strategy in animal health monitoring (Stärk 1996). There is a growing awareness, by farm operators and regulatory authorities here in Australia and overseas, for the need to undertake health monitoring and surveillance programmes of fish stocks (Anderson & Barney 1991; Midtlyng *et al.* 1992). Surveillance has implications beyond national needs of disease containment or eradication. As global trade increases, sanitary and phytosanitary obligations between

countries must be met (Chillaud 1996). The basis for determining freedom from disease in fish populations has been formulated as the International Aquatic Animal Health Code (Anon 1995) and seeks to harmonize health guarantees for international trade in live aquatic animals as well as aquatic animal products (Håstein 1996).

The ability to detect infected animals is an essential requirement of animal health monitoring and surveillance. A major problem of testing farmed and wild fish is the absence of adequate diagnostic tests to detect latent infections, a condition where the pathogen may reside asymptomatically in the host without apparent signs of disease. These fish, termed carriers, are a significant threat to other fish and seriously compromise the health status and security of the population in which the carriers reside. Many of the bacteria listed in Table 1.1 are capable of latent infection. The current strategy for detecting carriers is both expensive and time consuming to conduct as it relies on suppressing immune function with corticosteroids and isolating pathogens from any resulting diseased fish (Bullock & Stuckey 1975). This technique has been used as a disease control measures in Ireland (Smith 1991) and eastern Canada (Olivier, 1992) and has been instrumental in limiting the spread of furunculosis to sea cage farms. In the absence of a simple and sensitive detection procedure for carriers, there is a greater likelihood of inadvertent spread of disease by the movement of carrier fish between farms and from disease affected to disease free regions.

Concern has been expressed by the Office International des Épizooties (OIE) over the basis on which countries claim freedom from disease in respect of specific pathogens. Increasingly, countries will need to demonstrate the basis of their claim and this can only be provided if systematic surveys using sensitive procedures have been used. Using current bacteriological technologies, it will be considerably more difficult for countries to demonstrate freedom from certain diseases. As global trade develops, Australia will need to demonstrate freedom from disease not just as a marketing strategy but as an essential requirement of trade and as a means of protecting or limiting the spread of disease.

1.6 Disease detection strategies

Culture Detection of bacterial disease in fish largely relies on conventional bacteriological culture techniques. These methodologies are used where there is evidence of morbidity or mortality in fish and a likelihood of the pathogen being present in amounts which can be detected by culture. Identification of bacterial fish pathogens relies on phenotypic tests, principally biochemical, nutritional and physiological characteristics (Austin & Austin 1993, Frerichs 1993). The deficiencies of conventional testing are the lack of robustness in identification schemes for some bacterial genera and the length of time required to identify some species. Some biovars of *A. salmonicida* such as *A. salmonicida* ssp. "*Ierunnica*" can take up to 7 days to appear in culture (Carson, Pinkard & Schmidtke unpublished data) while identification of *Fx. maritimus* is not reliable in view of the uncertain classification of the species and the taxonomy of this group of bacteria (Bernardet *et al.* 1996). The potential for delay in identifying the pathogen may seriously compromise any possibilities of limiting the disease outbreak to the affected farm.

FAT & ELISA Immunological methods of detecting bacterial pathogens in fish have centred on direct visualization of bacteria by the fluorescent antibody test (FAT) or the colour reaction produced by solid phase enzyme linked immunosorbent assay (ELISA). FAT is a rapid procedure for use in acute phase infection where the pathogen is present in sufficient numbers to be detected by microscopy . Specificity for the test is usually high, particularly if a monoclonal antibody is used. Sensitivity is poor however, typically no better than $1x10^6$ bacteria g^{-1} although with sample concentration and examination of 100 fields of view, it is possible to detect at least $1x10^3$ cells g^{-1} of *Renibacterium salmoninarum* (Sakai *et al.* 1989). A greater level of sensitivity and economy of testing, particularly where large numbers of samples are involved, can be achieved using antigen capture ELISA. In this format, bacteria in fish tissues are first concentrated by an immobilised capture antibody and subsequently detected with a secondary enzyme linked antibody. Capture ELISA has been used to detect several bacterial fish pathogens including *A. salmonicida* (Bernoth 1990), *Vibrio anguillarum* (Romestand *et al.* 1993) and

R. salmoninarum (Gudmundsdottir *et al.* 1993). Despite the ability of capture ELISA to concentrate bacteria, the sensitivity of the system is typically no better than $1x10^5$ cells (Swaminathan & Feng 1994) although Hiney *et al.* (1994) were able to detect as little as $1x10^4$ cfu ml^{-1} of *A. salmonicida*.

Gene probes The rapid advancement of molecular biology techniques in recent years has led to the development of a number of powerful analytical technologies and in particular procedures involving manipulation and analysis of genetic material. Characterization of bacteria can be made on the basis of unique sequences of nucleotides on genomic DNA or ribosomal RNA of the cell. Short assemblages of nucleotides can be constructed as a probe to match a unique segment of genomic DNA. The choice of target sequences determines the specificity of the probes while the method of applying the probes determines their likely sensitivity. The most widely used methods for gene probes are as labelled probes for use in DNA-DNA or DNA-RNA hybridization or as primer pairs for use in the polymerase chain reaction (PCR) method.

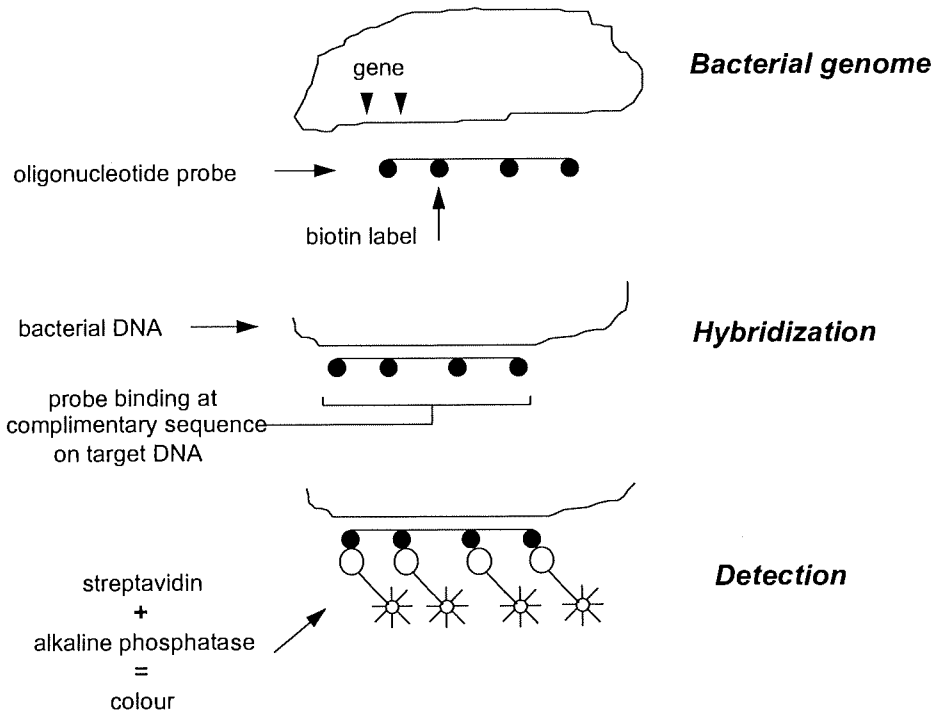

Figure 1.2 DNA-DNA (or RNA) hybridization process

One form of DNA hybridization utilizes a probe which is a short length of single stranded DNA (oligonucleotide probe) of known sequence of nucleic acids that are characteristic of the target bacterium. The probe is labelled with a radioactive marker or a chemical tag such as biotin which can be harnessed to an enzyme system to produce a colour reaction. DNA or RNA is extracted from bacteria or tissue samples and immobilised on a solid support such as a nylon membrane. Presence of the characteristic sequence in the DNA (Fig. 1.2) is revealed by binding of the oligonucleotide probe with its complimentary sequence on the target DNA. Presence of the hybridized DNA is revealed by development of a colour reaction or signal from a radioactive label attached to the probe.

The polymerase chain reaction is based on selective amplification of a unique segment of DNA by making multiple copies of the target sequence. Amplification will only occur if a pair of specific oligonucleotides (primer pair) have hybridized to the target sequence of DNA (Fig. 1.3). Successful amplification of DNA can lead to the formation of approximately a million copies of the target sequence within two or three hours of cycling (Saiki *et al.* 1988). Detection of the amplified sequence is achieved by electrophoretic separation of the DNA and visualization of the DNA by staining with ethidium bromide.

Application of gene probes to medical and veterinary disease diagnosis has greatly increased the sensitivity, specificity and speed of pathogen detection. Probes have been used as a means of rapid identification of bacteria which have been isolated by conventional culture techniques or for the direct detection of bacteria and viruses in host tissues. Use of this technology in the diagnosis of fish diseases has not received much attention although its potential as a tool has been recognised for some time (Anderson & Barney 1991; Vivarès & Guesdon 1992). Initial work in this area has centred largely on developing a suite of gene probes against the more commonly encountered bacterial fish pathogens (Table 1.2).

Selecting an appropriate target sequence of DNA in the bacterial genome is based on at least two factors: specificity and predictability. For the probe to

have any value as a diagnostic tool, it should be able to identify all known strains of a species and not react with any other near related species. Two different strategies have been used for probe development. Empirically

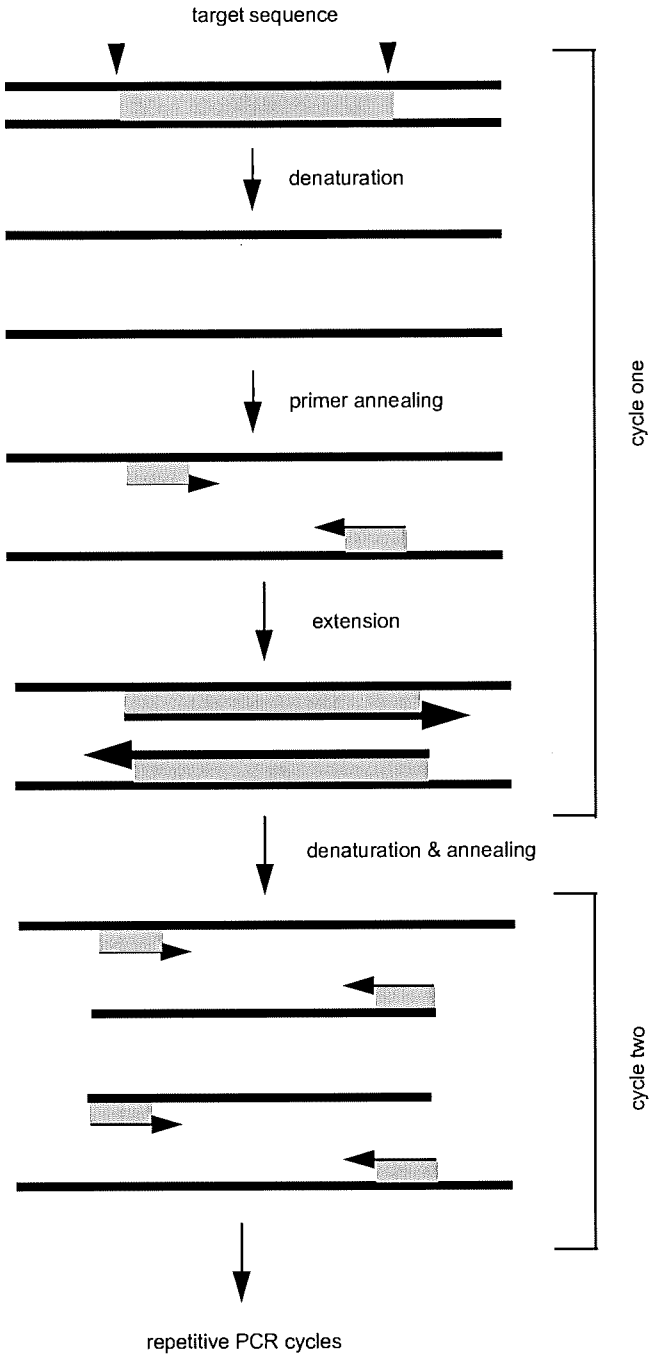

Figure 1.3 Schematic diagram of polymerase chain reaction (PCR) of a target sequence of DNA. The primers (→) define the sequence to be amplified. The three stages of a cycle are denaturation, primer annealing and primer extension. Primer extension occurs by the action of a heat stable polymerase which synthesises a complimentary piece of DNA from individual nucleotides using the intact complimentary strand of DNA as a template. The cycles are repeated to replicate the target sequence of DNA.

Pathogen	Target	Application	Reference
Aeromonas salmonicida	16S rRNA	PCR	Høie *et al.* 1996
	PAAS	PCR	Hiney *et al.* 1992
	*vap*A gene	PCR	Gustafson *et al.* 1992
	Asal-3 clone	PCR	Miyata *et al.* 1996
Aeromonas hydrophila	16S rRNA gene	PCR	Dorsch *et al.* 1994
	lip gene	PCR	Cascón *et al.* 1996
Aeromonas schubertii	16S rRNA gene	PCR	Ash *et al.* 1993
Flavobacterium psychrophilum	16S rRNA gene	PCR	Toyama *et al.* 1994
Mycobacterium marinum	16S rRNA gene	PCR	Knibb *et al.* 1993
Renibacterium salmoninarum	16S rRNA gene	PCR	Magnússon *et al.* 1994
	p57 gene	PCR	Brown *et al.* 1994
	pMAM29 clone	PCR	León *et al.* 1994a
	pMAM29 clone	hybridization	León *et al.* 1994b
	pRS47 clone	hybridization	Hariharan *et al.* 1995
Vagococcus salmoninarum	16S rRNA gene	hybridization	Williams & Collins 1992
Vibrio anguillarum	16S rRNA gene	hybridization	Martínez-Picado *et al.* 1994
	5S rRNA gene	hybridization	Ito *et al.* 1995
	pVaPL63 clone	hybridization	Powell & Loutit 1994
Vibrio ordalii	5S rRNA gene	hybridization	Ito *et al.* 1995
Vibrio penaeicida	16S rRNA gene	PCR	Genmoto *et al.* 1996
Vibrio trachuri	PST-I clone	PCR	Iwamoto *et al.* 1995
Vibrio vulnificus	23S rRNA gene	PCR	Arias *et al.* 1995
	haemolysin gene	PCR	Coleman *et al.* 1996
Yersinia ruckeri	A fragment	PCR	Argenton *et al.* 1996

Table 1.2 Gene probes for polymerase chain reaction or hybridization, for the detection of known bacterial pathogens of fish.

PAAS: probe assay *A. salmonicida* plasmid; *vap*A: surface array protein gene
lip: lipase gene

designed probes use information rich regions of the genome, but tend to be of unknown predictability and specificity. To adequately verify probes of this type, extensive testing with a range of strains and near related species must be undertaken, particularly if the probe is to be used for species identification. An alternative strategy is to develop directed probes which target conserved regions of the genome. Although this type of target sequence is stable and predictive it is often information poor and may be of low specificity. An advantage of using a conserved region however is that once probe specificity is established, the predicability of species identification is high.

Small-subunit ribosomal ribonucleic acid (rRNA) includes 5S, 16S and 23S rRNA and these combined, form the 70S ribosome, a complex cell organelle that mediates protein synthesis. The gene that codes for 16S rRNA is highly conserved (Pace 1973) and contains nucleotide sequences that are remarkably characteristic of the genus. Within this conserved gene of 1,540

nucleotides, there are hypervariable regions, comprising usually no more than 45 nucleotides, which contain characteristic signatures that define a species (Stackebrandt & Goebel 1994).

The predictive characteristic of the 16S rRNA gene has meant that many of the bacterial probes have been designed for this region of the genome (Table 1.2). Development of 16S rRNA probes has been facilitated by the extensive catalogue of sequence data from over 4000 bacterial species held in the GenBank Sequence Database (National Center for Biotechnology Information, Bethesda, Maryland, USA) that has been collected for taxonomic purposes and can be readily accessed by computer via the Internet.

In some instances, where there is insufficient information in the 16S rRNA gene to differentiate species, or where there is incomplete data sets for a genus, other regions of the genome have been selected. Only a few of the probes for fish pathogens are of an empirical, non-16S rRNA design. Notable exceptions are the probes for the *vap*A gene coding for the paracrystalline surface protein layer (A-layer) associated with virulence in *A. salmonicida* (Noonan & Trust 1995) and the p57 gene coding for a unique cell surface antigen in *R. salmoninarum* (Chien *et al.* 1992).

Probes have been used for the detection and identification of fish pathogens either by PCR or hybridization technologies. Most applications have used PCR, a reflection of its superior characteristics of ease of use, sensitivity and speed of result compared to hybridization. The limit of detection, without intermediary culture, cited for hybridization is variously reported to lie between 10^4 and 10^5 cells g^{-1} material (Sayler & Layton 1990; Swaminathan & Feng 1994) and requires at least 24 hours to obtain a result. PCR however with its high level amplification has the capacity empirically to detect a single genome and consequently detection levels by PCR are potentially much better than by hybridization. For many of the fish pathogens cited in Table 1.2, the detection limit can be as low as 1 genome equivalent (5 fg of DNA) for a 16S rRNA target in *A. salmonicida* (Gustafson *et al.* 1992) and as high as 180 genome equivalents (0.89 pg DNA) for the *lip* gene in *A. hydrophila* (Cascón *et al* 1996).

Most studies of fish pathogen probes have been concerned with their functionality and there are few reports concerning their application in routine surveillance of fish populations. The greatest emphasis has been placed on PCR for the detection of the significant fish pathogens *A. salmonicida* and *R. salmoninarum.* The PAAS primer set for *A. salmonicida* has been used in several studies to detect the pathogen not only in the environment but also in asymptomatic fish. The carrier rate in returning wild Atlantic salmon was determined to be 87% but the level of bacteria per fish was considered low, probably less than 1000 bacterial genomes per ml of blood (Mooney *et al.* 1995). No indication was given regarding the biological significance of the findings and it is noteworthy that the authors cite sensitivity as bacterial genomes since it is not possible to equate a genome equivalent with a viable functioning bacterial cell. The usefulness of PCR as a diagnostic tool has been assessed for *R. salmoninarum* (Brown *et al.* 1994). Comparisons were made between a fluorescent antibody test (FAT), enzyme linked immunosorbent assay (ELISA) and PCR of kidney and egg tissues. There was some variability between detection systems but there was a good correlation between PCR data from eggs and other tissues, irrespective of test method. The sensitivity of the PCR test in eggs was high and could detect as little as 2 bacteria per egg.

As a means of monitoring the environment, PCR represents a potentially useful tool. The ability to detect low levels of *A. salmonicida* in complex natural bacterial flora has been used to monitor the presence of *A. salmonicida* in aquatic microcosms (Deere *et al.* 1996) and fish farm effluent water (O'Brien *et al.* 1994). In the latter study, *A. salmonicida* was detected in effluent water from tanks of apparently healthy Atlantic salmon which developed furunculosis 14 days later. PCR, based on 16S rRNA primers, has been used to determine the residence time of a furunculosis vaccine in Atlantic salmon and to determine the distribution of killed cells within the kidney and spleen (Høie *et al.* 1996).

1.7 Perceived need for new diagnostic tests

Development of robust, reliable and sensitive techniques for fish pathogens has been recognised as a priority strategy by the CRC for Aquaculture (Health Focus Group report), the Standing Committee on Agriculture and Resource

Management (Fish Sub-Committee: Task Force on Managing Incursions) in their report of the Aquatic Animal Disease Contingency Planning Workshop and by the Australian Quarantine Inspection Service in their final report on the *Salmon Import Risk Analysis* (Anon 1997). All three organisations have identified the need to develop better diagnostic tools for fish disease diagnosis.

The paucity of adequate procedures in fish disease diagnostic techniques are recognised for:

- identification of bacterial pathogens
- characterisation methods
- rapid procedures for identification
- detection of pathogens in covertly infected fish

The absence of a systematic methodology not only compromises fish disease diagnosis, but is also a major contributing factor to the absence of adequate

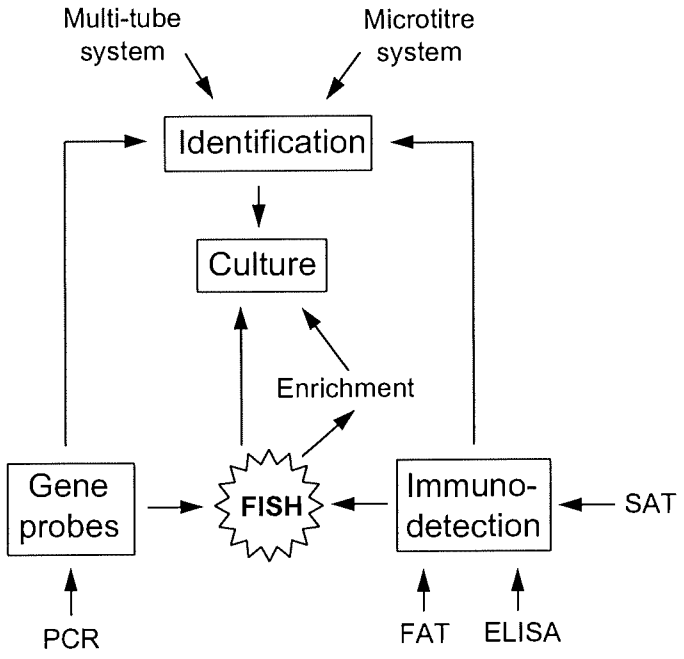

Figure 1.4 Inter-relationship and application of several systems for the detection of bacterial pathogens in fish. Gene probes for PCR can be used for direct detection of pathogens in fish or identification of cultures. FAT and ELISA are used almost exclusively for direct detection of bacteria in acute phase infection of fish, while SAT (slide agglutination test) is used with cultures. Identification systems using phenotypic characterisation employ either conventional tube tests or a microtitre tray format available for *Aeromonas* and *Vibrio* species; *Y. ruckeri*; *Carnobacterium piscicola*, *E. seriolicida* and *Vagococcus salmoninarum*.

data on many facets of fish diseases in Australia. Significant gaps in knowledge include:

- phenotypes of enzootic fish pathogens
- range of pathogens affecting farmed fish
- normal flora of farmed fish
- the nature of covert infection in farmed fish

The current suite of technologies and methodologies is based on conventional culture and some immunological methods, principally FAT and to a lesser extent ELISA. These procedures are targeted at diagnosis of acute phase infection where there is an abundance of pathogen. The techniques are in large measure complimentary (Fig. 1.4), but effective detection strategies may require application of more than one methodology.

Diagnostic techniques are focussed on detection, defined as presence or absence and identity of the pathogen. There is a major dichotomy in detection strategy according to the type of procedures used. **Vital** techniques employ culture to recover the target pathogen whereas **passive** procedures are culture independent and utilise various label systems to signal the presence of target bacteria. Limitations, severally or singly, which apply to existing vital and passive methods, are characterised by:

- poor detection sensitivity
- variable specificity of pathogen identification system
- slow growth rates in culture of some bacterial fish pathogens

FAT and ELISA, as passive systems for detecting several bacterial fish pathogens lack an adequate level of sensitivity for identifying pathogens in carrier fish, and depending upon the type of detection antibody, may also lack specificity. The range of pathogens which can be detected by passive systems is relatively small, confined to the major pathogens such as *A. salmonicida* and *R. salmoninarum*. The absence of suitable techniques for other pathogens limits the application of this tool for disease investigation adequacy of disease investigation.

Improved levels of specificity have been obtained with gene probes, and the potential to achieve useful levels of detection have been implied for gene

probes used in a PCR format. The perceived usefulness of gene probes as a powerful passive detection tool is evinced by the range of probes which have been developed for a variety of fish pathogens (Table 1.2). Like the immunological methods, the greatest emphasis has been placed on developing the utility of probes for *A. salmonicida* and *R. salmoninarum;* there has been little practical development yet of probes for other bacterial species.

Gene probes have the potential to greatly improve specificity and sensitivity for detecting bacterial fish pathogens. The technology can be applied to the direct detection of pathogen in fish tissue or can be used to obtain or confirm the identification of bacteria obtained by a vital technology such as culture. Important objectives for gene probe development that must be realised for the technology to have practical and sustaining benefits are:

- increased range of probes for bacterial fish pathogens
- detection limits lower than that achieved for FAT and ELISA
- assured species specificity

1.8 Project Objectives

The profound absence of adequate and comprehensive diagnostic techniques for fish diseases was identified as a serious and important deficiency in 1992. The need to develop and embrace new technologies is as important now as it was then and the objectives formulated for this project remain relevant for the Australian aquaculture industry.

Several bacterial diseases, enzootic in Australia, have had a demonstrated capacity to cause significance loss in farmed salmonids, or are known to pose serious threat of disease (Table 1.3). The ability to diagnose disease quickly and accurately is important for disease control and management. There is an additional dilemma: the listed pathogens are known to be carried

Pathogen	Disease
Aeromonas salmonicida (atypical)	Ulcer diseases, flounder and goldfish
Enterococcus seriolicida	Streptococcaceosis
Flexibacter maritimus	Cutaneous erosion disease
Yersinia ruckeri	Yersiniosis

Table 1.3 Enzootic pathogens known to cause disease or pose a threat of disease in Australian farmed salmonids

asymptomatically in fish and an ability to detect these bacteria is essential in making appropriate quarantine, health monitoring and treatment decisions. Isolation of pathogens is readily achievable in overtly diseased fish, but there is a lack of practical and sensitive techniques to detect these bacteria in other disease conditions (Table 1.4).

Pathogen	Culture	IFAT	ELISA	PCR
A. salmonicida	✓	✓	✓	✓
E. seriolicida	✓	✓	✗	✗
F. maritimus	✓	✗	✗	✗
Y. ruckeri	✓	✓	✓	✗

Table 1.4 Detection technologies available for major temperate fish pathogens in Australia

The objectives developed for the project are as listed (Table 1.5) and remain unchanged from the original proposal except for the inclusion of objective 4 that was added towards the end of the project.

No.	Objective
1.	Develop procedures for testing fish by molecular probes for the presence of bacterial fish pathogens.
2.	Develop methodologies using molecular probes for the rapid identification of bacterial fish pathogens recovered by conventional culture techniques.
3.	Develop molecular probes for enzootic strains of *Aeromonas salmonicida*, *Enterococcus seriolicida*, *Flexibacter maritimus* and *Yersinia ruckeri*.
4.	Develop a secondary confirmation system for PCR assays using internal probes and hybridization
5.	Formulate the developed test procedures for transfer to, and adoption by, veterinary diagnostic and research laboratories.

Table 1.5 Project objectives

Realisation of the objectives will result in the development of:

- rapid and accurate tools for the identification of bacterial fish pathogens
- methodologies for the rapid and accurate detection of some bacterial fish pathogens in fish tissue

Acquisition of these tools must be seen as a significant advance in disease diagnostic capability and a tangible means to maintaining Australia's disease free status in aquaculture and wild fisheries.

Section 2

Materials and Methods

2.1 Bacterial Cultures

Reference cultures, as species type strains, were obtained from culture collections as listed in Table 2.1.

Collection	Species	Strain number	Source
ATCC	*Aeromonas caviae*	15468	UTS
	Aeromonas eucrenophila	23309	UTS
	Aeromonas hydrophila	7966	UTS, ACM
	Aeromonas jandaei	49568	UTS
	Aeromonas media	33907	UTS
	*Aeromonas salmonicida**	14174†	CSIRO
	Aeromonas schubertii	43700	UTS
	Aeromonas sobria	43979	UTS
	Aeromonas veronii biotype *sobria*	9071	UTS
	Aeromonas veronii biotype *veronii*	35624	UTS
	Aeromonas trota	49567	UTS
	Carnobacterium piscicola	35586	ACAM
	Cytophaga marinoflava	75	ACAM
	Clostridium putrificum	25784	ACM
	Enterococcus seriolicida	49156	HWU
	Flavobacterium johnsoniae	17061	UNSW
	Haemophilus influenzae	33391	ACM
	Porphyromonas asaccharolytica	25260	ATCC
	Vibrio ordalii	33509	CSIRO
	Vibrio splendidus I	25914	ATCC
	*Yersinia ruckeril**	29473	CSIRO
NCFB	*Lactococcus garvieae*	2155	NCFB
	Lactococcus piscium	2778	NCFB
	Vagococcus salmoninarum	2777	NCFB
NCIMB	*Flavobacterium columnare*	2248	CSIRO
	Flexibacter maritimus	2154	CSIRO
	Flexibacter ovolyticus	13127	CSIRO
ACM	*Erwinia herbicola*	852	ACM
	Flavobacterium odoratum	3100	ACM

Table 2.1 Type strains used for development and testing of fish pathogen gene probes.

ACAM: Australian Collection of Antarctic Micro-organisms; ACM: Australian Collection of Micro-organisms, Queensland University; ATCC: American Type Culture Collection; CSIRO: Commonwealth Scientific & Industrial Research Organisation; HWU: Heriot-Watt University; NCFB: National Collection of Food Bacteria; NCIMB: National Collection of Industrial & Marine Bacteria; UNSW; University of New South Wales; UTas: University of Tasmania; UTS: University of Technology, Sydney

*Only as purified DNA; these strains are exotic to Australia

†Neotype strain

In addition to these reference strains, named isolates of the bacterial fish pathogens *A. salmonicida, E. seriolicida, Fx. maritimus* and *Y. ruckeri* from the collections of the Fish Health Unit of the Department of Primary Industry & Fisheries and the Fish Disease Laboratory, Australian Animal Health

Laboratory, CSIRO. These strains, listed in Tables 2.9 - 2.16, were used in verifying the utility of the gene probes. Additional species for verifying specificity of the probes are listed in Table 2.2.

Species	Strain number	Collection	Source
Aeromonas hydrophila	7965	ATCC	NS
Aeromonas bestiarum	14715	ATCC	NS
Aeromonas encheleia	35941	ATCC	NS
Aeromonas hydrophila	216, 219	SWB[1]	River water
Aeromonas hydrophila	37, 41, 48, 56, 65, 66, 67, 68, 69, 70, 71a, 72a, 73a	UTS[2]	NS
Citrobacter freundii	90/2624-18	DPIF	Angel fish
Enterococcus faecalis	ATCC 29212	Difco	NS
Enterococcus seriolicida	83/35051	CSIRO[3]	Rainbow trout
Enterococcus seriolicida	7876	VRIO[4]	Rainbow trout
Enterococcus seriolicida	NG8206	MU[5]	Yellowtail
Enterococcus seriolicida	HP9501-1	MU	Yellowtail
Escherichia coli	ATCC 25922	Difco	NS
Flexibacter maritimus	lyl-1	UNSW[6]	Japanese flounder
Flexibacter maritimus	NCIMB 2158	CSIRO	Dover sole
Flexibacter maritimus	JIP 32/91(5)	INRA[7]	Sea bass
Hafnia alvei	95/6404	DPIF	Atlantic salmon
Lactococcus garvieae	NCFB 2157	NCFB	Milk
Proteus vulgaris	96/5494	DPIF	Bovine faeces
Pseudomonas fluorescens	92/3556-2-16	DPIF	Atlantic salmon
Streptococcus iniae	95.41693/4A	QDPI[8]	Barramundi
Vibrio anguillarum	85/3475-1	DPIF	Rainbow trout
Yersinia enterocolitica	96/5440-1B	DPIF	Ovine faeces
Yersinia intermedia	92/4041	DPIF	Atlantic salmon
Yersinia pseudotuberculosis	96/5417-2	DPIF	Bovine faeces

Table 2.2 Bacterial species for verifying PCR primer specificity

ATCC: American Type Culture Collection; CSIRO: Commonwealth Scientific & Industrial Research Organisation; Difco Laboratories, Detroit, USA; DPIF: Department of Primary Industry & Fisheries; INRA: Institut National de la Recherche Agronomique, France; MU: Miyazaki University, Japan; NCFB: National Collection of Food Bacteria; SWB: Sydney Water Board; QDPI: Queensland Department of Primary Industry; VRIO: Veterinary Research Institute, Onderstepoort, South Africa; UNSW: University of New South Wales; UTS: University of Technology, Sydney. NS: not stated

[1]M. Dorsch; [2]J. Oakey; [3]N. Gudkovs; [4]R. Bragg; [5]T. Yoshida; [6]P. Beatson; [7]J-P Bernardet; [8]A. Thomas

All cultures were held in primary storage freeze-dried; secondary storage was at -80°C in peptone water + 8% v/v glycerol. Working cultures were maintained on appropriate culture media as listed in Table 2.3.

2.2 Sequence data

Availability of 16S rRNA sequence data for *A. salmonicida, E. seriolicida, Fx. maritimus* and *Y. ruckeri* was determined by searching the holdings of the

GenBank Sequence Database (National Center for Biotechnology Information, Bethesda, MD, USA). Recent additions had been made for *Fx. maritimus* (Gherna & Woese 1992) and *Y. ruckeri* (Ibrahim *et al.* 1993), generated in the course of taxonomic studies to determine the phylogeny of these two groups of bacteria. The sequence data for the 16S rRNA gene for both species were complete and suitable for identifying likely unique sequences for PCR that would be characteristic of the species.

Taxon	Medium	Incubation temp.
Aeromonas spp	Sheep's blood agar[1]	25°C
Clostridium spp	Sheep's blood agar, cooked meat medium	37°C
Cytophaga spp	Marine Ordal's medium	25°C
Enterobacteriaceae	Sheep's blood agar or peptone yeast extract agar	37/25°C[1]
Flavobacterium spp	YEPP medium	25°C
Gliding spp	Freshwater Ordal's medium	25°C
Flexibacter spp	Marine Ordal's medium	25°C
Haemophilus spp	Chocolate blood agar	37°C
Porphyromonas sp	Sheep's blood agar, cooked meat medium	37°C
Pseudomonas spp	Sheep's blood agar	25°C
Streptococcaceae	Sheep's blood agar	37/25°C[3]
Vibrio spp	*Vibrio* nutrient agar	25°C

Table 2.3 Maintenance media for fish pathogens and reference strains
[1]Media details: see Appendix; *Y. ruckeri* and *Y. intermedia*: 25°C; [3]*E. faecalis*: 37°C

Sequences for several strains and subspecies of *A. salmonicida* had been determined for the 16S rRNA gene (Martinez-Murcia *et al.* 1992). It was decided however, that since the strain of *A. salmonicida* isolated from greenback flounder appeared to be a unique strain (J. Carson & K. Pinkard unpublished data) and may be naturally occurring in Australia, sequencing of this strain was considered essential. No sequences had been lodged with GenBank for *E. seriolicida* and sequencing of the 16S rRNA gene of this species was also undertaken.

2.3 Sequencing

Strains Type strain ATCC 49156 of *E. seriolicida* and DPIF strain 93/1061-1 (designated type strain) of *A. salmonicida* isolated from greenback flounder, were used for sequencing.

DNA extraction *A. salmonicida* was grown in 500ml Brain Heart Infusion broth (Difco Laboratories, Detroit, MI, USA) at 25°C for 72 h the cells collected

by centrifugation and resuspended in saline-EDTA. Lysozyme was added to a final concentration of 2mg ml^{-1} and incubated for 1 h at 60°C. *E. seriolicida* was grown at 25°C in 250 ml Todd-Hewitt broth (Oxoid, Basingstoke, England) supplemented with 2 g l^{-1} DL-threonine, a Gram-positive cell wall modifier, added to facilitate cell lysis (Klaenhammer *et al.* 1978); after 18 h incubation, 0.12g l^{-1} crystalline penicillin G (Komatsu 1979) and 0.05g l^{-1} vancomycin were added. The cells were incubated for a further hour, collected by centrifugation and resuspended in saline-EDTA. Lysozyme was added to a final concentration of 10 mg ml^{-1} and incubated for 36 h at 60°C. DNA was extracted from the cell lysates by the general method of Marmur (1961). Integrity and purity of the DNA was determined on 0.8% agarose/TBE buffer gel containing ethidium bromide at a final concentration of 0.5 μg ml^{-1}.

Amplification of 16S rDNA The bacterial 16S rRNA gene was selectively amplified by polymerase chain reaction (Saiki *et al.* 1985). Highly conserved sequences at each end of the the gene were used as priming sites for synthetic oligonucleotides primer A and primer H (Edwards *et al.* 1989) (Table 2.4) and allows almost the entire gene of 1500 bases to be amplified. PCR was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Foster City, CA, USA) with Perkin-Elmer reagents. Ten replicate reactions, each 100 μl, were

Primer	Location	Sequence	Reference
A	8-27	AGAGTTTGATCCTGGCTCAG	Edwards *et al.* 1989
26	360-341	CCCACTGCTGCCTCCCGTAG	Edwards *et al.* 1989
19	519-536	CAGCAGCCGCGGTAATAC	Edwards *et al.* 1989
27	536-519	GTATTACCGCGGCTGCTG	Edwards *et al.* 1989
20	685-704	GTAGCGGTGAAATGCGTAGA	Embley *et al.* 1988
WS5	704-685	TCTA**T**GCATTTCACCGCTAC	Embley *et al.* 1988
23	907-926	AAACTCAAAGGAATTGACGG	Edwards *et al.* 1989
21	1100-1115	CAACGAGCGCAACCCT	Embley *et al.* 1988
WS6	1245-1226	**CTA**CCATTGTAGCACGTGTG	Stackebrandt & Charfreitag 1990
WS8	1392-1406	GTAACACACCGCCCGT	Embley *et al.* 1988
H	1541-1522	AAGGAGGTGATCCAGCCGCA	Edwards *et al.* 1989

Table 2.4 Oligodeoxynucleotide primers used for sequencing and PCR. Location by *E.coli* numbering (5' → 3') (Neefs *et al.* 1993). Nucleotides underlined and in bold differ from the published sequenced

undertaken using 10 ng template genomic DNA, 50 pmol of primers A and H, 10μl 10x PCR buffer II, 200μM each dNTPs, 1.0 mM $MgCl_2$, 2.5 units of *AmpliTaq*™ DNA polymerase and 18 MΩ purified water. Amplification

parameters were denaturation for 1 min at 94°C, primer annealing at 55°C for 2 min and extension for 6 min at 72°C (Edwards *et al.* 1989).

Long amplification sequences could not be achieved at the time, and consequently shorter lengths within the gene were amplified using secondary internal sense and anti-sense primers as listed in Table 2.4. The spatial relationship of the primers is given in Figure 2.1.

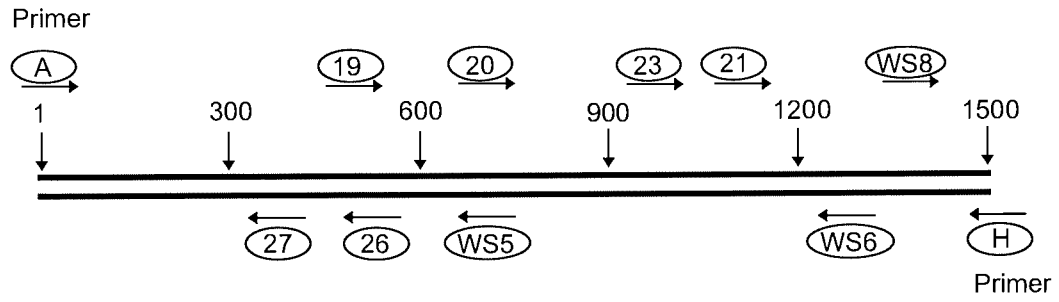

Figure 2.1 Target sites for internal sequencing primers on the 16S rRNA gene
1 is first base pair, 1500 is last base pair of the 16S rRNA gene

Purification of PCR product PCR products were separated on 1% agarose/TAE buffer gel containing ethidium bromide at a final concentration of 0.5 μg ml^{-1}. DNA of an appropriate size, visualized by UV transillumination, was excised from the gel and extracted from the agarose and subsequently purified using a GeneClean II® kit (BIO 101 Inc., Vista, CA, USA).

Sequencing of amplified 16S rDNA The dideoxynucleotide chain termination sequence method (Sanger *et al.* 1977), adapted for double stranded PCR product (Bachmann *et al.* 1990) and modified by Dobson (1993) was used to sequence the 16S rRNA gene. The methodologies were implemented using the Sequenase™ Version 2.0 Kit (United States Biochemical Corp., Cleveland, OH, USA) incorporating the Sequenase™ genetically engineered bacteriophage T_7 DNA polymerase. Amplified fragments of DNA were labelled with α-^{35}S-dATP (12.5 mCi ml^{-1}).

The sequencing primers were as listed in Table 2.1. For *A. salmonicida* primers A, H, 20, 26, WS5, 27, 19, 23, WS6 and 21 were used and for *E. seriolicida* primers A, H, WS5 19, 27, 21, 23 and WS8 were used. The

primers were used in combinations that ensured that regions of the 16S rRNA gene were sequenced at least twice and any ambiguous sequences were re-determined until a consistent result was obtained. Where this could not be achieved, an 'n' was recorded in the sequence to denote an unresolvable nucleotide.

The annealing reaction was achieved by combining 1 μl of sequencing primer (10 pmol μl^{-1}), 1 μl of DNA (1 μg μl^{-1}), 2 μl 5x Sequenase™ reaction buffer, 1 μl nonidet P40 detergent (20%) and 5 μl water (18 MΩ), boiling the mixture for 3 min and snap freezing in the vapour phase of liquid nitrogen.

Preliminary incorporation of the radioactive marker, α-^{35}S-dATP, into short extensions was achieved by the addition to 10 μl of annealing reactant of: 1 μl of 0.1M dithiothreitol, 2 μl of dGTP/dCTP/dTTP mix (0.5 mM each), 1.5 μl of α-^{35}S-dATP (12.5mCi ml^{-1}) and 2 μl of Sequenase™ version 2.0 diluted 1:8 in Sequenase™ buffer. Incorporation was allowed to occur at room temperature for between 3 for short inter-primer distances and 5 min for bigger distances between primers.

Termination reactions were performed by dividing the incorporation mixture into 4 equal parts of 3.5 μl. Each volume received 2.5 μl of one dideoxynucleotide, ddATP, ddCTP, ddGTP or ddTTP pre-warmed to 37°C. The mixtures were incubated at 37°C for 3 min and the reaction stopped by the addition of a formamide based stop solution (United States Biochemical).

Additional reactions were performed using the dGTP analogue dITP (deoxyinosine 5'-triphosphate) to promote denaturation of DNA secondary structures during electrophoresis (Sambrook *et al.* 1989).

Electrophoresis Separation of labelled DNA was performed by polyacrylamide gel electrophoresis in a temperature controlled Pharmacia LKB Macrophor sequencing system (Amrad, Melbourne). Labelled DNA was denatured at 90°C for 3 min and then separated in 6% denaturing (7M urea) polyacrylamide gel in 1x TBE buffer at 55°C at 1800V. All four ddNTP reactions were run alongside each other together with the reactions generated with dITP.

Lanes were loaded at 0 h and new lanes loaded after 3 h to give a total running time of 5 and 2 h respectively. The sequences, based on size, overlapped and gave a combined range of 200-300 bases of readable sequence per primer. Gels were fixed in 10% acetic acid, air dried and exposed for 18-24 h to XOMAT-AR film (Eastman Kodak, Rochester, NY, USA).

Sequence alignments Sequences were read visually and the data stored for analysis using Seqspeak 1.0 DNA Sequence Editor program (K. Conover 1991, Dalhousie University, Nova Scotia, Canada). Published sequences for species of the type genera were obtained in a pre-aligned form from the Ribosomal Database Project (RDP) (University of Illinois, Urbana-Champaign, Il, USA) (Olsen *et al.* 1991) and aligned manually with the new sequences using the HOMED program (Homologous Sequence Editor) accessed through ANGIS (Australian National Genomic Information Service).

Species	Strain/Accession no.
A. salmonicida ssp. *salmonicida*	RDP Arm.sp9533 = CDC 9701-84 = NCIMB 1102^T
A. salmonicida ssp. *achromogenes*	NCIMB 1110^T
A. salmonicida ssp. *masoucida*	NCMB 2020^T
A. hydrophila	ATCC 7966^T
A. trota	ATCC 49657^T
Aeromonas sp Hybridization Group 2	RDP Arm.sp9701 = CDC 9533-76
Enterococcus faecalis	RDP Eco.faecal
E. sulphureus	NCDO 2379^T
Escherichia coli	GSDB J01695
Flectobacillus glomeratus	ATCC 43844^T
Flexibacter. aggregans	ATCC 23162^T
Fx. maritimus	ATCC 43398^T
Lactococcus lactis ssp. *lactis*	GSDB M54921
L. lactis ssp. *cremoris*	ATCC 19257^T
L. garvieae	NCDO 2156^T
L. raffinolactis	NCDO 617^T
L. piscium	NCFB 2778^T
L. plantarum	NCDO 1869^T
Streptococcus bovis	ATCC 33317^T
Antarcticum vesiculatum	GenBank M61002
Y. enterocolitica	ATCC 9610^T
Y. intermedia	GenBank X75279
Y. ruckeri	ATCC 29473^T
Y. aldovae	ATCC 35236^T
Y. rohdei	ATCC 43380^T
Y. pestis	ATCC 19428^T

Table 2.5 Sequences used for probe alignments.
Strain suffix ^{T} denotes type strain; CDC = Communicable Disease Center, Atlanta, USA

The 16S rRNA gene sequence of *A. salmonicida* DPIF 93/1061-1, *E. seriolicida* ATCC 49156^T, *Fx. maritimus* NCIMB 2154^T and *Y. ruckeri* ATCC 29473^T were compared with sequences of other species as listed in Table 2.5; sequence data were obtained from EMBL (European Molecular Biology Laboratory), GSDB (Genome Sequence DataBase, National Center for Genome Resources, Santa Fe, New Mexico, USA) and RDP.

Probe/PCR primer design The alignments for *A. salmonicida* DPIF 93/1061-1, *E. seriolicida* ATCC 49156^T, *Fx. maritimus* NCIMB 2154^T and *Y. ruckeri* ATCC 29473^T were examined visually for regions of greatest dissimilarity compared to near relatives. Potential probe site sequences were then compared for similarity to sequences listed in RDP and GSDB within the categories of bacteriophage, bacteria, structural RNAs and viruses to establish the occurrence of homology within these biota and to identify sequences with high similarity. Searching was undertaken using the FAST A program based on the Lipman-Pearson algorithm (Lipman & Pearson 1985) and by BLAST (Basic Local Alignment Search Tool). The probes were selected on the basis of their suitability for amplification by the PCR process.

Internal probes for PCR verification. The 16S rRNA sequences of *E. seriolicida, Fx. maritimus* and *Y. ruckeri* were aligned with the sequences of the nearest genomic neighbours (Table 2.6) identified during PCR primer design. By manual inspection of the nucleotide sequence between respective

Species	Homology
Aeromonas salmonicida	100%
A. sobria	100%
A. eucrenophila	94%
A. jandaei	94%
A. schubertii	94%
A. veronii	94%
A. hydrophila	85%
A. caviae	76%
A. trota	76%
A. media	75%
Enterococcus seriolicida	100%
Carnobacterium piscicola	76%
Clostridium putrificum	76%

Species	Homology
Flexibacter maritimus	100%
Cytophaga marinoflava	65%
Flavobacterium odoratum	70%
Porphyromonas assaccharolytica	70%
Yersinia ruckeri	100%
Y. bercovieri	100%
Y. pestis	96%
Y. pseudotuberculosis	96%
Erwinia herbicola	96%
Proteus vulgaris	92%
Y. kristensenii	92%
Haemophilus influenzae	67%

Table 2.6 Species with close genomic sequence homology to PCR primers. % homology is in respect of the primer of closest match.

primer pairs, specific internal probes of between 26 and 33 base pairs were identified for the three bacteria. The uniqueness of these sequences was established by a BLAST search of GSDB and RDP.

2.4 rRNA Hybridization

RNA extraction Bacteria were grown in 10 ml of appropriate broth (Appendix) and cells from 2 ml of culture were collected by centrifugation, washed twice in 0.1M phosphate buffer pH 7.0 and the cells pelleted by centrifugation. Ribosomal RNA was extracted from the cells using a Rapid RNA Extraction Kit (Amresco, Solon, OH, USA). RNA was extracted from *E. seriolicida* by pre-treating the cells with lysozyme and mutanolysin. Pelleted cells were resuspended in 200 µl of 0.1M phosphate buffer pH 6 containing 1 mg lysozyme and 0.3 mg mutanolysin (Galán & Timoney 1988) and incubated at 37°C for 2.5 h with intermittent mixing. The cells were pelleted and RNA extracted with the Amresco kit. The precipitated RNA was dissolved in 50 µl of water. Contaminating DNA was removed by treating RNA extracts with RNase free DNase I (Sigma, St Louis, MI, USA) at a final concentration of 2µg ml^{-1} and incubating at 37°C for 90 min. RNA was precipitated in ice cold ethanol and collected by centrifugation. Purity of the preparations was determined by gel electrophoresis using 1% agarose, TBE buffer and ethidium bromide at a final concentration of 0.5 µg ml^{-1}. Total RNA concentration was determined by measuring OD_{260}.

Hybridization temperatures Empirical hybridization temperatures T_{HYB} and wash temperatures T_W were determined using the equation of Casey & Davidson (Sambrook *et al.* 1989) for DNA:RNA hybridizations. Starting point hybridization temperatures were calculated as: *A. salmonicida*: 54°C, *E. seriolicida*: 50°C, *Fx. maritimus*: 52°C and *Y. ruckeri*: 50°C.

Blotting Dot blots of purified RNA were prepared by vacuum blotting on nitrocellulose. A range of RNA concentrations were used: 20, 10, 5, 2.5, 1.25 µg, 625, 313 and 156 ng total RNA. The nitrocellulose was rehydrated with 10x SSC, and then each well rinsed with 500µl of 10x SSC. The RNA samples were denatured in formaldehyde/SSC buffer and 200 µl of sample added to a

slot. Each slot was washed twice with 100µl of 6xSSC under gentle vacuum. Binding of RNA was achieved by baking the nitrocellulose at 80°C for 2 h.

Probes The probes for *A. salmonicida* (probe As 2), *E. seriolicida* (Es 9), *Fx. maritimus* (Flex 3) and *Y. ruckeri* (Yr 1) were synthesized as desalted oligonucleotides with a biotin label (Bresatec, Adelaide). The sequences for the probes are given in Table 2.7.

Hybridization Blots were pre-hybridized in hybridization buffer (5 x SSC, 5 x modified Denhardt's solution, 0.5% SDS) containing 100 µg ml^{-1} of denatured salmon sperm DNA, at 40°C for two h in a Hybaid hybridization oven. Pre-warmed hybridization buffer containing either 100 or 200 ng ml^{-1} of biotinylated probe was added to the blots and hybridization allowed to proceed for 15 h at 40 or 45°C. Blots were washed once for 15 min with 2 x SSC, 0.1% SDS at room temperature followed by two washes of 15 min each of 0.1 x SSC, 0.1% SDS or the more stringent wash solution of 0.05% x SSC, 0.1% SDS. Wash temperatures ranged between 40 and 70°C.

Detection Hybridized RNA:DNA was detected on the blots using the BluGene® system (Gibco-BRL, Gaithersburg, MD, USA), a streptavidin-alkaline phosphatase reaction producing a blue colour with a nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate substrate.

2.5 Polymerase Chain Reaction

2.5.1 DNA Extraction

Rapid extraction Sufficient bacterial colony to fill the aperture of a 1 µl sterile disposable loop was taken from an agar plate, the cells suspended in 100 µl sterile 18 MΩ water in a microfuge tube, heated at 100°C in a thermal block for 10 min. and then cooled on ice for 5 min. Tubes were centrifuged at 15,000***g*** for 5 min. to pellet the cell debris and the supernatant containing crude DNA was transferred to a new tube and stored at -20°C.

Purified DNA Approximately $5x10^7$ cells, equivalent to 1 ml of overnight broth culture were pelleted by centrifugation at 3,000***g*** for 3 min. The cells were resuspended in 100µl of sterile 18 MΩ water, 900µl of DNAzol™ (Gibco-BRL) added and the cells lysed by gently pipetting the mixture. Released nucleic

acids were precipitated by the addition of 500µl of ethanol and pelleted by centrifugation for 10 min. at 15,000***g***. The DNA was washed twice in 95% ethanol, dried under vacuum and then dissolved in the minimum amount of sterile water or 10mM Tris buffer, pH 8.0 before storing at -20°C. *E. seriolicida* was lysed by suspending washed cells in 20µl of lysozyme (100 mg ml^{-1}), 20 µl of RNase (10mg ml^{-1}) and 45µl of proteinase K (20 mg ml^{-1}) and incubating at 37°C for 30 min. After incubation, 1 ml of DNAzol was added and extraction performed as described.

DNA extraction from fish tissues Atlantic salmon, 20-30g weight were obtained from a freshwater hatchery in Tasmania. The fish were dissected with aseptic precautions and 50 mg samples of skin, kidney, brain and gut tissues were collected. The samples were homogenised using disposable pestles for 1.5 ml microfuge tubes (Kontes, Vineland, NJ, USA). DNA was isolated from the tissues using a QIAamp® extraction system (QIAGEN, Melbourne) with the following modifications to enhance extraction. To a homogenised sample of 50mg of tissue, 320 µl of ATL buffer plus 40µl of Proteinase K (18mg ml^{-1}) were added and incubated with gentle mixing at 55°C for 15 h. Extraction then proceeded as described by QIAGEN. A double elution step to maximise DNA recovery was undertaken as recommended in the QIAGEN protocol. For tissue samples containing *E. seriolicida*, a preliminary cell lysis treatment was used to enhance DNA extraction from Gram-positive bacteria. To the homogenised tissue, 40µl of lysozyme (50mg ml^{-1}) was added and incubated at 37°C for 30 min. DNA extraction then proceeded as described. DNA was stored at -20°C.

Assessment of purity DNA concentration was determined by measuring absorbance at 260 nm in a GeneQuant spectrophotometer (Pharmacia Biotech, Amrad, Melbourne) and purity assessed visually following electrophoresis on 1% agarose gel in TAE buffer containing ethidium bromide (0.5µg ml^{-1}). Band sizes were estimated from DNA markers prepared from an *Eco* R1 digest of bacteriophage SPP-1 (Bresatec, Adelaide), size range 0.36-8.5 Kb.

2.5.2 PCR protocols

Un-nested, 'Standard' PCR A standardised PCR format was used for *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri*. The primer pairs for PCR are listed in Table 2.7

Species	Primer	Direction	Sequence 5' → 3'
Flanking primers	A	forward	AGA GTT TGA TCC TGG CTC AG
	785R	reverse	GTG GAC TAC CAG GGT ATC TAA TCC
A. salmonicida	As1	forward	TTT CGC GAT TGG ATG AA
	As2	reverse	TTG ACA CGT ATT AGG CGC CA
E. seriolicida	Es9	forward	CGA GCG ATG ATT AAA GAT AGC TTG CTA
	Es6	reverse	ATA AGA ATC ATG CGA TTC TCA
Fx. maritimus	rFlex1	forward	GGA ATG GCA TCG TTT TAA AG
	rFlex3	reverse	AAT ACC TAC TCG TAG GTA CG
Y. ruckeri	Yr2	forward	AAC CCA GAT GGG ATT AGC TAG TAA
	Yr1	reverse	GTT CAG TGC TAT TAA CAC TTA ACC C

Table 2.7 Primer sequences specific for the bacterial fish pathogens *A. salmonicida, E. seriolicida, Fx. maritimus* and *Y. ruckeri*

Each PCR contained the deoxynucleotides, adenine, thymine, guanine, cytosine, at a concentration of 200 μM each, 2.5 mM $MgCl_2$, PCR buffer IV (25mM ammonium sulphate, 75mM Tris-HCl [pH 9.0], 0.01% Tween 20) (Advanced Biotechnologies, Surrey, UK), 50 pmol each primer (de-salted and de-protected, Gibco-BRL), 1.0 U *Taq* DNA Polymerase (Advanced Biotechnologies) and the appropriate template DNA. The PCRs were performed as 20μl volumes in thin walled PCR tubes either in a Corbett Research FTS-960 (Mortlake, NSW) or an MJ Research PTC100-96V (Watertown, Mass., USA) thermocycler with heated lid. The reaction parameters included an initial 3 min denaturation at 94°C, followed by annealing for 30 s. at 65°C and extension for 2 min at 72°C followed by 36 cycles consisting of 94°C for 30 s, 65°C for 30 s and 72°C for 2 min, with an additional final extension at 72°C for 4 min. In determining specificity of the probes, the protocol was modified by reducing the concentration of primer to 40 pmol and using 'Red Hot Polymerase' (*Taq*) (Advanced Biotechnologies) at 0.5 U.

Un-nested *A. salmonicida* PCR The PCR conditions as described were used with the following modifications: magnesium ion concentration was reduced to 1.375 mM of $MgCl_2$, the annealing temperature reduced to 60°C and the total number of amplification cycles limited to 30.

Nested PCR To increase sensitivity, a nested PCR using the flanking primers A and 785r (Table 2.7) in the first round and species specific primer pairs in the second round were used. Flanking primer A (position 12-31 [*E. coli* numbering] on 16S rRNA gene) and 785r (position 819-842) extends from the beginning to approximately the centre of the 16S rRNA gene and includes the unique regions of the four bacterial pathogens. PCR components were as described for the standard PCR but the reaction parameters were modified by lowering the annealing temperature to 37°C; all other cycling conditions were as described for the standard PCR. In the second round of the PCR, 1μl of the first round reaction was used as the template DNA. The PCR conditions were as described for the Standard PCR using the species specific primers.

PCR integrity The quality of template DNA was assessed by PCR using the A, 785r primer set. DNA samples were only considered suitable if they could be amplified with this primer set.

PCR product Presence or absence of PCR product was determined by running a portion of the PCR reaction mixture in a 2% agarose gel containing 0.5μg ml^{-1} ethidium bromide in TAE or TBE buffer. To 20μl of PCR reaction mixture, 4μl of 6x gel loading buffer was added and depending on well size, either 6 or 8μl samples were used in 8 or 14 well combs respectively. Included with each gel were reference markers prepared from an *Hpa* II digest of the plasmid pUC19 (Bresatec) with a size range of 26-501 bp or a 100 bp ladder (Advanced Biotechnologies), size range 100-1000 bp. Gels were run in a Horizon 58 unit (Gibco-BRL) at 70V for 40 min. A PCR was considered positive when a band of the expected size was detected by transillumination UV light at 300 nm (Ultra-Lum, Carson, CA, USA). Expected band sizes are given in Table 2.8.

Species	Expected size
A. salmonicida	260 base pairs
E. seriolicida	143 base pairs
F. maritimus	285 base pairs
Y. ruckeri	223 base pairs
A-785r	~ 785 base pairs

Table 2.8 Expected band sizes of PCR products for target pathogens

Quality control For each assay, template DNA from the type strain as known positive control was included; for a negative control, template DNA was replaced with 18MΩ water and was always prepared as the last PCR reaction to be set up. To control contamination (Kwok & Higuchi 1989), all reactions were set up using barrier pipette tips, reagents and disposable plasticware, where possible, were autoclaved at 121°C for 30 min, reagents were aliquoted prior to use and zoned work areas were used within the laboratory.

2.5.3 Specificity

Intra-species The reliability of the primers with respect to strain variation was assessed for each species. Isolates/strains were drawn from the collections as described in Tables 2.1 and 2.2 and from the holdings of the Fish Health Unit, DPIF as given in Tables 2.9-2.12. Crude DNA extracts as described were prepared from 24-48 h plate cultures and un-nested PCR reactions undertaken with appropriate primer sets and PCR formats as described.

A. salmonicida	Host	*A. salmonicida*	Host
3339	goldfish	93/0956-2	greenback flounder
81:4320	goldfish	93/1061-1	greenback flounder
84:9062-B1	goldfish	93/1171-3	greenback flounder
85:5152	goldfish	93/1586	greenback flounder
85:9370-A	goldfish	93/6420-K	Atlantic salmon
87:1147	koi carp	93/6704-10	greenback flounder
87:1928-B	goldfish	940121	goldfish
87:4863-C	goldfish	AFHRL 1	goldfish
88:5350	goldfish	AFHRL 6	goldfish
890657	goldfish	ATCC 14174	brook trout neotype strain
910390-1P	goldfish	CSL 1107/1B	goldfish

Table 2.9 Bacterial strain numbers for *A. salmonicida* used in PCR specificity tests
Goldfish & carp strains from the Fish Disease Laboratory, AAHL, CSIRO

Y. ruckeri	Host	*Y. ruckeri*	Host
88/3873	Atlantic salmon	91/4311-A7	Atlantic salmon
88/4359-1K	Atlantic salmon	94/4853	Atlantic salmon
90/3988	Atlantic salmon	95/4881-2	Atlantic salmon
90/4162-K	Atlantic salmon	96/0283	Atlantic salmon
90/4411-3	Atlantic salmon	96/0977-2	Atlantic salmon
90/4455-2	Atlantic salmon	96/5134	Atlantic salmon
90/4505	Atlantic salmon	ATCC 29473^T	rainbow trout
91/4311-A10	Atlantic salmon		

Table 2.10 Bacterial strain numbers for *Y. ruckeri* used in PCR specificity tests

E. seriolicida	Host	*E. seriolicida*	Host
88/0598	rainbow trout	88/3625-3	rainbow trout
88/1400-1K	rainbow trout	88/3873-2	rainbow trout
88/1508-R	rainbow trout	88/3998-69	rainbow trout
88/1592-2	rainbow trout	89/1593	rainbow trout
88/1660-1	rainbow trout	90/3874	rainbow trout
88/1660-3	rainbow trout	90/723-134	rainbow trout
88/1740-6	rainbow trout	90-0723-113	rainbow trout
88/1937-3	rainbow trout	83/35051 (Victoria)	rainbow trout
88/2054-48	rainbow trout	VRI 7876	rainbow trout
88/2625-2	rainbow trout	NG 8206	yellowtail
88/2955-1	rainbow trout	HP 9501-1	yellowtail
88/3041-16	rainbow trout	ATCC 49156^T	yellowtail
88/3041-5	rainbow trout		

Table 2.11 Bacterial strain numbers for *E. seriolicida* used in PCR specificity tests

Fx. maritimus	Host	*Fx. maritimus*	Host
89/0148-1	rainbow trout	89/1579-2S	Atlantic salmon
89/0235-1	rainbow trout	89/1579-3G	Atlantic salmon
89/0235-3	rainbow trout	89/1579-3G	Atlantic salmon
89/0239-1	Atlantic salmon	89/1579-3S	Atlantic salmon
89/0282-1	rainbow trout	89/1763-2	Atlantic salmon
89/0282-2	rainbow trout	89/1763-5	Atlantic salmon
89/0329-1	Atlantic salmon	89/2244-2B	rainbow trout
89/0329-10	Atlantic salmon	89/3001-2	striped trumpeter
89/0329-12	Atlantic salmon	89/3001-4F	striped trumpeter
89/0329-2	Atlantic salmon	89/3001-5.2	striped trumpeter
89/0329-3	Atlantic salmon	89/3001-6.1	striped trumpeter
89/0329-4	Atlantic salmon	89/3001-7	striped trumpeter
89/0329-6	Atlantic salmon	89/4747	Atlantic salmon
89/0329-7	Atlantic salmon	89/4913-6	Atlantic salmon
89/0329-8	Atlantic salmon	89/4943-3	Atlantic salmon
89/0329-9	Atlantic salmon	89/4983-3	Atlantic salmon
89/039-5	Atlantic salmon	90/1445	Atlantic salmon
89/0528-1	Atlantic salmon	91/0126	Atlantic salmon
89/0528-4	Atlantic salmon	91/0247	Atlantic salmon
89/0547-3-1	Atlantic salmon	97/0401-1	greenback flounder
89/0547-61	Atlantic salmon	97/0415-1	greenback flounder
89/0547-73	Atlantic salmon	97/0497-E1	Atlantic salmon
89/0578-3	Atlantic salmon	97/0497-E2	Atlantic salmon
89/0578-5	Atlantic salmon	CRC-1	Atlantic salmon
89/0699	Atlantic salmon	JIP 32/91 (5)	sea bass
89/1002	Atlantic salmon	lyl-1	Japanese flounder
89/1288-8	rainbow trout	NCIMB 2154^T	Red Sea bream
89/1579-2G	Atlantic salmon	NCIMB 2158	Dover sole

Table 2.12 Bacterial strain numbers for *Fx. maritimus* used in PCR specificity tests

Near related and inter-species The specificity of the probes was also assessed using phenotypically similar bacteria, near related species from the same genus or bacteria identified as having some genotypic similarity in

respect to specific 16S rRNA primer sequence (Table 2.6). For each of the four bacteria, the near related species are listed in Tables 2.13 - 2.16.

Species	Strain no.	Species	Strain no.
A. bestiarum	ATCC 14715	*A. jandaei*	ATCC 49568
A. caviae	ATCC 15468^T	*A. media*	ATCC 33907^T
A. encheleia	ATCC 35941	*A. salmonicida*	93/0956-2
A. eucrenophila	ATCC 23309^T	*A. schubertii*	ATCC 43700^T
A. hydrophila	ATCC 7966^T	*A. sobria*	ATCC 43979^T
A. hydrophila	ATCC 7965	*A. trota*	ATCC 49567^T
A. hydrophila	SWB 216	*A. veronii* var. *sobria*	ATCC 9071^T
A. hydrophila	SWB 219	*A. veronii* var. *veronii*	ATCC 35624^T
A. hydrophila	UTS 37	Other relevant species	
A. hydrophila	UTS 41	*E. seriolicida*	ATCC 49156^T
A. hydrophila	UTS 48	*E. coli*	ATCC 25922
A. hydrophila	UTS 56	*Flav. columnare*	NCIMB 2248^T
A. hydrophila	UTS 65	*Fx. maritimus*	NCIMB 2154^T
A. hydrophila	UTS 66	*Hafnia alvei*	95/6404
A. hydrophila	UTS 67	*Proteus rettgeri*	96/5494
A. hydrophila	UTS 68	*Ps. fluorescens*	92/3556-2-16
A. hydrophila	UTS 69	*Vag. salmoninarum*	NCFB 2777^T
A. hydrophila	UTS 70	*Vibrio anguillarum*	85/3475-1
A. hydrophila	UTS 71a	*Vibrio ordalii*	ATCC 33509^T
A. hydrophila	UTS 72a	*Y. intermedia*	92/4041
A. *hydrophila*	UTS 73a	*Y. ruckeri*	ATCC 29473^T

Table 2.13 Bacteria for testing specificity of *A. salmonicida* PCR primer set

Species	Strain no.	Species	Strain no.
Carn. piscicola	ATCC 35586^T	*Ent. faecalis*	ATCC 29212
Clostridium putrificum	ATCC 25784^T	*Flav. columnare*	NCIMB 2248^T
Lactococcus garvieae	NCFB 2155^T	*Fx. maritimus*	NCIMB 2154^T
Lactococcus garvieae	NCFB 2157	*Lact. piscium*	NCFB 2778^T
Other relevant species		*Ps. fluorescens*	92/3556-2-16
A. hydrophila	ATCC 7966^T	*Strep. iniae*	QDPI 95.41693/4A
A. salmonicida	93/0956-2	*Vag. salmoninarum*	NCFB 2777^T
E. coli	ATCC 25922	*Y. ruckeri*	ATCC 29473^T
E. seriolicida	ATCC 49156^T		

Table 2.14 Bacteria for testing specificity of *E. seriolicida* PCR primer set

Species	Strain no.	Species	Strain no.
Citrobacter freundii	90/2624-18	Other relevant species	
E. coli	ATCC 25922	*A. salmonicida*	93/0956-2
Erwinia herbicola	ACM 852^T	*Carn. piscicola*	ATCC 35586^T
Haem. influenzae	ATCC 33391^T	*E. seriolicida*	ATCC 49156^T
Hafnia alvei	95/6404	*Flav. columnare*	NCIMB 2248^T
Proteus rettgeri	96/5494	*Fx. maritimus*	NCIMB 2154^T
Y. enterocolitica	96/5440-1B	*Vag. salmoninarum*	NCFB 2777^T
Y. intermedia	92/4041	*Vibrio anguillarum*	85/3475-1
Y. pseudotuberculosis	96/5417-2	*Y. ruckeri*	ATCC 29473^T

Table 2.15 Bacteria for testing specificity of *Y. ruckeri* PCR primer set

Species	Strain no.	Species	Strain no.
Cy. marinoflava	ACAM 75	*Flexibacter ovolyticus*	NCIMB 13127^T
FCLB mucoid	89/2244-9	*Por. assaccharolytica*	ATCC 25260^T
FCLB mucoid	89/2756-1	Other relevant species	
FCLB mucoid	CRC-2	*A. salmonicida*	93/0956-2
FCLB mucoid	89/2244-8	*E. coli*	ATCC 25922
FCLB mucoid	89/3001-2	*E. seriolicida*	ATCC 49156^T
FCLB mucoid	89/2758	*Flav. columnare*	NCIMB 2248^T
FCLB mucoid	89/2244-6	*Flav. johnsoniae*	ATCC 17061^{Co-T}
FCLB mucoid	96/5171	*Fx. maritimus*	NCIMB 2154^T
FCLB mucoid	96/5258-1A	*Ps. fluorescens*	92/3556-2-16
FCLB mucoid	96/5258-2A	*Vibrio anguillarum*	85/3475-1
FCLB mucoid	96/5258-2A	*Vibrio. ordalii*	ATCC 33509^T
FCLB mucoid	96/5258-2B	*Vibrio splendidus* I	ATCC 25914^T
Flav. odoratum	ACM 3100	*Y. ruckeri*	ATCC 29473^T

Table 2.16 Bacteria for testing specificity of *Fx. maritimus* PCR primer set.
FCLB: Unidentified *Flexibacter-Cytophaga*-like bacteria

Crude or purified DNA was extracted from the bacteria as described and amplified by PCR using the primer sets as indicated in Tables 2.13-2.16. For species which had a measure of homology for at least one 16S rRNA primer (listed in Table 2.6), specificity was also assessed by nested PCR. Agarose gels were examined for the presence or absence of bands and where bands were evident, size estimated using the reference DNA markers.

DNA detection limit Purified DNA from *A. salmonicida* (DPIF 93/1061-1), *E. seriolicida* (ATCC 49156^T), *Fx. maritimus* (DPIF 89/4942-2) and *Y. ruckeri* (DPIF 94/1738-1) were used to determine the detection limit for the PCR reaction. Decimal dilutions of DNA were made in 18 MΩ water to give a range of dilutions from 1 $\mu g\ \mu l^{-1}$ to 1 $fg\ \mu l^{-1}$. Standard PCR and nested PCR were used with the varying concentrations of template DNA. Detection limits were expressed as genome equivalents by relating DNA concentration to the weight of DNA in a bacterial cell. Mean genome size for Gram negative and Gram positive bacteria (Trevors 1996) was converted from kbp to fg using a conversion factor of 1 kbp ~ $635x10^3$ daltons (Sambrook *et al.* 1989) and 1 dalton = $1.65x10^{-9}$ fg (Budavari 1989). The ability of bovine serum albumin to improve sensitivity (Kraeder 1996) was assessed by the addition of BSA (New England Biolabs, Beverley, MD, USA) at a final concentration of 1.5 $ng\ \mu l^{-1}$.

Detection limits in the presence of fish tissue were determined by seeding tissue homogenates with a 100µl of known concentration of bacterial cells. Cell

number of a stock suspension was determined microscopically using a Helber counting chamber and serial decimal dilutions then prepared to give a range of final concentrations between 0 and 1×10^8 cells g^{-1} tissue. Tissues tested included: skin, brain, kidney and gut. Following seeding, DNA was extracted as described.

2.5.4 Restriction digests of *Aeromonas* spp. PCR product

Sequences between primer sites A and As 2 for *A. hydrophila* type strain (ATCC 7966) and *A. salmonicida* (DPIF 93/1061-1) were inspected for restriction sites using the GCG program *Mapsort* accessed through ANGIS. By manual inspection of the data, a four base pair difference in sequence between positions 137 and 140 was identified together with an appropriate restriction endonuclease. For *A. salmonicida* but not *A. hydrophila*, amplified DNA can be cut with the restriction endonuclease *Bst*Y1 at the recognition site G▼GATC.

A nested PCR comprising a first round with primers A and 785r and a second round with primers A and As 2 was made with six aeromonads (Table 2.17).

Species	Strain no.
A. bestiarum	ATCC 14715
A. hydrophila	ATCC 7965
A. hydrophila	ATCC 7966^T
A. hydrophila	SWB 216
A. hydrophila	SWB 219
A. salmonicida	DPIF 93/1061-1

Table 2.17 *Aeromonas* species for restriction endonuclease testing with *Bst*Y1

PCR amplified DNA was digested at 60°C for one hr with the restriction endonuclease *Bst*Y1 (New England Biolabs, Beverley, MA, USA) using the manufacturer's protocol and buffer system. The digested DNAs were separated with 2% agarose/TAE buffer containing ethidium bromide at a final concentration of 0.5 μg ml^{-1}.

2.5.5 Partial sequencing of *Aeromonas hydrophila* 16S rRNA gene

A semi-nested PCR using first round primers A and 785r followed by a second round of PCR with the flanking primer A and the *A. salmonicida* primer As2 was performed using *A. hydrophila* ATCC 7965 and *A. bestiarum* (ATCC 14715). The PCR product was separated by electrophoresis with 2%

agarose/TAE buffer and excised from the agarose. The 473 bp product was extracted from the agarose and purified with a QIAex II gel extraction kit (QIAGEN). The purified DNA was cloned using the pGEM vector cloning system (Promega Corp., Madison, WI, USA), and the plasmids purified with a Wizard Miniprep kit (Promega). Sequences of the insertion were then generated with an Applied Biosystems model 377A automated sequencer (Perkin-Elmer Applied Biosystems) by using the dye primer cycle sequencing ready reaction (-21 M13 forward and M13 reverse) kit (Perkin-Elmer). For *A. hydrophila* strains SWB 216, 219, the region of interest was amplified using primers A and 785r and the PCR product labelled directly using the PRISM ready reaction dideoxy termination cycle sequencing kit (Perkin-Elmer).

2.5.6 Verification of PCR product with labelled internal probes

PCR Un-nested and nested PCR was performed in 96 well PCR microtitre plates using *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* template and primer pairs as described. A range of template DNA concentrations of 100, 10, 1 *n*g, 100, 10, 1 *p*g and 100 *f*g were used to assess the ability of hybridization to improve the detection limit of the PCR. Each PCR reaction mixture was made up to 50µl with 2x SSC and the DNA then denatured at 96°C for 10 min to remove any secondary structure and cooled rapidly on ice for 10 min; the volume was then made up to 100µl with 20x SSC.

Blotting Dot blots of denatured PCR DNA were prepared by vacuum blotting on Hybond-N+ nylon membrane (Amersham Life Science, Sydney). The membrane was pre-wetted in 10x SSC and the excess removed under vacuum in a Bio-Dot dot blot apparatus (Bio-Rad, Hercules, CA, USA) followed by rehydration of each well with 500µl of 10x SSC. PCR samples for probing (20µl of PCR reactant, denatured and made up to 100µl in SSC) were loaded onto the membrane, a gentle vacuum applied and then each well rinsed with 400µl of 10x SSC. The membrane was removed from the dot blotter, immersed in a denaturing solution (1.5M NaCl, 0.5M NaOH) for 5 min and then in a neutralising solution (1.5M NaCl, 0.5M Tris, 0.001M EDTA, pH 7.2) for 1 min. and air dried. The DNA was covalently bonded to the nylon membrane by baking at 80°C for 2 hours.

Probes The internal probes for *E. seriolicida, Fx. maritimus* and *Y. ruckeri* were synthesized as de-salted oligonucleotides with a biotin label (Gibco-BRL).

Hybridization Blots were pre-hybridized for 30 min at the hybridisation temperature (range: 40°C and 60°C) in hybridization buffer (5x SSC, 5x Denhardt's solution (2% glycine, 2% Ficoll 400, 2% PVP), 0.5% SDS); initial temperatures were: *E. seriolicida*: 50°C, *Fx. maritimus*: 52°C and *Y. ruckeri*: 50°C (see section *2.4 rRNA Hybridization*). Following pre-hybridization, salmon sperm DNA, denatured by boiling for 5 min, was added to the hybridization buffer at a concentration of 20μg ml^{-1}. The probes, denatured to remove secondary structure by boiling for 5 min. and cooled rapidly on ice, were then added to the preheated hybridization buffer at a concentration of 200-500 $ngml^{-1}$ and hybridization allowed to proceed for 15 h. The blots were then washed twice in 2x SSC, 0.1%SDS at room temperature for 5 min and twice in 0.1x SSC, 0.1%SDS at 40°C for 20 min.

Detection The hybridized biotinylated probes were detected on the blots using the BluGene® system (Gibco-BRL) as described by the manufacturer.

2.6 Working protocols

Full working protocols for the PCR assays together with reagent preparation are given in Section 6, the Workshop Notes.

Section 3

Results

3.1 Develop molecular probes for enzootic strains of *Aeromonas salmonicida*, *Enterococcus seriolicida*, *Flexibacter maritimus* and *Yersinia ruckeri*

3.1.1 Sequence data

E. coli numbering (Gutell, 1993) is used to describe sites and sequences on the 16S rRNA gene; approximate alignments for the four pathogens and *E. coli* are shown in Figure 3.1. The sequence for *Aeromonas salmonicida* DPIF 93/1061-1 (see Figure 3.1) is lodged with GenBank as *Aeromonas* sp. accession number L31915. Alignments with other aeromonads established a 100% sequence homology with *A. salmonicida* ssp. *achromogenes* and *A. salmonicida* ssp. *masoucida* and a 99.9% homology with *A. salmonicida* ssp. *salmonicida* (Martinez-Murcia *et al.* 1992).

```
[          1                    Primer A                          50]
A. salm     .......... ..GAGTTTGA TCATGGCTCA GATTGAACGC TGGCGGCAGG
E. seriol   .......... .......... .......... .....AACGC TGGCGGCGTG
Fx. marit   ~NATACAATG AAGAGTTTGA TCCTGGCTCA GGATGAACGC TAGCGGCAGG
Y. ruckeri  .......... .......... ....GGCTCA GATTGAACGC TGGCGGCAGG
E. coli     ~~~~AAATTG AAGAGTTTGA TCATGGCTCA GATTGAACGC TGGCGGCAGG

[         51                                                    100]
A. salm      CCTAACACAT GCAAGTCGAG CGGCAGCGG- ---GAAAGTA GCTTGCTACT
                                        Primer Es 9
E. seriol    CCTAATACAT GCAAGTCGAG CGAT-----G ATTAAAGATA GCTTGCTATT
Fx. marit    CTTAACACAT GCAAGTCGAG GGGTAACAT- ------TGTA GCTTGCTACA
Y. ruckeri   CCTAACACAT GCAAGTCGAG CGGCAGCGG- ----AAAGTA GCTTGCTACT
E. coli      CCTAACACAT GCAAGTCGAA CGGTAACAG- ----GAAGAA GCTTGCTTCT

[        101                                                    150]
A. salm      TTT----GCC GGCGAGCGGC GGACGGGTGA GTAATGCCTG GGG-ATCTGC
E. seriol    TTTATGA--- -AG-AGCGGC GAACGGGTGA GTAACGCGTG GGAAATCTGC
Fx. marit    -------GAT GACGACCGGC GCACGGGTGC GTAACGCGTA TAGAATCTGC
Y. ruckeri   TT-----GCC GGCGAGCGGC GGACGGGTGA GTAATGTCTG GGG-ATCTGC
E. coli      TT-----GCT GACGAGTGGC GGACGGGTGA GTAATGTCTG GGA-AACTGC

[        151                                                    200]
A. salm      CCAGTCGAGG GGGATAACAG TTGGAAACGA CTGCTAATAC CGCATACG--
E. seriol    CGAGTAGCGG GGGACAACGT TTGGAAACGA ACGCTAATAC CGCATAACAA
Fx. marit    CTTCTACAGA GGGATAGCCT TTAGAAATGA AGATTAATAC CTCATAACAC
Y. ruckeri   CTGATGGAGG GGGATAACTA CTGGAAACGG TAGCTAATAC CGCATAAC--
E. coli      CTGATGGAGG GGGATAACTA CTGGAAACGG TAGCTAATAC CGCATAAC--

[        201                                                    250]
A. salm      ------CC-T ACGGGG---- ----GAAAGG AGGGGACCTT CGGGCCTTTC
                   Primer Es 6
E. seriol    TGAG-AATCG CATGATTCTT ATTTAAAAGA AGC-----AA TT---GCTTC
                  Primer rFlex 1
Fx. marit    TTTGGAATGG CATCGTTTTA AAGTTAAAGA --------TT TA------TC
Y. ruckeri   ------CTCG CAAGAG---- ----CAAAGT GGGGGACCTT CGGGCCTCAC
E. coli      ------GTCG CAAGAC---- ----CAAAGA GGGGGACCTT CGGGCCTCTT
```

```
[          251                                                        300]
                Primer As 1
A. salm       GCGATTGGAT GAACCCAGGT GGGATTAGCT AGTTGGTGGG GTAATGGCTC
E. seriol     ACTACTTGAT GATCCCGCGT TGTATTAGCT AGTTGGTAGT GTAAAGGNCN
Fx. marit     GGTAGAAGAT GACTATGCGT CCTATTAGCT AGATGGTAAG GTAACGGCTT
                                  Primer Yr 2
Y. ruckeri    GCCATCGGAT GAACCCAGAT GGGATTAGCT AGTAAGTGGG GTAATGGCTC
E. coli       GCCATCGGAT GTGCCCAGAT GGGATTAGCT AGTAGGTGGG GTAACGGCTC

[          301                                                        350]
A. salm       ACCAAGGCGA CGATCCCTAG CTGGTCTGAG AGGATGATCA GCCACACTGG
E. seriol     ACCAAGGCGA TGATACATAG CCNNCCTGAG AGGGTGATCG GCCACACTGG
Fx. marit     ACCATGGCAA CGATAGGTAG GGGTCCTGAG AGGGAGATCC CCCACACTGG
Y. ruckeri    ACCTAGGCGA CGATCCCTAG CTGGTCTGAG AGGATGACCA GCCACACTGG
E.coli        ACCTAGGCGA CGATCCCTAG CTGGTCTGAG AGGATGACCA GCCACACTGG

[          351                                                        400]
A. salm       AACTGAGACA CGGTCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG
E. seriol     GACTGAGACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTA GGGAATCTTC
Fx. marit     TACTGAGACA CGGACCAGAC TCCTACGGGA GGCAGCAGTG AGGAATATTG
Y. ruckeri    AACTGAGACA CGGTCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG
E.coli        AACTGAGACA CGGTCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG

[          401                                                        450 ]
A. salm       CACAATGGGG GAAACCCTGA TGCAGCCATG CCGCGTGTGT GAAGAAGGCC
E. seriol     GGCAATGGGG GCAACCCTGA CCGAGCAACG CCGCGTGAGT GAAGAAGGTT
Fx. marit     GGCAATGGAG GCAACTCTGA CCCAGCCATG CCGCGTGCAG GAAGACTGCC
Y. ruckeri    CACAATGGGC GCAAGCCTGA TGCAGCCATG CCGCGTGTGT GAAGAAGGCC
E.coli        CACAATGGGC GCAAGCCTGA TGCAGCCATG CCGCGTGTAT GAAGAAGGCC

[          451                                                        500]
                                                          Primer As 2
A. salm       -TTCG-GGTT GTAAAGCACT TTCAGCGAGG AGGAAAGGTT GGCGCC-TAA
E. seriol     -TTCG-GATC GTAAAACTCT GTTGTTAGAG AAGAACGTTA AGTAGAGTGG
                                                       Primer rFlex 3
Fx. marit     CTAT-GGGTT GTAAACTGCT TTTATACAGG AAGAAACGTA CCTA---CGA
                                                          Primer Yr 1
Y. ruckeri    -TTCG-GGTT GTAAAGCACT TTCAGCGAGG AGGAA-GGGT TAAGTGTTAA
E.coli        -TTCG-GGTT GTAAAGTACT TTCAGCGGGG AGGAA-GGGA GTAAAGTTAA

[          501                                                        550]
A. salm       TACGTGTCAA CTGTGACGTT ACTCGCAGAA GAAGCACCGG CTAACTCCGT
E. seriol     AAAATTACTT AAGTGACGGT ATCTAACCAG AAAGGGACGG CTAACTACGT
Fx. marit     G----TAGGT ATTTGACGGT ACTGTAAGAA TAAGGACCGG CTAACTCCGT
Y. ruckeri    TAGCACTGAA CATTGACGTT ACTCGCAGAA GAAGCACCGG CTAACTCCGT
E.coli        TACCTTTGCT CATTGACGTT ACCCGCAGAA GAAGCACCGG CTAACTCCGT

[          551                                                        600]
A. salm       GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG
E. seriol     GCCAGCAGCC GCGGTAATAC GTAGGTCCCA AGCGTTGTCC GGATTTATTG
Fx. marit     GCCAGCAGCC GCGGNNATAC GGAGNGTCCN AGCGTTATCC GGAATCATTG
Y. ruckeri    GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG
E.coli        GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG

[          601                                                        650]
A. salm       GGCGTAAAGC GCACGCAGGC GGTTGGATAA GTTAGATGTG AAAGCCCCGG
E. seriol     GGCGTAAAGC GAGCGCAGGT GGTTTCTTAA GTCTGATGTA AAAGGCAGTG
Fx. marit     GGTTTAAAGG GTCCGCAGGC GGTCGATTAA GTCAGAGGTG AAATCCCATA
Y. ruckeri    GGCGTAAAGC GCACGCAGGC GGTTTGTTAA GTCAGATGTG AAATCCCCGA
E.coli        GGCGTAAAGC GCACGCAGGC GGTTTGTTAA GTCAGATGTG AAATCCCCGG
```

```
[         651                                                        700]
A. salm      GCTCAACCTG GGAATTGCAT TTAAAACTGT CCAGCTAGAG TCTTGTAGAG
E. seriol    GCTCAACCAT TGTGT-GCAT TGGAAACTGG GAGACTTGAG TGCAGGAGAG
Fx. marit    GCTTAACTAT GGAACTGCCT TTGATACTGG TTGACTTGAG TAATACGGAA
Y. ruckeri   GCTTAACTTG GGAACTGCAT TTGAAACTGG CAAGCTAGAG TCTTGTAGAG
E.coli       GCTCAACCTG GGAACTGCAT CTGATACTGG CAAGCTTGAG TCTCGTAGAG

[         701                                                        750]
A. salm      GGGGGTAGAA TTCCAGGTGT AGCGGTGAAA TGCGTAGAGA TCTGGAGGAA
E. seriol    GAGAGTGGAA TTCCATGTGT AGCGGTGAAA TGCGTAGATA TATGGAGGAA
Fx. marit    GTAGATAGAA TATGTAGTGT AGCGGTGAAA TGCATAGATA TTACATAGAA
Y. ruckeri   GGGGGTAGAA TTCCAGGTGT AGCGGTGAAA TGCGTAGAGA TCTGGAGGAA
E.coli       GGGGGTAGAA TTCCAGGTGT AGCGGTGAAA TGCGTAGAGA TCTGGAGGAA

[         751                                                        800]
A. salm      TACCGGTGGC GAAGGCGGCC CCCTGGACAA AGACTGACGC TCAGGTGCGA
E. seriol    CACCGGAGGC GAAAGCGGCT CTCTGGCCTG TAACTGACAC TGAGGCTCGA
Fx. marit    TACCGATTGC GAAGGCAGTC TACTACGTAT TTACTGACGC TCATGGACNA
Y. ruckeri   TACCGGTGGC GAAGGCGCCC CCCTGGACAA AGACTGACGC TCAGGTGCGA
E.coli       TACCGGTGGC GAAGGCGGCC CCCTGGACGA AGACTGACGC TCAGGTGCGA

[         801                          Primer 785r                   850]
A. salm      AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
E. seriol    AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
Fx. marit    AAGCGTGGGG AGCGAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
Y. ruckeri   AAG-GTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCTGTAAA
E.coli       AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA

[         851                                                        900]
A. salm      CGATGTCGAT TTGGAGGCTG TGTC-CTTGA -GACGTGGCT TCCGGAGCTA
E. seriol    CGATGAGTGC TAGCTGTAGG GAGCT-ATA- AGTTCTCTGT AGCGCAGCTA
Fx. marit    CGATGGACAC TAGTTGTTGG GA----AATG ---TCTCAGT GACTAAGCGA
Y. ruckeri   CGATGTCGAC TTGGAGGTTG TGCC-CTTGA -GGCGTGGCT TCCGGAGCTA
E.coli       CGATGTCGAC TTGGAGGTTG TGCC-CTTGA -GGCGTGGCT TCCGGAGCTA

[         901                                                        950]
A. salm      ACGCGTTAAA TCGACCGCCT GGGGAGTACG GCCGCAAGGT TAAAACTCAA
E. seriol    ACGCATTAAG CACTCCGCCT GGGGAGTACG ACCGCAAGGT TGAAACTCAA
Fx. marit    AAGTGATAAG TGTCCCACCT GGGGAGTACG ATCGCAAGAT TGAAACTCAA
Y. ruckeri   ACGCGTTAAG TCGACCGCCT GGGGAGTACG GCCGCAAGGT TAAAACTCAA
E.coli       ACGCGTTAAG TCGACCGCCT GGGGAGTACG GCCGCAAGGT TAAAACTCAA

[         951                                                       1000]
A. salm      ATGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA
E. seriol    AGGAATTGAC GGNGGCCNGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA
Fx. marit    AGGAATTGAC GGGGGCCNGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA
Y. ruckeri   ATGAATTGAC GGGGGCCCGC ACAAGCGGTG AAGCATGTGG TTTAATTCGA
E.coli       ATGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA

[        1001                                                       1050]
A. salm      TGCAACGCGA AGAACCTTAC CTGGCCTTGA CATG-TCTGG -AATCCTGTA
E. seriol    AGCAACGCGA AGAACCTTAC CAGGTCTTGA CATA-CTCGT GCTATCCTTA
Fx. marit    TGATACGCGA GGAACCTTAC CAGGGCTTAA ATGTGGAATG -ACAGGGCTA
Y. ruckeri   TGCAACGCGA AGAACCTTAC CTACTCTTGA CATC-CACAG -AACTTGGCA
E.coli       TGCAACGCGA AGAACCTTAC CTGGTCTTGA CATC-CACGG -AAGTTTTCA

[        1051                                                       1100]
A. salm      GAGATACGGG AGTGCCTTCG GGAA-TCAGA ACACAGGTGC TGCATGGCTG
E. seriol    GAGATNAGGA GTT-CCTTCG GG-ACACGGG ATACAGGTGG TGCATGGTTG
Fx. marit    GAGATAGCNT TT--TCTTCG GA---CATTT CACAAGGTGC TGCATGGTTG
Y. ruckeri   GAGATGCCTT GGTGCCTTCG GGAA-CTGTG AGACAGGTGC TGCATGGCTG
E.coli       GAGATGAGAA TGTGCCTTCG GGAA-CCGTG AGACAGGTGC TGCATGGCTG
```

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[        1101                                                    1150]
A. salm      TCGTCAGCTC GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA
E. seriol    TCGTCAGCTC GTGTCGTGAG ATGTTNGGTT AAGTCCCGCA ACGAGCGCAA
Fx. marit    TCGTCAGCTC GTGCCGTGAG GTGTCAGGTT AAGTCCTATA ACGAGCGCAA
Y. ruckeri   TCGTCAGCTC GTGTTGTGAA ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA
E.coli       TCGTCAGCTC GTGTTGTGAA ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA

[        1151                                                    1200]
A. salm      CCCCTGTCCT TTGTTGCCAG CACGTAATGG TGGGAACTCA AGGGAGACTG
E. seriol    CCCTTATTAC TAGTTGCCAT CA-TTAAGT- TGGGCACTCT AGTGAGACTG
Fx. marit    CCCCTATTGT TAGTTGCTAG CAGGTAAAGC TGAGGACTCT AGCGAGACTG
Y. ruckeri   CCCTTATCCT TTGTTGCCAG CACGTAATGG TGGGAACTCA AGGGAGACTG
E.coli       CCCTTATCCT TTGTTGCCAG CGGTCCGG-C CGGGAACTCA AAGGAGACTG

[        1201                                                    1250]
A. salm      CCGGTGATAA ACCG-GAGGA AGGTGGGGAT GACGTCAAGT CATCATGGCC
E. seriol    CCGGTGATNA ACCG-GAGGA AGGTGGGGAT GACGTCAAAT CATCATGCCC
Fx. marit    CCGGTG-CAA ACCGCGAGGA AGGTGGGGAT GACGTNNAAT CATCACGGCC
Y. ruckeri   CCGGTGACAA ACCG-GAGGA AGGTGGGGAT GACGTCAAGT CATCATGGCC
E.coli       CCAGTGATAA ACTG-GAGGA AGGTGGGGAT GACGTCAAGT CATCATGGCC

[        1251                                                    1300]
A. salm      CTTACGGCCA GGGCTACACA CGTGCTACAA TGGCGCGTAC AGAGGGCTGC
E. seriol    CTTATGACCT GGGCTACACA CGTGCTACAA TGGATGGTAC AACGAGTCGC
Fx. marit    CNTACGTCCT GGGCTACACA CGTGCTACAA TGGTATGGAC AATGAGCAGC
Y. ruckeri   CTTACGAGTA GGGCTACACA CGTGCTACAA TGGCAGATAC AAAGTGAAGC
E.coli       CTTACGACCA GGGCTACACA CGTGCTACAA TGGCGCATAC AAAGAGAAGC

[        1301                                                    1350]
A. salm      AAGCTAGCGA TAGTGAGCGA ATCCCAAAAA GCGCGTCGTA GTCCGGATCG
E. seriol    CAACCCGCGA GGGTGCGCTA ATCTCTTAAA ACCAATCTCA GTTCGGATTG
Fx. marit    CATCTGGCAA CAGAGAGCGA ATCTAC-AAA CCATATCACA GTTCGGATCG
Y. ruckeri   GAACTCGCGA GAGCAAGCGG ACCACATAAA GTCTGTCGTA GTCCGGATTG
E.coli       GACCTCGCGA GAGCAAGCGG ACCTCATAAA GTGCGTCGTA GTCCGGATTG

[        1351                                                    1400]
A. salm      GAGTCTGCAA CTCGACTCCG TGAAGTCGGA ATCGCTAGTA ATCGCGAATC
E. seriol    CAGGCTGCAA CTNGCCTGCA TGAAGTCGGA ATCGCTAGTA ATCGCGGATC
Fx. marit    GAGTCTGCAA CTCGACTCCG TGAAGCTGGA ATCGCTAGTA ATCGGATATC
Y. ruckeri   GAGTCTGCAA CTCGACTCCA TGAAGTCGGA ATCGCTAGTA ATCGTAGATC
E.coli       GAGTCTGCAA CTCGACTCCA TGAAGTCGGA ATCGCTAGTA ATCGTGGATC

[        1401                                                    1450]
A. salm      AGA-ATGTCG CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
E. seriol    AGC-ACGCCG CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
Fx. marit    AGCCATGATC CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
Y. ruckeri   AGA-ATGCTA CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
E.coli       AGA-ATGCCA CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA

[        1451                                                    1500]
A. salm      CACCATGGGA GTGGGTTGCA CCAGAAGTAG ATAGCTTAAC CTTCGGGAGG
E. seriol    CACCACGGAA GTTGGGAGTA CCCAAAGTAG GTTGCCTAAC CGCAAGGAGG
Fx. marit    AGCCATGGAA GCNGGTTGTA CCTGAAGTTG G-----NTAC CGCAAGGAG-
Y. ruckeri   CACCATGGGA GTGGGTTGCA AAAGAAGTAG GTAGCTTAAC CTTCGGGAGG
E.coli       CACCATGGGA GTGGGTTGCA AAAGAAGTAG GTAGCTTAAC CTTCGGGAGG
```

Figure 3.1 16S rRNA sequence alignments for *Aeromonas salmonicida, Enterococcus seriolicida, Flexibacter maritimus* and *Yersinia ruckeri.*
Primer sequences marked in bold and underlined; N, not determined

The 16S rRNA gene sequence for *E. seriolicida* ATCC 49156, the type strain, is lodged with GenBank as *Enterococcus seriolicida*, accession number

L32813 and is shown in Figure 3.1. The sequence was aligned with other near related enterococci and lactococci and there was a high level of similarity to several species of lactococci especially *L. cremoris* (94.2%), *L. raffinolactis* (94.5%), *L. piscium* (92.1%), *L. lactis* ssp. *lactis* (92.5%) and *L. garvieae* (99.4%). The sequence data for *L. garvieae* is based on the type strain NCFB (NCDO) 2156 lodged with GenBank as accession number X54262. The sequence heterogeneity of *E. seriolicida* and *L. garvieae* is based on 7 base pair differences as listed in Table 3.1.

Position	*L. garvieae*	*E. seriolicida*
191	U	G
193	G	C
194	G	A
1027	Deletion	C
1091	G	U
1092	G	A
1203	C	G

Table 3.1 Sequence variation between *L. garvieae* and *E. seriolicida*

Unlike *A. salmonicida* and *E. seriolicida* which were sequenced for this project, 16S rRNA gene sequences for *Fx. maritimus* and *Y. ruckeri* were published respectively by Gherna & Woese in 1992 and Ibrahim *et al.* in 1993 and also lodged with GenBank. The sequence for *Fx. maritimus*, based on the type strain, is aligned with the other fish pathogens in Figure 3.1; the GenBank accession number is M64629. The phylogenetic analysis of the 16S rRNA sequences place *Fx. maritimus* within the *Cytophaga* subgroup, comprised predominantly of species of *Cytophaga*. The *Cytophaga* sub-group possesses a unique rRNA sequence that distinguishes it from other subgroups namely *Sphingobacter*, *Flexibacter*, *Flavobacter* and *Bacteroides*. Within the subgroup *Cytophaga*, the *Fx. maritimus* cluster also has a unique rRNA signature (Gherna & Woese 1992) and is evidence of the singular properties of this taxon.

The 16S rRNA sequence for the type strain of *Y. ruckeri* is aligned in Figure 3.1; the GenBank accession number is X75275. Within the genus *Yersinia* there is a high level of sequence similarity that ranges from 96.9-99.8%; the closest relative genus is *Hafnia alvei* with an average sequence similarity of

96.4%. Five sub lines are recognised within the genus, of which one is occupied solely by *Y. ruckeri*. *Yersinia* species with closest sequence similarity to *Y. ruckeri* are *Y. pseudotuberculosis* (98.3%), *Y. frederiksenii* (98.2%) and *Y. pestis* (98%) while outside the genus, species of high sequence similarity include *H. alvei* (95.9%) and *Citrobacter freundii* (95.6%).

3.1.2 Probe/PCR primer selection

Probes suitable for rRNA hybridization and PCR are listed in Table 3.2 and their spatial relationship in the 16S rRNA sequence given in Figure 3.2, an approximate alignment of the four fish pathogens with *E. coli*. The position of

Species	Primer	Sequence 5' → 3'	Position	Site
A. salmonicida	As1 →	TTT CGC GAT TGG ATG AA	218-234	H8
	As2 ←	TTG ACA CGT ATT AGG CGC CA	479-459	V3
E. seriolicida	Es9 →	CGA GCG ATG ATT AAA GAT AGC TTG CTA	63-89	V1
	Es6 ←	ATA AGA ATC ATG CGA TTC TCA	200-180	V2
Fx. maritimus	rFlex1 →	GGA ATG GCA TCG TTT TAA AG	187-208	V2
	rFlex3 ←	AAT ACC TAC TCG TAG GTA CG	498-473	V3
Y. ruckeri	Yr2 →	AAC CCA GAT GGG ATT AGC TAG TAA	233-256	H8
	Yr1 ←	GTT CAG TGC TAT TAA CAC TTA ACC C	471-455	V3

Table 3.2 Primer sequences specific for the bacterial fish pathogens *A. salmonicida, E. seriolicida, Fx. maritimus* and *Y. ruckeri*
Direction arrows: → forward primer; ← reverse primer;
V1, 2, 3: variable regions corresponding to helices 6, 10-11, 17-18; H8, helix 8

the probe sequences, using *E. coli* numbering (Gutell 1993), was established by individual manual alignment. The location of the probes in the 16S rRNA gene in relation to established variable regions was established using the

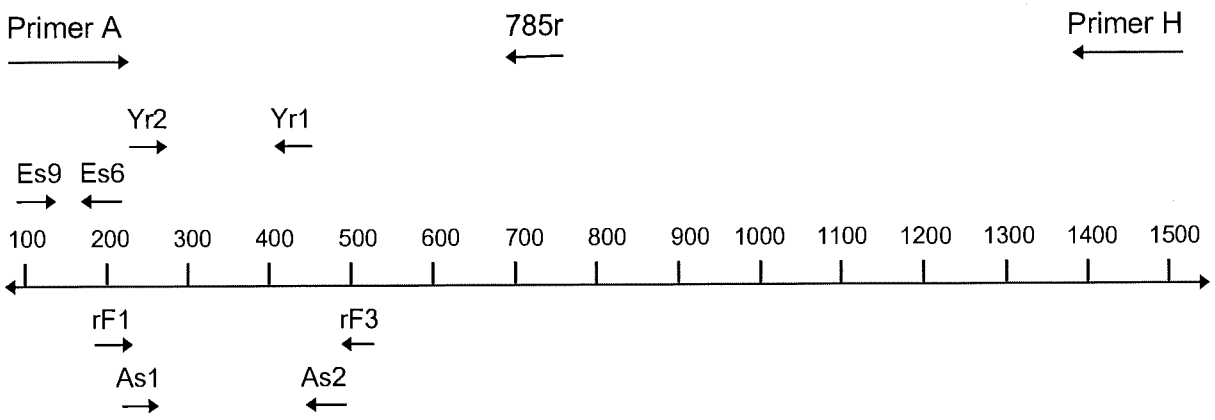


Figure 3.2 Map of PCR primer sets on the 16S rRNA gene: *A. salmonicida* (As1 As2), *E. seriolicida* (Es9 Es6), *F. maritimus* (rFlex 1 rFlex3) and *Y. ruckeri* (Yr2 Yr1

notation of Neefs *et al.* (1990, 1993) and is given in Table 3.2. Theoretical specificity of the probes was determined by looking for sequence homology at an intra- and inter-genus level for bacteria and for bacteriophage, structural RNA and virus sequences held in the RDP and GSDB databases. No exact matches were established for any primer pair for the four bacteria although for the As1 primer for *A. salmonicida* there is 100% sequence homology with *Aeromonas sobria* and for Yr2 there is 100% homology with *Yersinia bercovieri*. The nearest matches to the primers are listed in Tables 3.3 - 3.6.

Primer	Species	No. bases different to primer
As 1	*A. sobria*	0
	A. allosaccharophila	1
	A. euchrenophila	1
	A. jandaei	1
	A. schubertii	1
	A. veronii	1
	Vibrio sp. DB 6075	1
	Vibrio sp. DB 9606	1
	Vibrio sp. DS 512	1
	A. caviae	4
	A. hydrophila	4
	A. media	4
	A. trota	4
As 2	*A. hydrophila*	3
	A. media	3

Table 3.3 Species with sequence homology closest to the *A. salmonicida* PCR primers

Primer	Species	No. bases different to primer
Es 6	*Carnobacterium gallinarum*	5
	C. piscicola	5
	Clostridium novyi	5
	Cl. putrificum	5
	Cl. sporogenes	5
	Cl. botulinum	5
	Lactobacillus maltaromicus	5
	Flavobacterium columnare	6
Es 9	*Borrelia hermsii*	6
	Bacillus pabuli	7

Table 3.4 Species with closest sequence homology to the *E. seriolicida* PCR primers

Primer	Species	No. bases different to primer
Flex 1	*Flavobacterium odoratum*	6
	Cytophaga marinoflava	7
Flex 3	*Porphyromonas asaccharolytica*	6

Table 3.5 Species with closest sequence homology to *Fx. maritimus* PCR primers

Primer	Species	No. bases different to primer
Yr 1	*Haemophilus aegyptius*	8
	H. influenzae	8
	H. paragallinarum	8
	Pasteurella volantium	10
	Vibrio vulnificus	10
	Yersinia aldovae	10
	Y. kristensenii	10
	V. parahaemolyticus	11
	Yersinia bercovieri	12
Yr 2	*Yersinia bercovieri*	0
	Erwinia herbicola	1
	Y. pestis	1
	Y. pseudotuberculosis	1
	Yersinia kristensenii	2
	Proteus vulgaris	2
	Aeromonas allosaccharophila	3
	A. euchrenophila	3
	A. jandaei	3
	A. salmonicida ssp. *achromogenes*	3
	A. salmonicida ssp *masoucida*	3
	A. salmonicida ssp *salmonicida*	3
	A. sobria	3
	A. veronii	3
	Citrobacter freundii	3
	Escherichia coli	3
	Hafnia alvei	3
	Plesiomonas shigelloides	3
	Serratia marcescens	3
	Yersinia aldovae	3
	Y. enterocolitica	3
	Y. fredricksenii	3

Table 3.6 Species with closest sequence homology to *Y. ruckeri* PCR primers

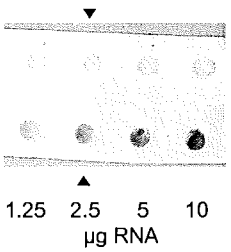
3.2 Develop methodologies using molecular probes for the rapid identification of bacterial fish pathogens recovered by conventional culture techniques

3.2.1 rRNA Hybridization for rapid identification

Fig 3.3 rRNA extracted from *Flexibacter maritimus*

The technique was limited to testing with *A. salmonicida* and *Y. ruckeri*. Useable yields of RNA comprising 16S and 23S fractions (Figure 3.3) could be obtained with the Amresco Rapid RNA Extraction kit.

Although the probes have a high level of specificity, developing sufficiently stringent conditions so that species could be differentiated was not wholly achievable. Initial hybridization conditions were at T_{HYB} 40°C and T_{WASH} 55°C and 500ng ml^{-1} of probe. Under these conditions the As2 probe hybridized to *A. salmonicida* RNA as well as to *Y. ruckeri* RNA; similarly the Yr1 probe hybridized equally well to *Y. ruckeri* RNA and *A. salmonicida* RNA across a range of RNA concentrations. Under conditions of increasing stringency achieved by first raising T_{WASH} to 70°C and then by reducing the salts concentration in the wash buffer and lowering probe concentration to 100 ng ml^{-1}, some differentiation between *A. salmonicida* and *Y. ruckeri* could be achieved. Under these more stringent conditions, the optimum concentration of template RNA was 2.5µg dot^{-1}, T_{HYB} 40°C, T_{WASH} 55°C and wash hybridization buffer of 0.1% SSC. At these optima, *A. salmonicida* rRNA, probed with the *Y. ruckeri* probe Yr1, barely produced a colour reaction (Figure 3.4), however *A. salmonicida* probe



A. salmonicida template rRNA

Y. ruckeri template rRNA

Figure 3.4 rRNA dot blot hybridization with *Yersinia ruckeri* probe Yr 1
Template RNA concentration given on bottom scale. Optimum differential concentration marked by triangle

As2 hybridized more strongly to *Y. ruckeri* rRNA than the homologous rRNA from *A. salmonicida* (Figure 3.5). No further work was undertaken with probes As2 or Yr1; the performance of probes, rFlex 3 or Es9 was not evaluated.

3.2.2 PCR for rapid identification

Test format The PCR formats developed for the four fish pathogens utilise standard reagents and equipment. The test is highly portable and has been shown to run without modification in at least three different laboratories (University of Tasmania, Department of Primary Industry & Fisheries, Tasmania and the Australian Animal Health Laboratory, CSIRO [N. Gudkovs & H. Byers, pers. comm.]). The test produces unambiguous quantities of amplified DNA detectable by gel electrophoresis; expected band sizes are given in Table 3.7.

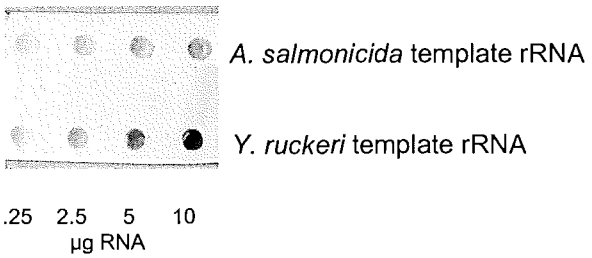


Figure 3.5 rRNA dot blot hybridization with *Aeromonas salmonicida* probe AS 2

Species	Expected size
A. salmonicida	260 base pairs
E. seriolicida	143 base pairs
F. maritimus	285 base pairs
Y. ruckeri	223 base pairs
A-785r	~ 785 base pairs

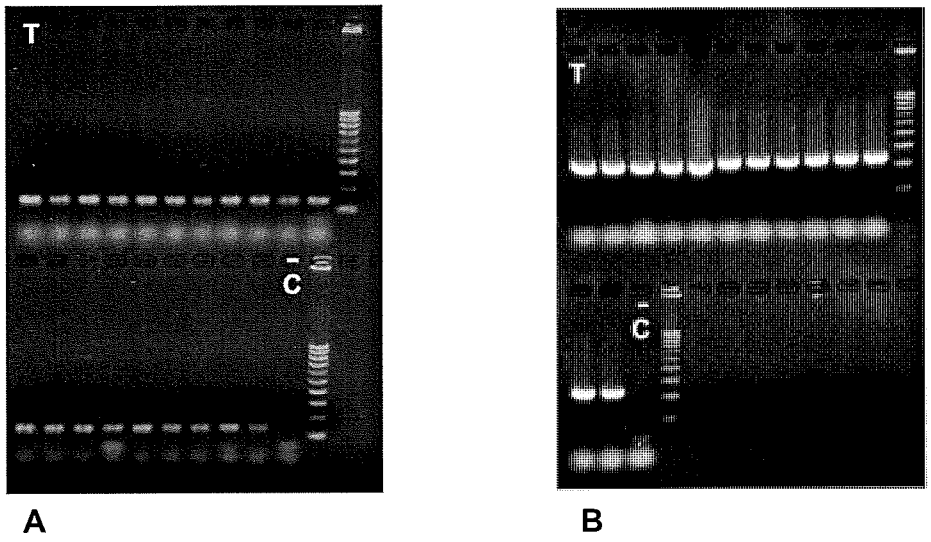
Table 3.7 Expected PCR band sizes

Intra-species verification The fidelity of the PCR primer sets to detect multiple isolates and/or strains of the species was determined by performing PCR tests against libraries of the four different species (Table 2.9 - 2.12); the identity of the species had been confirmed previously by phenotypic characterisation. The summarised findings are given in Table 3.8; all isolates

Species	No. isolates	Type strain	Exotic & enzootic strains	No. positive
A. salmonicida	22	✓	✓	22/22
E. seriolicida	25	✓	✓	25/25
Fx. maritimus	56	✓	✓	56/56
Y. ruckeri	15	✓	✓	15/15

Table 3.8 Intra-species fidelity test of PCR

including type strains, reacted positively by PCR with the appropriate primer pair and produced unambiguous bands of an expected band size (Figure 3.6).



A B C

Figure 3.6 PCR intraspecies fidelity

A: *Enterococcus seriolicida*
B: *Yersinia ruckeri*
C: *Flexibacter maritimus*

Each lane represents a separate isolate

Label: -C negative control lane
T type strain
A + B: 100-1000 bp markers
C: pUC19 marker, 501/489, 404, 331, 242, 190 bp

Inter-species verification The ability of the PCR primer sets to function only with the target species was tested in two ways. PCR tests were performed with phenotypically similar species, or species likely to be encountered alongside the target species in salmonid fish. The second form of testing was

A. salmonicida As 1/As 2	***E. seriolicida*** Es 6/Es 9	***Fx. maritimus*** rFlex 1/rFlex 3	***Y. ruckeri*** Yr 1/Yr 2
Aeromonas caviae	*Carn. piscicola*	*Cytophaga marinoflava*	*Aeromonas salmonicida*
A. euchrenophila	*Clost. putrificum*	*Flav. odoratum*	*Citrobacter freundii*
A. hydrophila	*Flav. columnare*	*Porph. asaccharolytica*	*Erwinia herbicola*
A. jandaei			*Escherichia coli*
A. media			*Haemophilus influenzae*
A. schubertii			*Hafnia alvei*
A. trota			*Yersinia enterocolitica*
A. veronii			*Y. pseudotuberculosis*

Table 3.9 Bacterial species with close sequence homology to the target primer sets and tested for specificity by PCR assay.

undertaken using species that had a similar genotype to the primer sets of the target species as identified by FAST A sequence homology comparisons using GenBank and RDP databases. Bacteria of closest sequence homology to the primers are given in Tables 3.3 - 3.6 and the species selected for testing are as indicated in Table 3.9.

***Aeromonas salmonicida* PCR primer set As1/As2** The range of bacteria tested is given in Table 2.13. By un-nested PCR, the strains listed in Table 3.10 produced bands either at 260 bp., the expected size for

Species	Strain no.	Observed band size
A. bestiarum	ATCC 14715	260 bp
A. hydrophila	ATCC 7965	260 bp
A. hydrophila	SWB 216	260 bp
A. hydrophila	SWB 219	260 bp
A. hydrophila	UTS 67	260 bp
Hafnia alvei	DPIFT 95/6404	> 1000 bp
Yersinia intermedia	DPIFT 92/4041	> 1000 bp

Table 3.10 Near related species producing bands by PCR with *Aeromonas salmonicida* PCR primer set As1/As2

A. salmonicida, or at a band size of >1000 bp. None of the other *Aeromonas* sp. including the type strain of *A. hydrophila* ATCC 7966 produced bands with the *A. salmonicida* primer set As1/As2 (Figure 3.7). Problems of specificity

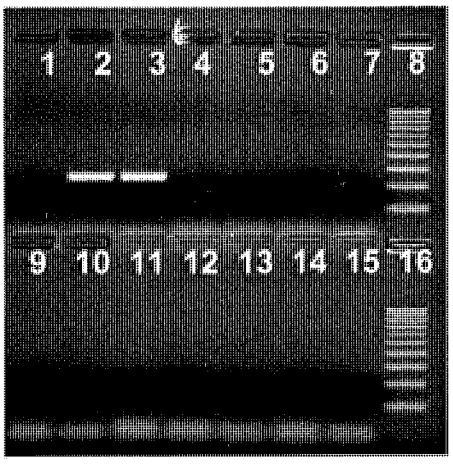


Figure 3.7 PCR of *Aeromonas* sp. with primer set As 1/As 2

Lane 1: *Aeromonas hydrophila* ATCC 7966^T
Lane 2: *A. bestiarum* ATCC 14715
Lane 3: *A. salmonicida* ATCC 14174
Lane 4: *A. caviae* ATCC 15468^T
Lane 5: *A. media* ATCC 33907^T
Lane 6: *A. eucrenophila* ATCC 23309^T
Lane 7: *A. sobria* ATCC 43976^T
Lane 8: 100 bp markers
Lane 9: *A. veronii* biotype *sobria* ATCC 9071^T
Lane 10: *A. jandaei* ATCC 49568^T
Lane 11: *A. veronii* biotype *veronii* ATCC 35624^T
Lane 12: *A. encheleia* ATCC 35941
Lane 13: *A. schubertii* ATCC 43700^T
Lane: 14: *A. trota* ATCC 49567^T
Lane 15: negative control
Lane 16: 100 bp markers

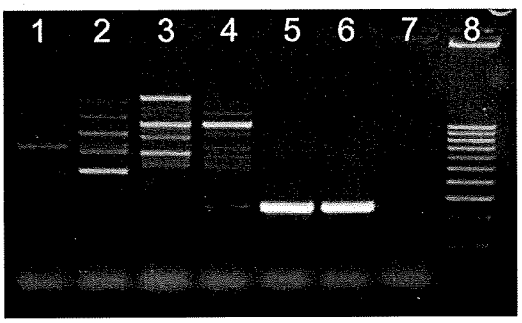


Figure 3.8 PCR of *Aeromonas* sp. with primer set As 1/As 2 for 35 cycles, low Mg^{++} concentration

Lane 1: *Aeromonas hydrophila* ATCC 7966^T
Lane 2: *A. caviae* ATCC 15468^T
Lane 3: *A. eucrenophila* ATCC 23309^T
Lane 4: *A. sobria* ATCC 43976^T
Lane 5: *A. bestiarum* ATCC 14715
Lane 6: *A. salmonicida* ATCC 14174
Lane 7: negative control
Lane 8: 100 bp markers

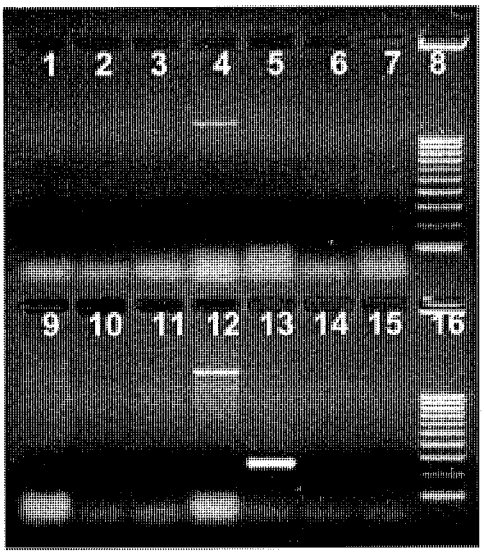


Figure 3.9 *Aeromonas salmonicida* PCR.

Non-specific bands >1000 bp marked by triangle

Lane 1: *Yersinia ruckeri* ATCC 29473^T
Lane 2: *Enterococcus seriolicida* ATCC 49156^T
Lane 3: *Flexibacter maritimus* NCIMB 2454^T
Lane 4: *Hafnia alvei* 95/6404
Lane 5: *Proteus rettgeri* 96/5494
Lane 6: *Vagococcus salmoninarum* NCFB 2777^T
Lane 7: *Pseudomonas fluorescens* 92/3556-2-16
Lane 8: 100 bp markers
Lane 9: *Vibrio anguillarum* 85/3475-1
Lane 10: *Flavobacterium columnare* NCIMB 2248^T
Lane 11: *Vibrio ordalii* ATCC 33509^T
Lane 12: *Yersinia intermedia* 92/4041
Lane 13: *Aeromonas salmonicida* 93/0956-1
Lane: 14: *E. coli* ATCC 25922
Lane 15: negative control
Lane 16: 100 bp markers

associated with the *A. salmonicida* primer set are seen in Figure 3.8 where simply increasing the number of amplification cycles from 30 to 35 reveals a number of non-specific bands when testing *Aeromonas* species other than *A. salmonicida*. The >1000 bp bands that occurred with *H. alvei* and *Y. intermedia* (Figure 3.9) are considered to be the result of non-specific amplification caused by high template DNA concentration. For *H. alvei* and *Y. intermedia*, the template concentrations were 362 and 535 ng μl^{-1} respectively and exceeded the recommended concentration of 10-200 ng DNA μl^{-1} for PCR reactions (Atlas & Bej 1994). In the nested format of the PCR, only *A. salmonicida* produced a 260 bp band; none of the other aeromonads tested (Table 3.9) produced bands of any size.

***Enterococcus seriolicida* PCR primer set Es6/Es9** The range of bacteria tested by this PCR is given in Table 2.14. By un-nested PCR none of the bacteria produced an observable band of amplified DNA (Figure 3.10). More stringent testing was undertaken with *Lactococcus garvieae* and *Clostridium putrificum*, species with a close homology to the primer set Es6/Es9. By un-nested PCR, *L. garvieae* (NCFB 2155^T) produced a 143 bp band at 1, 10 and 100 ng of template DNA concentration; by comparison *Cl. putrificum* did not produce any bands of amplified DNA. By nested PCR, *L. garvieae* still produced a band at 143 bp across the range of template DNA concentrations tested, as did *Cl. putrificum* at the highest template concentration of 100 ng but not at the lower concentrations of 1 and 10 ng template DNA.

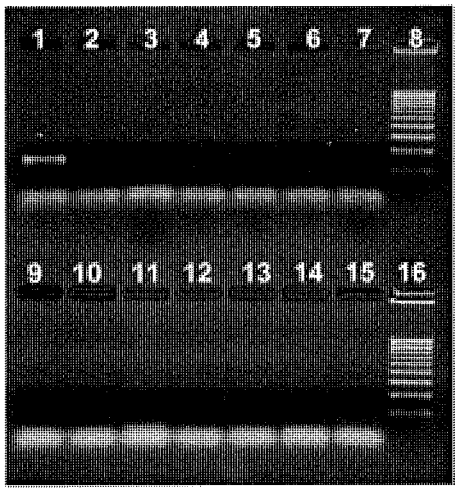


Figure 3.10 Specificity of *Enterococcus seriolicida* primer set Es 6/Es 9

Lane 1: *Enterococcus seriolicida* ATCC 49156^T
Lane 2: *Vagococcus salmoninarum* NCFB 2777^T
Lane 3: *Carnobacterium piscicola* ATCC 35586^T
Lane 4: *Lactococcus piscium* NCFB 2778^T
Lane 5: *Streptococcus iniae* QDPI 95.41693/4A
Lane 6: *Enterococcus faecalis* ATCC 29212
Lane 7: *Yersinia ruckeri* ATCC 29473^T
Lane 8: 100 bp markers
Lane 9: *Aeromonas salmonicida* 93/0956-1
Lane 10: *A. hydrophila* ATCC 7966^T
Lane 11: *Pseudomonas fluorescens* 92/3556-2-16
Lane 12: *Flavobacterium columnare* NCIMB 2248^T
Lane 13: *E. coli* ATCC 25922
Lane: 14: *Flexibacter maritimus* NCFB 2154^T
Lane 15: negative control
Lane 16: 100 bp markers

***Flexibacter maritimus* PCR primer set rFlex 1/rFlex 3** The taxonomy of this group of bacteria is not well described and the relationship of near related taxa has not been clearly established in all cases. Despite this measure of uncertainty, the inter-species and inter-genus specificity of the rFlex primer set is extremely good. None the of the near related bacteria listed in Table 2.15 produced detectable bands of amplified DNA (Figure 3.11). This set of bacteria included a number of unidentified *Flexibacter-Cytophaga*-like bacteria found in association with sea-farmed Atlantic salmon as well as the three genomically similar species *Flavobacterium odoratum,*

Cytophaga marinoflava and the anaerobe *Porphyromonas assaccharolytica*. Neither of these three bacteria produced bands by nested PCR, nor at different template concentrations of 1ng, 10ng or 100ng DNA.

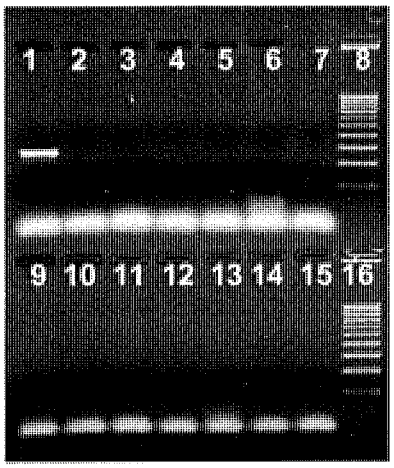


Figure 3.11 Specificity of *Flexibacter maritimus* primer set rFlex 1/rFlex 3

Lane 1: *Flexibacter maritimus* NCFB 2154^T
Lane 2: *Flavobacterium columnare* NCIMB 2248^T
Lane 3: *Flavobacterium columnare* NCIMB 2248^T
Lane 4: *Cytophaga marinoflava* ACAM 75^T
Lane 5: *Flexibacter ovolyticus* NCIMB 13127^T
Lane 6: *Vibrio anguillarum* 85/3475-1
Lane 7: *Vibrio ordalii* ATCC 33509^T
Lane 8: 100 bp markers
Lane 9: *Vibrio splendidus* I ATCC 25914^T
Lane 10: *Yersinia ruckeri* ATCC 29473^T
Lane 11: *Aeromonas salmonicida* 93/0956-1
Lane 12: *Enterococcus seriolicida* ATCC 49156^T
Lane 13: *Pseudomonas fluorescens* 92/3556-2-16
Lane: 14: *E. coli* ATCC 25922
Lane 15: negative control
Lane 16: 100 bp markers

***Yersinia ruckeri* PCR primer set Yr1/Yr2** Although there is a high level of sequence homology between species of the genus *Yersinia*, the primer set for *Y. ruckeri* was able to reliably differentiate between near related species (Figure 3.12). The discriminatory properties of the PCR extended to other members of the Enterobacteriaceae; none of the near or distantly related species (Table 2.15) produced detectable bands in the assay. In un-nested and

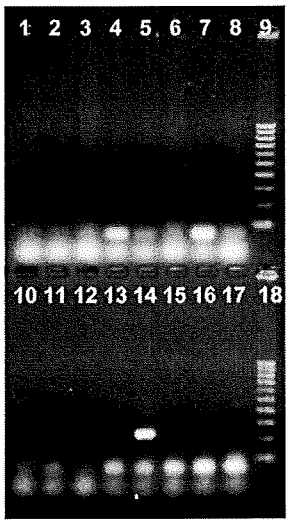


Figure 3.12 Specificity of *Yersinia ruckeri* primer set Yr 1/Yr 2

Lane 1: *Hafnia alvei* 95/6404
Lane 2: *Citrobacter freundii* 90/2624-18
Lane 3: *Yersinia intermedia* 92/4041
Lane 4: *E. coli* ATCC 25922
Lane 5: *Proteus rettgeri* 96/5494
Lane 6: *Vibrio anguillarum* 85/3475-1
Lane 7: *Flavobacterium columnare* NCIMB 2248^T
Lane 8: *Yersinia enterocolitica* 96/5440-1B
Lane 9: 100 bp markers
Lane 10: *Yersinia pseudotuberculosis* 96/5417-2
Lane 11: *Flexibacter maritimus* NCIMB 2154^T
Lane 12: *Enterococcus seriolicida* ATCC 49156^T
Lane 13: *Aeromonas salmonicida* 93/0956-1
Lane: 14: *Yersinia ruckeri* ATCC 29473^T
Lane 15: *Vagococcus salmoninarum* NCFB 2777^T
Lane 16: *Carnobacterium piscicola* ATCC 35586^T
Lane 17: negative control
Lane 18: 100 bp markers

nested PCR assessments of the primer set using template DNA from *Haemophilus influenzae* and *Erwinia herbicola*, species with some sequence homology to primers Yr 1 and Yr2, no reactions were detectable at any of the template concentrations of 1 ng, 10 ng or 100 ng.

***Aeromonas hydrophila* 16S partial sequence analysis** All the aeromonads given in Table 3.11, except for the type strain of *A. hydrophila* ATCC 7966^T, produced a PCR product of 260 bp, the expected band size for *A. salmonicida*. By nested PCR using a first round with primers A/785r and a second round with primer A and the *A. salmonicida* specific primer As2 all the

Species	Strain no.	A→As 2 473 bp	*Bsf*Y1 digest product
A. bestiarum	ATCC 14715	✓	350 + 123 bp
A. hydrophila	ATCC 7965	✓	350 + 123 bp
A. hydrophila	ATCC 7966^T	✗	None
A. hydrophila	SWB 216	✓	350 + 123 bp
A. hydrophila	SWB 219	✓	350 + 123 bp
A. salmonicida	DPIF 93/1061-1	✓	350 + 123 bp

Table 3.11 *Aeromonas* species for restriction endonuclease testing with *Bsf*Y1

strains except for the type strain of *A. hydrophila* produced an expected band size of 473 bp. Except for the type strain of *A. hydrophila* all other strains were digested with *Bsf*Y1 to produce two fragments of 350 and 123 bp (Figure 3.13) as predicted, as well as some undigested 473 bp PCR product. The

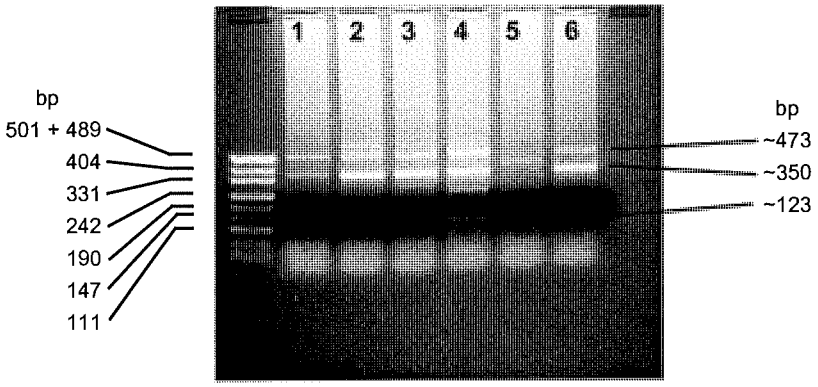


Figure 3.13 *Bsf*Y1 restriction endonuclease digest of primer A to As 2 PCR amplified DNA

Lane 1: *A. salmonicida* 93/1061-1
Lane 2: *A. hydrophila* ATCC 7965
Lane 3: *A. hydrophila* SWB 216
Lane 4: *A. hydrophila* SWB 219
Lane 5: *A. hydrophila* ATCC 7966^T
Lane 6: *A. bestiarum* ATCC 14715
Markers: pUC19

significance of the ~400 bp band for the type strain of *A. hydrophila*, evident in lane 5, is not clear but may represent some artefact of the restriction digest since no bands of any sort were evident in a gel (not shown) run following the second round of the nested PCR prior to digestion with *Bst*Y1.

Partial and complete sequences between primer sites A and As2 for several of the *A. hydrophila* strains are shown in Figure 3.14.

```
 [            1                    Primer A                          50]
Armsalmo      .......... ..GAGTTTGA TCATGGCTCA GATTGAACGC TGGCGGCAGG
Armhydro7966  .......... ..GAGTTTGA TCATGGCTCA GATTGAACGC TGGCGGCAGG
Armhydro7965  .......... .......... .......... .......... .GGCGGTAGG
Armbs         .......... TAGAGTTTGA TCATGGCTCA GATTGARCGC TGGCGGCAGG
ArmhydroSWB1  .......... TAGAGTTTGA TCATGGCTCA GATTGAACGC TGGCGGCAGG
ArmhydroSWB2  .......... TAGAGTTTGA TCATGGCTCA GATTGAACGC TGGCGGCAGG
E.coli        ~~~~AAATTG AAGAGTTTGA TCATGGCTCA GATTGAACGC TGGCGGCAGG

[          51                                                      100]
Armsalmo      CCTAACACAT GCAAGTCGAG CGGCAGCGGG AAA-GTAG-CT TGCTACTTT
Armhydro7966  CCTAACACAT GCAAGTCGAG CGGCAGCGGG AAA-GTAG-CT TGCTACTTT
Armhydro7965  CCT-ACCCNT TNNNGTCNAG CGGCANCGGG AAAANNAN-CT TGCTACTTT
Armbs          CCTAACACAT GCAAGTCGAG CGGCAG-GGG AAA-GTAG-CT TGCTACTTT
ArmhydroSWB1  CCTAACACAT GCAAGTCGAG CGGCAG-GNG AAA-GTAG-CT TGCTACTTT
ArmhydroSWB2  CCTAACACAT GCAAGTCGAG CGGCAGCGGG AAA-GTAG-CT TGCTACTTT
E.coli        CCTAACACAT GCAAGTCGAA CGGTAACAGG AA--GAAG-CT TGCTTCTTT

[          101                                        BstYI         150]
Armsalmo      TGCCGGCGAG CGGCGGACGG GTGAGTAATG CCTGGGGATC TGCCCAGTCG
Armhydro7966  TGCCGGCGAG CGGCGGACGG GTGAGTAATG CCTGGGAAAT TGCCCAGTCG
Armhydro7965  TGCCGGCGAG CGGCGGACGG GTGAGTAATG CCTGGGGATC TGCCCAGTCN
Armbs         TGCCGGCGAG CGGCGGACGG GTGAGTAATG CCTGGGGATC TGCCCAGTCG
ArmhydroSWB1  TGCCGGCGAG CGGCGGACGG GTGAGTAATG CCTGGGGATC TGCCCAGTCG
ArmhydroSWB2  TGCCGGCGAG CGGCGGACGG GTGAGTAATG CCTGGGGATC TGCCCAGTCG
E.coli        -GCTGACGAG TGGCGGACGG GTGAGTAATG TCTGGGAAAC TGCCTGATGG

[          151                                                     200]
Armsalmo      AGGGGGATAA CAGTTGGAAA CGACTGCTAA TACCGCATAC G-CCTACGGG
Armhydro7966  AGGGGGATAA CAGTTGGAAA CGACTGCTAA TACCGCATAC G-CCTACGGG
Armhydro7965  AGGGGGATAA CAGTTGGAAA CGACTGCTAA TACCGCATAC NCCCTACGGG
Armbs         AGGGGGATAA CAGTTGGAA- CGACTGCTAA TACCGCATAC GCCCTACGGG
ArmhydroSWB1  AGGGGGATAA CAGTTGGAAA CGACTGCTAA TACCGCATAC GCCCTACGGG
ArmhydroSWB2  AGGGGGATAA CAGTTGGAAA CGACTGCTAA TACCGCATAC G-CCTACGGG
E.coli        AGGGGGATAA CTACTGGAAA CGGTAGCTAA TACCGCATAA CGTCGCAAGA

[          201                                                     250]
                                          Primer As 1
Armsalmo      GGAAAGGAGG GGACCTTCGG GCCTTTCGCG ATTGGATGAA CCCAGGTGGG
Armhydro7966  GGAAAGCAGG GGACCTTCGG GCCTTGCGCG ATTGGATATG CCCAGGTGGG
Armhydro7965  GGAAAGGAGG GGACCTTCGG GCCTTTCGCG ATTGGATGAA CCCAGGTGGG
Armbs         GGAAAGGAGG GGACCTTCGG GCCTTTCGCG ATTGGATGAA CCCAGGTGGG
ArmhydroSWB1  GGAAAGGAGG GGACCTTCGG GCCTTTCGCG ATTGGATGAA CCCAGGTGGG
ArmhydroSWB2  GGAAAGGAGG GGAC-TTCGG GCCTTTCGCG ATTGGATGAA CCCAGGTGGG
E.coli        CCAAAGAGGG GGACCTTCGG GCCTCTTGCC ATCGGATGTG CCCAGATGGG
```

```
 [          251                                                    300]
Armsalmo      ATTAGCTAGT TGGTGGGGTA ATGGCTCACC AAGGCGACGA TCCCTAGCTG
Armhydro7966  ATTAGCTAGT TGGTGAGGTA ATGGCTCACC AAGGCGACGA TCCCTAGCTG
Armhydro7965  ATTAGCTAGT TGGTGGGGTN TTTGCTCACC AAGGCNACNA NCCCNATCTG
Armbs         ATTAGCTAGT TGGTGGGGTA ATGGCTCACC AAGGCGACGA TCCCTAGCTG
ArmhydroSWB1  ATTAGCTAGT TGGTGGGGTA ATGGCTCACC AAGGCGACGA TCCCTAGCTG
ArmhydroSWB2  ATTAGCTAGT TGGTGGGGTA ATGGCTCACC AAGGCGACGA TCCCTAGCTG
E.coli        ATTAGCTAGT AGGTGGGGTA ACGGCTCACC TAGGCGACGA TCCCTAGCTG

[          301                                                    350]
Armsalmo      GTCTGAGAGG ATGATCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC
Armhydro7966  GTCTGAGAGG ATGATCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC
Armhydro7965  GTCTGAGAGG ATCATCANCC ACACTGGAAC TTANACACCG TCCACACNCC
Armbs         GTCTGAGAGG ATGACCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC
ArmhydroSWB1  GTCTGAGAGG ATGATCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC
ArmhydroSWB2  GTCTGAGAGG ATGATCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC
E.coli        GTCTGAGAGG ATGACCAGCC ACACTGGAAC TGAGACACGG TCCAGACTCC

[          351                                                    400]
Armsalmo      TACGGGAGGC AGCAG-TGGG GAATATTGCA CAATGGGGGA AACCCTGATG
Armhydro7966  TACGGGAGGC AGCAG-TGGG GAATATTGCA CAATGGGGGA AACCCTGATG
Armhydro7965  CTCNGGAGGC NCCCCCTGGG GAATTTTTNC NCCCCGCCGG ..........
Armbs         TACGGGAGGC AGCAG-TGGG GAATATTGCA CAATGGGGG. ..........
ArmhydroSWB1  TACGGGAGGC AGCAG-TGGG GAATATTGCA CAATGGGGGA AACCCTGATG
ArmhydroSWB2  TACGGGAGGC AGCAG-TCCC CAATATTGCA CAATGGGGGA AACCCTGATG
E.coli        TACGGGAGGC AGCAG-TGGG GAATATTGCA CAATGGGCGC AAGCCTGATG

[          401                                                    450]
Armsalmo      CAGCCATGCC GCGTGTGTGA AGAAGGCCTT CGGGTTGTAA AGCACTTTCA
Armhydro7966  CAGCCATGCC GCGTGTGTGA AGAAGGCCTT CGGGTTGTAA AGCACTTTCA
Armhydro7965  .......... .......... .......... .......... ..........
Armbs         .......... .......... .......... .......... ..........
ArmhydroSWB1  CAGCCATGCC GCGTGTGTGA AGAAGGCCTT CGGGTTGTAA AGCACTTTCA
ArmhydroSWB2  CAGCCATGCC GCGTGTGTGA AGAAGGCCTT CGGGTTGTAA AGCACTTTCA
E.coli        CAGCCATGCC GCGTGTATGA AGAAGGCCTT CG-GTTGTAA AGTACTTTCA

[          451                                                    500]
                                  Primer As 2
Armsalmo      GCGAGGAGGA AAGGTTGGCG CC-TAATACG TGTCAACTGT GACGTTACTC
Armhydro7966  GCGAGGAGGA AAGGTTGATG CC-TAATACG TATCAACTGT GACGTTACTC
Armhydro7965  .......... .......... .......... .......... ..........
Armbs         .......... .......... .......... .......... ..........
ArmhydroSWB1  GCGAGGAGGA AAGGTTGGCG CC-TAATACG TGTCAA.... ..........
ArmhydroSWB2  GCGAGGAGGA AAGGTTGGCG CC-TAATACG TGTCAA.... ..........
E.coli        GCGGGGAGGA A-GGGAGTAA AGTTAATACC TTTGCTCATT GACGTTACCC
```

Figure 3.14 Parial sequences for *Aeromonas hydrophila* strains

Armsalmo: *A. salmonicida* 93/1061-1; Armhydro 7966: *A. hydrophila* ATCC 7966^T; Armhydro 7965: *A. hydrophila* ATCC 7965; Armbs: *A. bestiarum* ATCC 14715; ArmhydroSWB1: *A. hydrophila* SWB 216; ArmhydroSWB2: *A. hydrophila* SWB 219;Sequence differences between strains are underlined; N - missing value; primer sites in bold

From the alignments it is clearly evident that the sequences of the strains of *A. hydrophila* which amplified with the *A. salmonicida* primer sets are identical

to the sequences of *A. salmonicida* not only at the primer sites As1 and As2 but also at the restriction digest site G▼GATC between positions 137 and 140. *A. hydrophila* ATCC 7966, the type strain, has a different sequence from *A. salmonicida* and the other strains of *A. hydrophila* at primer sites As1 and As2 and accounts for the absence of PCR product with this strain of *A. hydrophila* when tested with the *A. salmonicida* primer set.

DNA detection limit The sensitivity of the PCR assays for the four target pathogens was determined with purified template DNA by nested and un-nested PCR formats (Figures 3.15 and 3.16). Consideration was also given to

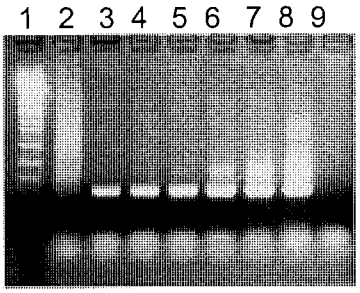


Figure 3.15 DNA detection threshold assay for *Aeromonas salmonicida* 93/0956-1, nested PCR

Lane 1: pUC 19 markers; Lane 2: 1 *fg*
Lane 3: 10*fg*; Lane 4: 100 *fg*
Lane 5: 1 *pg*; Lane 6: 10 *pg*
Lane 7: 100 *pg*; Lane 8: 1 *ng*
Lane 9: negative control

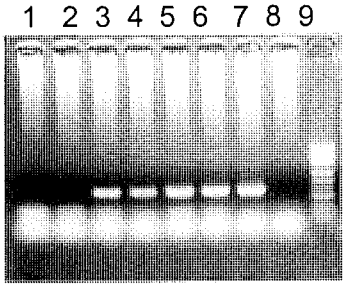


Figure 3.16 DNA detection threshold assay for *Enterococcus seriolicida* ATCC 49156^T, nested PCR

Lane 1: 1 *fg* Lane 2: 10*fg*
Lane 3: 100 *fg*; Lane 4: 1 *pg*
Lane 5: 10 *pg*; Lane 6: 100 *pg*
Lane 7: 1 *ng*; Lane 8: negative control
Lane 9: pUC 19 markers

the consistency of detecting low levels of template DNA. Overall the *reliable* detection limit for all four pathogens by un-nested PCR is 1 ng DNA (2.75 x 10^5 GE) (derived from Trevors 1996) and for nested PCR 10 pg (2.75 x 10^3 GE). These conservative estimates of detection limit are much in excess of the *potential* detection limits which were achieved but not consistently (Table 3.12).

Species	Type	Detection level	Genome equivalents
A. salmonicida	Nested	10 *fg*	2 cells
E. seriolicida	Un-nested	1 *ng*	300,000 cells
	Nested	100 *fg*	30 cells
Fx. maritimus	Un-nested	10 *pg*	2,500 cells
	Nested	1 *pg*	250 cells
Y. ruckeri	Un-nested	1*ng*	250,000 cells
	Nested	10 *pg*	2,500 cells

Table 3.12 Detection limits for nested and un-nested PCR formats

In one trial, the capacity of BSA to ameliorate the effects of PCR inhibitors was assessed with *Fx. maritimus*. In a standard un-nested PCR without BSA the detection limit was 10 pg but in the presence of BSA, there was a ten fold increase in the detection limit which was lowered to 1 pg DNA, a genome equivalent of only 250 cells.

3.3 Develop procedures for testing fish by molecular probes for the presence of bacterial fish pathogens

The bacterial pathogens were seeded at varying concentrations into selected tissues to reflect the known or likely distribution of the pathogen in fish; these are summarised in Table 3.13. Nested and un-nested PCR formats were used

Species	kidney	brain	skin	gut
A. salmonicida	✓	✗	✓	✗
E. seriolicida	✓	✓	✗	✗
Fx. maritimus	✗	✗	✓	✗
Y. ruckeri	✓	✗	✗	✓

Table 3.13 Tissues seeded for determining detection limits by PCR

for all species except for *E. seriolicida* because of the uncertain specificity of the nested format in regard to *Clostridium putrificum*. The summarised data is given in Table 3.14. For all data sets given, the no DNA negative control and un-seeded tissue samples were negative. A typical titration assay is given in Figure 3.17. For *A. salmonicida* and *Y. ruckeri*, the endpoints could not be established in the nested PCR assays because positive bands were detected at all seeded concentrations as well as the un-seeded tissue samples though not in the no-DNA controls (Figure 3.18).

Species	Tissue	Un-nested	Nested	Tissue	Un-nested	Nested
A. salmonicida	kidney	NT	$^{\dagger}1x10^{1}$	skin	NT	$^{\dagger}1x10^{1}$
E. seriolicida	kidney	$1x10^{7}$	NT	brain	$1x10^{5}$	NT
Fx. maritimus	skin	$1x10^{5}$	$1x10^{5}$	NT	NT	NT
Y. ruckeri	kidney	$1x10^{4}$	$^{\dagger}1x10^{1}$	gut	$1x10^{3}$	$^{\dagger}1x10^{1}$

Table 3.14 PCR detection limits in fish tissues. Values as cells g^{-1} tissue
NT: not determined; †: endpoint less than theoretical detection limit

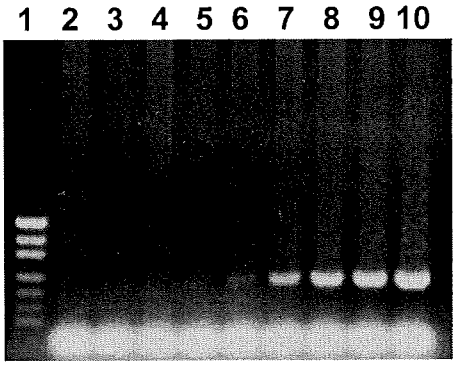


Figure 3.17 Un-nested PCR of Atlantic salmon kidney tissue seeded with *Yersinia ruckeri*

Lane 1: pUC19 markers; Lane 2: un-seeded tissue
Lane 3: $1x10^1$ cells g^{-1}; Lane 4: $1x10^2$ cells g^{-1}
Lane 5: $1x10^3$ cells g^{-1}; Lane 6: $1x10^4$ cells g^{-1}
Lane 7: $1x10^5$ cells g^{-1}; Lane 8: $1x10^6$ cells g^{-1}
Lane 9: $1x10^7$ cells g^{-1}; Lane 10: $1x10^8$ cells g^{-1}

Arrow: positive PCR band, detection threshold value

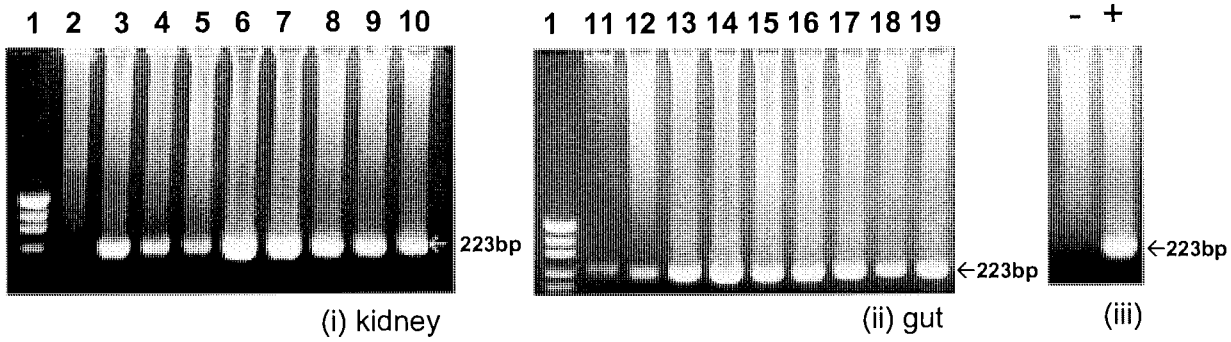


Figure 3.18 Nested PCR of Atlantic salmon tissues seeded with *Yersinia ruckeri*

(i) Lane 1: pUC19 markers; Lane 2: un-seeded kidney tissue
Lanes 3-10 seeded kidney tissue: $1x10^1$ - $1x10^8$ cells g^{-1}
(ii) Lane 1: pUC19 markers; Lane 11: un-seeded gut tissue
Lanes 12-19 seeded gut tissue: $1x10^1$ - $1x10^8$ cells g^{-1}
(iii) Positive and negative controls

Theoretical detection limit Based on tissue sample size, the extraction process and PCR format, the theoretical detection limit was determined as $2x10^3$ cell g^{-1}. The limit value is based on the following data and assumptions: the maximum tissue sample size which can be processed by the QIAamp® kit (Qiagen) is 50 mg with a final extraction volume of 100µl. To detect at least this number of bacteria the following assumptions were made: each bacterial cell contains only one copy of the 16S rRNA gene, there are no PCR inhibiting substances and each microlitre of extract contains the one copy of the target gene.

3.4 Develop a secondary confirmation system for PCR assays using internal probes and hybridization

3.4.1 Identification of internal probe sequences

The 16S rRNA sequences of *E. seriolicida, Fx. maritimus* and *Y. ruckeri* were extracted from ANGIS and aligned with the sequences of the nearest genomic neighbours (Tables 3.3-3.6). Specific internal probes of between 26 and 33 base pairs were identified for the three bacteria and the uniqueness of their sequences established by a BLAST (Basic Local Alignment Search Tool) search of the ribosomal database. The nearest sequence to *E. seriolicida* was a *Pseudomonas* species (strain GR-04, GSDB X86626) (87% similarity); to *Fx. maritimus, Flectobacillus glomeratus* (GSDB G174588) (85% similarity); and

Enterococcus seriolicida

```
 [          51                          Primer Es9                    100 ]
C.pputrifi  TAACACATGC AAGTCGAGCG ATG------- --AAG--TTC CTTCGGGAA-
L.maltarom  TAATACATGC AAGTCGAACG CA-----CG- AAGTTGAAGA GCTTGCTCTT
Eco.seriol  TAATACATGC AAGTCGAGCG AT-------G ATTAAAGATA GCTTGCTATT
Lcc.garvie  TNATACATGC AAGTCGAGCG AT-------G ATTAAAGATA GCTTGCTNTT

[         101           Esprb                                       150 ]
C.pputrifi  -CGG------ ---ATTAGCG GCGGACGGGT GAGTAACACG TGGGCAACCT
L.maltarom  TAACG-AAG- ---TG-AGTG GCGGACGGGT GAGTAACACG TGGGTGACnn
Eco.seriol  TTTATGA--- ---AG-AGCG GCGAACGGGT GAGTAACGCG TGGGAAATCT
Lcc.garvie  TTTATGA--- ---AG-AGCG GCGAACGGGT GAGTAACGCG TGGGAAATCT

[         151                                                       200 ]
C.pputrifi  GCCTTATAGA GGGGAATAGC CTTCCGAAAG GAAGATTAAT ACCGCATAAG
L.maltarom  GCnnATTAGA GGGGGATAAC ATTCGGAAAC GGATGCTAAT ACCGCATAGT
Eco.seriol  GCCGAGTAGC GGGGGACAAC GTTTGGAAAC GAACGCTAAT ACCGCATAAC
Lcc.garvie  GCCGAGTAGC GGGGGACAAC GTTTGGAAAC GAACGCTAAT ACCGCATAAC

[         201        Primer Es6                                     250 ]
C.pputrifi  ATTGTACGTT CGCATGAAGT AGCAAT-TAA AGGA------ --GCAA----
L.maltarom  TTCAGGR-AT CGCATGAT-T CTTGAAGGAA AGGTGGC--- --TTCG---G
Eco.seriol  AATGAG-AAT CGCATGATTC TTATTT-AAA AGAAGC---- --AATT----
Lcc.garvie  AATGAG-AAT CGCATTATTG TTNTTT-GAA AGAAGC---- --AATT----
```

Flexibacter maritimus

```
 [         201  Primer  rFlex 1            Fxmprb                    250 ]
Cy.marinof  TGATTTGGCA TCATTTTAAT AGTAAAG-GT TA-CGGTACA AGATGAGCAT
F.odoratum  ATGAATGGCA TCGTTTRTAT AATAAAGATT TATCGGTAAA AGATGGGCAT
Flx.marit   TGGAATGGCA TCGTTTTAAA GTTAAAGATT TATCGGTAGA AGATGACTAT
Ppm.asacch  TTAGATCCCA TGAGAAGAGG AGGAAAGATT AATCGCTAAG AGATGGGCCT

[         251                                                       300 ]
Cy.marinof  GCGTCCTATT AGCTAGTAGG TGTGGTAACG GCACACCTAG GCAACGATAG
F.odoratum  GCGTATCATT AGCTAGTTGG TGTGGTAACG GCATACCAAG GCTnCGATGA
Flx.marit   GCGTCCTATT AGCTAGATGG TAAGGTAACG GCTTACCATG GCAACGATAG
Ppm.asacch  GCGTTCCATT AGCTAGTTGG TAAGGTAACG GCTTACCAAG GCAACGATGG
```

```
[         301                                                    350 ]
Cy.marinof  GTAGGGGTCC TGAGAGGGAG ATCCCCCACA CTGGTACTGA GACACGGACC
F.odoratum  TTAGGGGTCC TGAGAGGGAG ATCCCCCACA CTGGTACTGA GACACGGACC
Flx.marit   GTAGGGGTCC TGAGAGGGAG ATCCCCCACA CTGGTACTGA GACACGGACC
Ppm.asacch  ATAGGGGGAC TGAGAGGTTG ACCCCCCACA TTGACACTGA GATACGGGTC

[         351                                                    400 ]
Cy.marinof  AGACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGACAAT GGGCGCAAGC
F.odoratum  AGACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT GGAGGCAACT
Flx.marit   AGACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGGCAAT GGAGGCAACT
Ppm.asacch  NAACTCCTAC GGGAGGCAGC AGTGAGGAAT ATTGGTCAAT GGGCGAGAGC

[         401                                                    450 ]
Cy.marinof  CnnATCCAGC CATGCCGCGT GCAGGAAGAC TGCCCTAT-G GGTTGTAAAC
F.odoratum  CTGAACCAGC CATGCCGCGT GCAGGATGAC GGTCCTAT-G GATTGTAAAC
Flx.marit   CTGACCCAGC CATGCCGCGT GCAGGAAGAC TGCCCTAT-G GGTTGTAAAC
Ppm.asacch  CTGAACCAGC CAAGTCGCGT GAAGGAAGAC TGCCCGCAAG GGTTGTAAAC

[         451                    Primer rFlex 3              500 ]
Cy.marinof  TNCTTTTATA CGGGAAGAAT AAGGTCTACG AGTAGGCTGA TGACGGTACC
F.odoratum  TGCnTTTGTA CGGGAAGAAA TGTAATTACG TGTAATTATT TGACGGTACC
Flx.marit   TGCTTTTATA CAGGAAGAAA CGTACCTACG AGTAGGTATT TGACGGTACT
Ppm.asacch  TNCTTTTGTA TGGGATTAAA GTCACCTACG TGTAGGTNTT TGCAGTTACC
```

Yersinia ruckeri

```
 [         201                                  Primer Yr1    250 ]
H.influenz  GCGGGACTGA GAGGCCGCAT GCCATAGGAT GAGCCCAAGT GGGATTAGGT
Yer.bercov  GGGGGACCTT CGGGCCTCAC GCCATCGGAT GAACCCAGAT GGGATTAGCT
Yer.kristn  GGGGGACCTT AGGGCCTCAC GCCATCGGAT GTGCCCAGAT GGGATTAGCT
Yer.pestis  GGGGGACCTT AGGGCCTCAC GCCATCGGAT GAACCCAGAT GGGATTAGCT
Yer.rucker  GGGGGACCTT CGGGCCTCAC GCCATCGGAT GAACCCAGAT GGGATTAGCT

[         251                                                    300 ]
H.influenz  AGTTGGTGGG GTAAATGCCT ACCAAGCCTG CGATCTCTAG CTGGTCTGAG
Yer.bercov  AGTAAGTGGG GTAATGGCTC ACCTAGGCGA CGATCCCTAG CTGGTCTGAG
Yer.kristn  AGTAAGTGGG GTAATGGCTC ACCTAGGCGA CGATCCCTAG CTGGTCTGAG
Yer.pestis  AGTAGGTGGG GTAATGGCCC ACCTAGGCGA CGATCCCTAG CTGGTCTGAG
Yer.rucker  AGTAAGTGGG GTAATGGCTC ACCTAGGCGA CGATCCCTAG CTGGTCTGAG

[         301                                                    350 ]
H.influenz  AGGATGACCA GCCACACTGG AACTGAGACA CGGTCCAGAC TCCTACGGGA
Yer.bercov  AGGATGACCA GCCACACTGG AACTGAGACA CGGTCCAGAC TCCTACGGGA
Yer.kristn  AGGATGACCA NCCACACTGG ACCTGAGACA CGGTCCAGAC TCCTACGGGA
Yer.pestis  AGGATGACCA GCCACACTGG AACTGAGACA CGGTCCAGAC TCCTACGGGA
Yer.rucker  AGGATGACCA GCCACACTGG AACTGAGACA CGGTCCAGAC TCCTACGGGA

[         351                                                    400 ]
H.influenz  GGCAGCAGTG GGGAATATTG CGCnATGGGG GGAACCCTGA CGCAGCCATG
Yer.bercov  GACANCAGTG GGGAATATTG CACAATGGGC GCAAGCCTGA TACACCCATA
Yer.kristn  GGCAGCAGTG GGGAATATTG CACAATGGGC GCAAGCCTGA TGCAGCCATG
Yer.pestis  GGCAGCAGTG GGGAATATTG CACAATGGGC GCAAGCCTGA TGCAGCCATG
Yer.rucker  GGCAGCAGTG GGGAATATTG CACAATGGGC GCAAGCCTGA TGCAGCCATG
 [         401                                          Yrprb    450 ]
H.influenz  CCGCGTGAAT GAAGAAGGCC TTCGGGTTGT AAAGTTCTTT CGGTATTGAG
Yer.bercov  CCGCGTGTGT GNAGAAGNCC TTCGGGNTGT AAAGCACTTT CACCGAGGAG
Yer.kristn  CCGCGTGTGT GAAGAAGGCC TTCGGGTTGT AAAGCACTTT CAGCGAGGAG
Yer.pestis  CCGCGTGTAT GAAGAAGGCC TTCGGGTTGT AAAGTACTTT CAGCGAGGAG
Yer.rucker  CCGCGTGTGT GAAGAAGGCC TTCGGGTTGT AAAGCACTTT CAGCGAGGAG
```

```
[          451              Primer Yr2                              500 ]
H.influenz  GAAGGTTGAT GTGTTAATAG CACATCAAAT TGACGTTAAA TACAGAAGAA
Yer.bercov  GAAGNCAGTC GTGTTAATAG CACGATTGAT TGNCGTTACT CGCAGAAGAA
Yer.kristn  GAAGGCAATC GTGTTAATAG CACGGTTGAT TGACGTTACT CGCAGAAGAA
Yer.pestis  GAAGGGGTTG AGTTTAATAC ACTCAATCAT TGACGTTACT CGCAGAAGAA
Yer.rucker  GAAGGGTTAA GTGTTAATAG CACTGAACAT TGACGTTACT CGCAGAAGAA
```

Figure 3.19 Sequence alignments for the bacterial fish pathogens *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* with genomically similar species and the position of the internal PCR hybridization probes.

C.putrific: *Clostridium putrificum*; Cy. marinof: *Cytophaga marinoflava* Eco.seriol: *Enterococcus seriolicida*; F.odoratum: *Flavobacterium odoratum*; Flx.marit: *Flexibacter maritimus*; H.influenz: *Haemophilus influenzae*; L.maltarom: *Lactobacillus maltaromicus*; Lcc.garvie: *Lactococcus garvieae*; Ppm.asacch: *Porphyromonas asaccharolytica*; Yer.bercov: *Yersinia bercovieri*; Yer.kristn: *Yersinia kristensenii*; Yer.pestis: *Yersinia pestis*; Yer.rucker: *Yersinia ruckeri*.

n: nucleotide unknown; primer sites in bold italics; probe sites underlined and bold

to *Y. ruckeri*, *Rhanella aquatilis* (X79939) (96% similarity). The probes (Table 3.15) were considered to have sufficient distinguishing characteristics for use as internal verification of amplified PCR product; their approximate alignments with near related species is given in Figure 3.19.

Species	Probe sequence 5' → 3'
E. seriolicida	TTT TTA TGA AGA GCG GCG AAC GGG T
Fx. maritimus	AAA GTT AAA GAT TTA TCG GTA GAA GAT GAC TAT
Y. ruckeri	GCA CTT TCA GCG AGG AGG AAG GGT TAA

Table 3.15 Internal verification probes for PCR amplified DNA

3.4.2 Internal probe specificity

The internal probes designed for *E. seriolicida, Fx. maritimus* and *Y. ruckeri*, were able to hybridize to their respective DNAs generated by un-nested PCR. The probes only hybridized with their homologous PCR amplified DNA and not with DNA amplified by the other two bacteria. The optimum conditions for hybridization were found to be a probe concentration of 500 ng ml^{-1}, a T_{HYB} of 40-42°C and a T_{WASH} of 40°C. Although hybridization dots were clearly evident none of the signals were particularly strong nor was it possible to achieve consistent results. In the nested PCR format for *E. seriolicida* and *Cl. putrificum* the internal probe *Esprb* hybridised with PCR product generated from *E. seriolicida* as well as *Cl. putrificum*. Increasing stringency of the hybridization

conditions by raising hybridization temperature to 60°C failed to provide conditions which differentiated the two species.

3.4.3 DNA detection limit determined by dot blot hybridization of PCR reactions

It was possible to lower the detection limit for *E. seriolicida* in fish tissue using the *Esprb* probe. In an un-nested PCR the detection limit was determined at 100 ng but with hybridization 1 ng of template DNA was detected, a 100 fold improvement. Improved sensitivity was not seen with the remaining two probes. In a comparative assays of PCR and dot blot hybridization for *Fx. maritimus*, it was possible to detect 1 pg of DNA by nested PCR alone but only 10 pg of DNA by hybridization with the internal probe. A similar observation was noted for *Y. ruckeri* where the detection limit by PCR alone was 10 pg but with hybridization the detection level was 100 pg.

3.5 Formulate the developed test procedures for transfer to, and adoption by, veterinary diagnostic and research laboratories

Protocols were developed for performing nested and un-nested PCR for the rapid identification of pure cultures of the four bacterial pathogens *Aeromonas salmonicida, Enterococcus seriolicida, Flexibacter maritimus* and *Yersinia ruckeri*. In addition, formats were developed for the direct detection of these bacteria in fish tissue. The protocols were designed so that the technology could be readily adopted by laboratories which either had no experience in these type of assays or were familiar with the technology but required specific information regarding the assays.

A manual was prepared in a format that provided step-by-step guidance of the techniques and with sufficient background information for laboratories to use the procedures in isolation and without the need to obtain data or methodologies from other sources. The protocols were tested *de novo* with staff at the Department of Primary Industry and Fisheries, Tasmania and the Fish Disease Laboratory of the Australian Animal Health Laboratory, CSIRO. The final form of the manual was made following this trial technology transfer and is given in full in the Appendix (Section 6).

State	Institution	No. Participants
Tasmania	University of Tasmania	3
	CSIRO, Division of Fisheries	2
Victoria	CSIRO, Australian Animal Health Laboratory	2
	Department of Agriculture	1
Queensland	Department of Primary Industry, Queensland	1
Western Australia	Department of Agriculture	1
South Australia	Primary Industries, South Australia	1

Table 3.17 Institutions participating in the technology transfer workshop

The PCR technology was transferred to laboratories by running a three day laboratory based workshop with an emphasis on participants undertaking all the techniques developed during the project. The workshop was promoted through the Co-operative Research Centre for Aquaculture's Internet news group, by direct mailing of Australian members of the European Association of Fish

Day 1	
Introduction	Jeremy Carson
Molecular Probes and PCR (lecture)	John Bowman
PCR of bacterial colonies	Michelle Williams
• DNA extraction	
• Set up nested and un-nested PCR	
PCR of seeded fish tissue	Michelle Williams
• DNA extraction (first part)	
Summary	
Day 2	
PCR for disease detection (lecture)	Jeremy Carson
Practical aspects of PCR (lecture)	Michelle Williams
PCR of bacterial colonies	Michelle Williams
• gel electrophoresis	
PCR of seeded fish tissue	Michelle Williams
• DNA extraction (second part)	
• Set up PCR	
PCR of bacterial colonies	Michelle Williams
• determine concentration of extracted DNA	
• gel electrophoresis of extracted DNA	
Summary	
Day 3	
PCR of seeded fish tissue	Michelle Williams
• Gel electrophoresis of PCR products	
Summary	

Table 3.18 Workshop programme

Pathologists and state Government diagnostic veterinary laboratories. The institutions listed in Table 3.17 sent scientific staff who were involved either in fish disease diagnostic services, fish disease research or fisheries research.

The workshop was conducted at the laboratories of the Department of Agricultural Sciences at the University of Tasmania in Hobart. The course each day began with a lecture regarding aspects of the technology, followed by practical work during most of the day and finishing with a summary discussion session. An outline of the programme is given in Table 3.18. Staff involved in teaching the course were: Michelle Williams (University of Tasmania), Dr John Bowman (University of Tasmania), Sharee McCammon (University of Tasmania) and Dr Jeremy Carson (Department of Primary Industry & Fisheries, Tasmania).

Course Content	**Approval**	
• Was the mix of practical, lecture and discussion appropriate?	86%	
• Was sufficient explanation of the technology provided in the lectures?	80%	
• Did the lectures or discussions provide new insights or information?	84%	
• Did the practicals demonstrate the technology sufficiently?	84%	
• Did the prepared notes provide sufficient information?	88%	
Instruction		
• Was the technology presented clearly in the workshop?	86%	
• Explanations to questions:	80%	
• Technology transfer:	72%	
Application of the Technology	**Comment**	
• Would you make use of the technologies described during the workshop?	Yes:	80%
	No:	20%
If **yes**, what do you propose to use the techniques for?	Disease diagnosis:	20%
	Identification:	40%
	Teaching:	10%
	Not stated:	30%
• If you don't have an immediate application for the technology will you use it in:	6 months:	20%
	12 months:	10%
	2 years:	10%
	Not stated:	60%
• What obstacles, if any, are there to adopting the technology?	Cost:	30%
	Resources:	10%
	No. of samples:	20%
	Not stated:	40%
Organisation	**Approval**	
• Information describing the course:	85%	
• Organisation and conduct of the course:	94%	
• Facilities used for the workshop:	94%	
Overall		
• How would you rate the course overall?	90%	

Table 3.19 Participant responses about technology transfer workshop

Feedback about the workshop was obtained from participants by anonymous questionnaire; the response rate was 91%. The findings are summarised in Table 3.19. Approval values were calculated by assigning a value of 1-5 to the responses given by the participants, 5 was excellent, 1 was poor and expressing the total for each question as a percentage of the maximum score. Overall there was a high level of satisfaction with the technology, its suitability for disease diagnosis and a stated commitment to using it.

Section 4

Discussion and Conclusions

4.1 Develop molecular probes for enzootic strains of *Aeromonas salmonicida, Enterococcus seriolicida, Flexibacter maritimus* and *Yersinia ruckeri*

4.1.1 Basis of probe design

A directed probe strategy that nominated *a priori*, the 16S rRNA gene as the target sequence was used to develop the diagnostic molecular probes for the four bacterial fish pathogens. This approach was chosen because of the inherent efficiency and predicability of directed probes, in contrast to a random probe strategy using target sequences anywhere on the bacterial genome. Random probes, although capable of sub-species or strain identification if suitably constructed, require extensive verification *a posteriori* to determine their utility (Priest & Austin 1993).

The 16S rRNA gene is highly stable and is the basis on which diagnostic probes may be discriminatory at the genus as well as species level (Barry *et al.* 1990). The gene contains sequences of several types: regions which are highly conserved across genera: sequences characteristic of the genus: and sequences associated with a species (Priest & Austin 1993). The amount of information and its usefulness depends on several factors but principally the phylogenetic age of the taxon. In taxa of greater phylogenetic age, there is greater variability in sequence data amongst species (Fox *et al.* 1980; Stackebrandt 1988), but in more recently evolved genera such as *Aeromonas* (Ruimy *et al.* 1994), sequence variation is low and discrimination between species may not always be achievable (Stackebrandt & Goebel 1994). In most cases however there is usually sufficient sequence variation in 16S rRNA to identify unique signatures that can be used for reliable identification of the species (Priest and Austin 1993).

4.1.2 Aeromonas salmonicida

The sequence data for the genus *Aeromonas* show a high level of overall similarity (98-100%) between species (Martinez-Murcia *et al.* 1992) indicating that there may be little scope for developing species specific probes. In the V2 variable region of the 16S rRNA gene, Barry *et al.* (1990) found that sequences between *A. salmonicida, A. hydrophila, A. media* and *A. veronii* were almost

identical but in the V6 region, differences ranged from 2 to 45 base pairs. Further studies involving 12 species of *Aeromonas* was able to demonstrate that these species had unique 16S rDNA sequences in the V3 region, despite the recent phylogenetic origins of the genus (Martinez-Murcia *et al.* 1992). The usefulness of the observed sequence differences between species was further evaluated by Dorsch *et al.* (1994) who was able to identify unique sequences corresponding to 10 species or species complex in the V3 region of the 16S rRNA gene.

The sequence data for *A. salmonicida* strain DPIF 93/1061-1 was aligned with the *A. salmonicida* subspecies and found to have 100% homology with *A. salmonicida* ssps. *achromogenes* and *masoucida* and represents further evidence that this isolate from native greenback flounder is a *bona fide* biovar of *A. salmonicida*. The PCR primer set identified in this study were located in helix 8 (adjacent to V2) and V3 region, loci that contain species linked variation (Martinez-Murcia *et al.* 1992; Dorsch *et al.* 1994). Of the two loci, helix 8 contains the least sequence variation and is regarded as a moderately conserved region although containing some marked variability (Neefs *et al.* 1993). At this site primer As1 is completely homologous with *A. sobria* while for most other aeromonads there is only one base pair difference. This is not the case for primer As2 in the V3 region where the closest sequence homology of 85% (3 bp difference) is with *A. hydrophila.* The choice of primers for *A. salmonicida* is circumscribed by the paucity of sequence variation in the 16S rRNA gene and it is not surprising that similar primer sets have been independently identified (Dorsch *et al.* 1994; Høie *et al.* 1996). The similarity of the probe sequences is given in Table 4.1.

Source	Primer/Probe	Sequence 5' → 3'
FRDC 93/128	As1 (forward)	TTT CGC GAT TGG ATG AA
Høie *et al.* 1996	Forward	G GCC TTT CGC GAT TGG ATG A
FRDC 93/128	As2 (reverse)	TTG ACA CGT ATT AGG CGC CA
Høie *et al.* 1996	Reverse	TCA CAG TTG ACA CGT ATT AGG CGC
Dorsch *et al.* 1994	oligonucleotide	TTG ACA CGT ATT AGG CGC CA

Table 4.1 16S rRNA sequences for *A. salmonicida* probes

Although primer As1 is very similar (94-100% homology) to some species of *Aeromonas*, there were no apparent similarities to known sequences for other bacteria. The ability of the *A. salmonicida* primer set to distinguish species of the genus rests largely with primer As2. The predicted specificity was observed when the primer set was tested by PCR except in the case of some strains of *A. hydrophila*. Høie *et al.* (1996) found that their primer set could not reliably distinguish *A. salmonicida* from *A. hydrophila* although Dorsch *et al.* (1994) however assumed their probe was specific for *A. salmonicida* and identified several aeromonads from river water by probe alone. Subsequent phenotypic characterisation of two of their isolates, SWB 216, SWB 219 (J. Carson and T. Wagner unpublished data), established unequivocally that they were *A. hydrophila* and not *A. salmonicida*.

On the basis of these reports and findings, there was sufficient evidence to suggest that an *A. salmonicida* primer set based on the 16s rRNA gene may lack sufficient sequence variation to discriminate between *A. hydrophila* and *A. salmonicida*. With the primer set As1/As2, and using either a nested PCR format or the stringent un-nested format, a PCR product of 260 bp, the expected amplicon size for *A. salmonicida*, occurred with 4/17 strains of *A. hydrophila*; significantly, there was no reaction with the type strain of *A. hydrophila* ATCC 7966^T. The only other aeromonad that produced a 260 bp amplicon with As1/As2 was *A. bestiarum* (hybridization group 2) ATCC 14715. Høie *et al.* (1997) tested this strain as *A. hydrophila* ATCC 14715 and also found that it produced a PCR amplicon with their *A. salmonicida* primer set, which is almost identical to As1/As2 (Table 4.1).

To determine the sequence identity of the 260 bp amplicon produced with the cross reacting strains of *A. hydrophila*, a 430bp amplicon of DNA was amplified between primer site A and As2 and digested with the restriction endonuclease *Bst*Y1. All the 430bp amplicons were digested indicating that these 4 strains of *A. hydrophila* and *A. bestiarum* have sequence homology with *A. salmonicida* in this region of the 16S rRNA gene. Subsequent sequencing of the 16S rRNA between primer sites A and As2 of the *A. hydrophila* strains and *A. bestiarum*, showed that these strains have almost

total sequence homology with *A. salmonicida*. The sequence homology observed between *A. salmonicida* and *A. bestiarum* (formerly *Aeromonas* HG2) confirms an earlier report of sequence homology between *A. bestiarum* (CIP 7430 = ATCC 51108^T) and the three extant sub-species of *A. salmonicida* ssp. *salmonicida, achromogenes* and *masoucida* (Martinez-Murcia *et al.* 1992). This homology of the 16S rRNA sequence between primer sites As1 and As2 for *A. salmonicida*, *A. bestiarum* and some strains of *A. hydrophila* accounts for the 260bp amplicon produced by PCR with this primer set. The type strain of *A. hydrophila* ATCC 7966^T however does not have sequence homology with *A. salmonicida* and does not react with primer set As1/As2.

The failure of the As1/As2 primer pair to reliably differentiate between *A. salmonicida*, *A. bestiarum* and some strains of *A. hydrophila* is a result of the sequence homology that *A. bestiarum* and some strains of *A. hydrophila* have with *A. salmonicida*. The 16S rRNA gene was chosen as a strategy for directed probe design because of the high probability of species specificity attached to this gene. In most cases, sequence variation is sufficiently high between species to ensure reliable differentiation. Where a genus lacks phylogenetic 'depth' as in the case of *Aeromonas* (Martinez-Murcia *et al.* 1992), there may be insufficient sequence variation to distinguish species. High sequence homology between species that have DNA-DNA hybridization values less than 70%, the criterion accepted by convention for differentiating species, has been reported for *Bacillus globisporus* and *B. psychrophilus* (Fox *et al.*, 1992). The close sequence homology (>99.5%) between these two species is constant and quite different to the sequence homology between *A. salmonicida* and *A. hydrophila* which appears to be variable. In the latter case, the type strain of *A. hydrophila* ATCC 7966^T has a sequence homology that is recognisably different from *A. salmonicida* and yet some strains of *A. hydrophila* have an identical sequence to *A. salmonicida* between helices 1 to 11 of the 16S rRNA which encompass the critical species differentiation sites at helix 8 (As1) and the variable region V3 (As2). The occurrence of sequence variability in small sub unit rRNA is generally assumed to be low and yet in one retrospective study, over 14% of strains examined had a sequence variation between 1 and

2%, equivalent to 15-30bp across the 16S rRNA gene (Clayton *et al.* 1995). Clearly the assumption of the type strain as the genomic representative of the species and intraspecies sequence constancy can not be made without first testing an adequate range of strains. As shown here, strain variation, particularly of those species of shallow divergence, needs to be considered in primer design. The primer set for *A. salmonicida* was developed assuming a high level of intraspecies constancy for *A. hydrophila*. The primer set was validated by its ability to reliably distinguish between *A. hydrophila* and *A. salmonicida* but only when the genotype of *A. hydrophila* strains is identical to the type strain. The shared sequence homology that exists between *A. salmonicida* and some strains of *A. hydrophila* as well as *A. bestiarum* limits the usefulness of the primer set. It has a valid role nevertheless as a screening tool for *A. salmonicida*. Any positive findings will require verification by some alternative means or tested further with other *A. salmonicida* specific PCR probes.

Directed and random probes have been developed for *A. salmonicida* outside the 16S rRNA construct, but each has some limitations of performance in regard to specificity. A directed PCR probe targeting the *vap*A gene that codes for expression of the paracrystalline surface protein layer (A-protein) virulence factor (Gustafson *et al.* 1992) has been developed and can detect strains of *A. salmonicida* that have the *vap*A gene irrespective of whether or not the A-layer protein is expressed by the cell. The 70 kDa serine protease gene, another virulence factor in *A. salmonicida*, has been used to develop a hybridization probe (Whitby *et al.* 1992) although its usefulness as a detection probe may limited in that not all biovars or strains of *A. salmonicida* carry the gene (Whitby *et al.* 1992). The remaining four *A. salmonicida* probes were all derived by random probe strategy. For two of these probes, PAAS (Hiney *et al.* 1992) (4.2 mDa plasmid, Mooney *et al.* 1995) and the Sørum 3.4 mDa plasmid (Høie *et al.* 1997), the target sequence has been identified as extra-chromosomal plasmids of *A. salmonicida* ssp. *salmonicida*, but for the Asal-3 clone derived PCR primer set specific for *A. salmonicida* ssp. *salmonicida* (Miyata *et al.* 1996) and the *A. salmonicida* hybridization probes 15e1 and 15e4

(Oakey *et al.* 1998), the identity and location of the target sequences is unknown.

All these directed and random generated probes, apart from the 16s rRNA primers As1/As2 and Høie *et al.* (1997), are reported as being specific for *A. salmonicida* and do not appear to cross react with other aeromonads or species commonly encountered in fish. Although the inter-species specificity appears to be high for these probes, the strain specificity is less satisfactory. Byers *et al.* (1997) screened a collection of 286 isolates of *A. salmonicida* comprising typical and atypical strains and found that the *vap*A primers detected 92% of strains while the PAAS primer identified 94% of strains. It is apparent that both the 16S rRNA primers and probes targeting *A. salmonicida* specific sequences have limitations through either false positives (16S rRNA) or false negatives (PAAS and *vap*A).

4.1.3 *Enterococcus seriolicida*

The sequence data determined for *E. seriolicida* clearly showed that the species had a characteristic 16S rRNA signature of the genus *Lactococcus* and construction of a phylogenetic tree places the species on a major branch shared with *Lactococcus lactis*, composed of its two subspecies *lactis* and *cremoris* (Miller 1995). Sequence similarity amongst the species ranges between 92.4% (*L. garvieae* and *L. piscium*) and 96.7% (*L. piscium* and *L. plantarum*) indicating a measure of phylogenetic depth to the genus (Williams *et al.* 1990). The closest sequence homology to *E. seriolicida* is *L. garvieae* at 99.5%, representing 7 base pairs scattered across the 16S rRNA gene. These differences are too few to be considered a meaningful basis for differentiation (Fox *et al.* 1992). The phenotypes for both species are almost identical (J. Carson & L. Schmidtke unpublished data) and is further evidence to suggest that *E. seriolicida* and *L. garvieae* are the one species. Resolution of the classification of *E. seriolicida* was reached following DNA-DNA hybridization studies which showed a level of re-association between the two species that ranged between 73 and 100% (Eldar *et al.* 1996), so confirming that *E. seriolicida* is a junior synonym of *L. garvieae*. For the purposes of this report, the name *E. seriolicida* will continue to be used, but its recent classification is

acknowledged and the name *L. garvieae* should be used in future to describe Australian isolates of *E. seriolicida*.

A species specific primer set was identified for *E. seriolicida* in the variable regions V1 and V2. The closest sequence homologies to the primers were never more than 76% representing 5 base pair differences found amongst species of the genus *Carnobacterium* and *Clostridium*. Another PCR primer set based on the 16S rRNA sequence has been identified (Zlotkin *et al.* 1998) and shares partial similarity to the primer set Es9/Es6. The forward primer, pLG-1 is

Source	Primer	Sequence 5' → 3'
FRDC 93/128	Es6 (forward[§])	TGA GAA TCG CAT GAT TCT TAT
Zlotkin *et al.* 1995	pLG-1 (forward)	CA TAA CAA TGA GAA TCG C
Zlotkin *et al.* 1995	pLG-2 (reverse)	CAA CCC GCG AGG GTG C

Table 4.2 16S rRNA sequences for *E. seriolicida* probes
[§]Es6 primer in reverse notation

located across part of the reverse primer site Es6 (Table 4.2) in the V2 region at position 167 to 203 (*E. coli* numbering). The reverse probe pLG-2 is located between 1300 and 1284 in the V8 variable region and the PCR fragment produced by the primer pair is 1,100 bp, considerably larger than the 143 bp fragment produced by the Es9/Es6 primers. The pLG primers were tested for specificity with single strains of *Lactococcus lactis, Streptococcus iniae, Streptococcus difficile, Lactococcus piscium, Vagococcus salmoninarum* and *A. salmonicida*; no amplification occurred with these species but no other specificity testing was reported. Intra-species specificity was confirmed by testing 35 strains of *L. garvieae* (syn. *E. seriolicida*) from Europe, Asia and Australia; all strains produced a 1,100 bp fragment with the pLG primers.

4.1.4 *Flexibacter maritimus*

Recent taxonomic studies have resolved some of the uncertainty surrounding the classification of yellow pigmented Gram-negative rods (Bernardet *et al.* 1996), confirmed the status of several genera, and led to the allocation of many species to these revised taxa. Although *Fx. maritimus* did not fall within the definitions of these re-modelled genera, its identity as a taxon was confirmed and its relationship to its near neighbours established. The

phylogenetic relationship of *Fx. maritimus* has established the species as part of the *Cytophaga* sub-group of the cytophaga-flavobacter-bacteroides phylum (Gherna & Woese (1992). Within the *Cytophaga* subgroup, a *Fx. maritimus* cluster could be identified based on an invariate sequence signature. Although the taxonomic position of *Fx. maritimus* based on phenotype may not yet be resolved, there is sufficient genotypic evidence however to identify the species as a discrete taxonomic unit.

The primer set rFlex1/rFlex3 is located on two known variable regions, V2 and V3 and share little homology with other species of known sequence. Only three species were identified that had some similarity to the primers, *Flavobacterium odoratum, Cytophaga marinoflava* and *Porphyromonas* (syn. *Bacteroides*) *asaccharolytica* but only at 70% sequence homology, equivalent to a 6 base pair difference between *Fx. maritimus* and these three species. A primer pair for PCR based on the 16S rRNA gene (Table 4.3) was identified by

Source	Primer	Sequence 5' → 3'
Carson *et al.* 1998	rFlex1 (forward)	GGA ATG GCA TCG TTT TAA AG
Wakabayashi *et al.* 1995	MAR1 (forward)	A ATG GCA TCG TTT TAA A
Carson *et al.* 1998	rFlex3 (reverse)	AAT ACC TAC TCG TAG GTA CG
Wakabayashi *et al.* 1995	MAR2 (reverse)	CGC TCT CTG TTG CCA GA

Table 4.3 16S rRNA sequences for *Fx. maritimus* probes

Wakabayashi *et al.* (1995) and shares some attributes to the rFlex primers described in this study. Primer rFlex1 is identical to MAR1, located in the V2 region, but the reverse primers rFlex3 and MAR2 are not the same. The MAR2 primer lies approximately between position 1304 and 1288 (*E. coli* numbering) in the V8 region and would produce an amplicon of ~1100 bp, considerably larger than the 285 bp fragment formed by the rFlex primer pair. Specificity of the MAR primers was not reported except that it differentiated *Fx. maritimus* from other un-named species.

4.1.5 *Yersinia ruckeri*

The genus *Yersinia* forms a discrete cluster of species within the gamma subgroup of the *Proteobacteria*, based on 16S rRNA phylogenetic analysis (Ibrahim *et al.* 1993). Within the genus, 5 sub-lines can be identified, one of which contains a single species, *Y. ruckeri*. The sequence homology for the

group is high ranging from 99.6% for *Y. intermedia* and *Y. mollarettii* to 96.6% for *Y. ruckeri* and *Y. enterocolitica*. Despite the closeness of sequence data for the 16S rRNA gene, a unique primer set Yr2 and Yr1 could be identified that differentiated *Y. ruckeri* from other yersinae, other members of the Enterobacteriaceae and other normal flora associated with fish.

Primer Yr2, the forward primer, is located at helix 8, adjacent to the V2 variable region. Although helix 8 is more conserved, and hence has less inter-species sequence variation, there was sufficient data to identify a probe with features characteristic of *Y. ruckeri*. The sequence of primer Yr2 is however not unique; it is identical to *Y. bercovieri* and is only one base pair different from *Y. pestis* and *Y. pseudotuberculosis*. Despite the close sequence homology between *Y. ruckeri* and these other three *Yersinia* species at the Yr2 locus, specificity of the primer set was achieved as a result of the low sequence homology of the Yr1 probe. Functionality, of the primer set is achieved through primer Yr1, located in the V3 region, which has a sequence homology of 68% (8 bp difference) to three *Haemophilus* species and a sequence homology to *Y. aldovae* of 60% (10 bp difference). The specificity of the Yr2/Yr1 primer set was verified by their inability to anneal during PCR with species having some sequence homology to *Y. ruckeri* or other yersinae.

A primer set has been developed for *Y. ruckeri* using the RAPD technique (random amplification of polymorphic DNA) to identify a species specific region on the genome (Argenton *et al.* 1996). A specific 555 bp fragment 'A' appears to be unique to *Y. ruckeri* and forms the basis of a primer pair YER1 and YER2, which produces an amplicon 512 bp in size. The sequence does not correspond to any region in the 16S rRNA gene or any other reported sequence in the EMBL data bank. The primers have been tested with three strains of *Y. ruckeri* and one isolate each of *A. salmonicida*, *A. hydrophila*, *Vibrio anguillarum, Proteus vulgaris, E. coli* and *Renibacterium salmoninarum*; apart from the target species which reacted positively, none of the other species produced bands. These primers are random generated probes and their utility and specificity awaits more extensive testing. In comparison, the

Yr2/Yr1 primer pair for *Y. ruckeri*, based on the 16S rRNA gene have already been shown to be specific and can be used with a high level of assurance.

Objective 1: Develop molecular probes for enzootic strains of *Aeromonas salmonicida*, *Enterococcus seriolicida*, *Flexibacter maritimus* and *Yersinia ruckeri*

Summary

- PCR primers have been developed based on 16S rRNA sequences
- 16S rRNA primers inherently have high intra- and inter-species specificity, an essential characteristic for the detection of unknown biovars of the target pathogens
- The primer sets are species specific for *Enterococcus seriolicida, Flexibacter maritimus* and *Yersinia ruckeri*
- The As1/As2 primers are specific for *Aeromonas salmonicida* and its biovars, but will cross with react some strains of *Aeromonas hydrophila* (HG 1) and *Aeromonas bestiarum* (HG 2); there is no cross reaction with other *Aeromonas* species
- The 16s rRNA based primers for *A. salmonicida* could be used for screening purposes in the knowledge that all strains of *A. salmonicida* are likely to be detected, but there will be a measure of uncertainty due to false positive reactions from cross reacting aeromonads
- Non-16S rRNA probes for *A. salmonicida* do not cross react with other aeromonads but will lead to under reporting (false negatives) if used for screening

4.2 Develop methodologies using molecular probes for the rapid identification of bacterial fish pathogens recovered by conventional culture techniques

Identification of *A. salmonicida* and *Fx. maritimus* by phenotype poses some difficulties, particularly where a rapid identification is required, because both species are relatively slow growing and tests to differentiate the species are still not well defined. Rapid presumptive techniques for the identification of suspect colonies often relies on serological tests such as slide agglutination or fluorescent antibody tests. Robust discriminatory antisera have been developed for *Y. ruckeri* and *E. seriolicida* although there are some problems nevertheless since several serotypes are recognised for these two species, each requiring a separate diagnostic antiserum. For *A. salmonicida*, polyclonal antisera can cross react with *A. hydrophila* while monoclonal antibodies for *A. salmonicida*, although highly specific, may not necessarily react with all biovars (J. Young & N. Gudkovs, pers.comm.). Rapid identification of *Fx. maritimus* is particularly problematic: the serological homogeneity of the species is not known and phenotypic tests may take up to two weeks incubation to complete.

Identification of a species by genotype is an attractive proposition, particularly if an intra-strain and species genomic signature can be identified, since several potentially rapid procedures are available. Selection of suitable techniques was based on criteria of:

- ease of execution
- speed of test
- specificity and sensitivity
- interpretation
- cost of equipment
- likely availability of equipment in diagnostic laboratories
- avoidance of assays requiring radioactive labels

Based on these criteria, two test formats were considered: rRNA hybridization with non-radioactive labelled oligonucleotide probes and the polymerase chain reaction.

4.2.1 rRNA hybridization

Probing of ribosomal RNA with labelled oligonucleotides can be undertaken with minimal equipment and with the use of a dot blotter, multiple

samples can be handled conveniently. Although the technique is not particularly sensitive, this was not important in this particular format since the test was intended to be used with colonies. Since the target was ribosomal RNA, it has a high copy number in bacteria and would be present in excess.

Despite the use of kits to simplify the test, rRNA hybridization was abandoned as a rapid, reproducible test for the identification of bacterial colonies. Further work was discontinued because of the difficulty in obtaining conditions under which the probes would be specific for the target sequences and not hybridize with non-target sequences. Test formats were developed for *Y. ruckeri* and *A. salmonicida* with varying degrees of success. The *Y. ruckeri* system using the Yr1 probe known to be specific for *Y. ruckeri* and share little sequence homology with other bacteria, could differentiate between *A. salmonicida* and *Y. ruckeri* if stringent conditions were adopted and concentration of the template controlled. A similar performance could not be obtained with the As2 probe for *A. salmonicida* which even under very stringent conditions hybridized equally well with *A. salmonicida* as well as *Y. ruckeri* rRNA.

Although a hybridization system was developed for *Y. ruckeri* with demonstrated specificity at least in regard to *A. salmonicida*, it was not considered expedient to continue optimising the system for the remaining species. Failure to identify appropriate test conditions for the *A. salmonicida* probe are not readily apparent; probe specificity of As2 in relation to *Y. ruckeri* was confirmed by sequence alignments and the hybridization conditions were similar to those used successfully with *Y. ruckeri* and species specific oligonucleotide probes for the fish pathogens *Renibacterium salmoninarum* (Hariharan *et al.* 1995) and *Vibrio anguillarum* (Powell & Loutit 1994).

The hybridization protocol, despite the use of RNA extraction kits and colour development kits, took in excess of 18 hr to obtain a result. Set against the speed and relative simplicity of PCR it was hard to justify this method as a working identification protocol. Although PCR requires specialist equipment, the cost of this has decreased sharply recently and many research and diagnostic laboratories are equipped with thermocyclers and gel electrophoresis units. Set

against the ready access to PCR hardware, the ease of the test format and speed of PCR, hybridization as a primary test technology for rapid identification of bacterial cultures cannot be supported.

4.2.2 Polymerase chain reaction

Important attributes of the PCR test that make it most suitable as an identification test are speed and very small sample size. Coupled with appropriately designed primers and assay conditions, the test can be both highly specific and very reproducible, characteristics that make PCR a widely used tool in disease diagnosis and bacterial identification (Markham 1993; Persing 1993a).

Parameter	Range	PCR	*A. salmonicida* PCR
$MgCl_2$	0.5-3.0 mM	2 mM	1.375 mM
Primers	0.3-3.0 ea	2 µM ea	2 µM ea
dNTPs	20-200mM ea	200 µM ea	200 µM ea
Polymerase	0.2-0.5U $20\mu l^{-1}$	0.5 U $20\mu l^{-1}$	0.5 U $20\mu l^{-1}$
Cycles	30-50	35	30

Table 4.4 Typical variable parameters and range for PCR

The overall parameters for the PCR assays are within the range of conditions established for this type of test (Table 4.4) (Persing 1993b; Cha & Thilly 1995). Primer design represents a compromise between selecting a sequence that is unique for the target species and uniformity of nucleotide composition in respect of G+C ratios and melting temperature (T_m) of the primer pairs. Primer T_m, (Table 4.5) is the temperature where 50% of the primer strands are bound to target template DNA. The melting temperature is affected by oligonucleotide sequence, concentration and solute composition. Primers for the four bacterial

Species	Primer	Length	G+C ratio	Annealing T°C	T_m nn	T_m AT/GC
A. salmonicida	As1	17 bp	41%	60°C	57°C	48°C
	As2	20 bp	50%	60°C	62°C	60°C
E. seriolicida	Es9	27 bp	41%	65°C	65°C	76°C
	Es6	21 bp	33%	65°C	55°C	56°C
Fx. maritimus	rFlex1	20 bp	40%	65°C	57°C	56°C
	rFlex3	20 bp	45%	65°C	49°C	58°C
Y. ruckeri	Yr2	24 bp	42%	65°C	59°C	68°C
	Yr1	25 bp	40%	65°C	58°C	70°C

Table 4.5 Characteristics of PCR primer sets.
T_m nn: melting temperature by nearest neighbour method
T_m AT/GC: melting temperature by 4(G+C)+2(A+T) method

pathogens ranged from 17 to 27 nucleotides in length. Primers at this size tend to be very sequence specific if the annealing temperature is close to their T_m value (Persing 1993b; Dieffenbach *et al.* 1995). Different methods are available for determining T_m values; the nearest neighbour method is considered more accurate although the empirical A+T/G+C method of Suggs *et al.* (1988) is satisfactory for oligonucleotides less than 20 bases in length. For *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* an optimal annealing temperature of 65°C was established for all three bacteria. This temperature is above the T_m values established by the nearest neighbour method but is closer to the values established by the A+T/G+C method of computation. Nearest neighbour T_m is an indication of possible annealing temperature although the final value may exceed the computed T_m by as much as 12°C (Newton 1995). Irrespective of which T_m value is used, the singular and important characteristic is that the elevated annealing temperatures selected for the PCR assays impose a high level of stringency and therefore specificity on the test, an essential requirement for bacterial species identification. Another important factor in regard to PCR performance is the relative uniformity of G+C ratios of the primer pairs. For *Fx. maritimus* and *Y. ruckeri*, the G+C ratios are similar (Table 4.5), but are less well matched for *A. salmonicida* and *E. seriolicida*. These differences in nucleotide composition can lead either to reduced specificity or efficiency since big differences in G+C ratios may result in disparate melting temperatures and hence annealing temperatures. Overall, the primers selected for the assays represent a good compromise between T_m and G+C ratio.

The uniform PCR assay parameters established for *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* could not be achieved for *A. salmonicida*. Specificity could be obtained by reducing the concentration of $MgCl_2$ to 1.375 mM and limiting the number of amplification cycles to 30 (Roux 1995). This strategy raised the stringency of primer annealing but to obtain any amplification with these conditions, the temperature was reduced to 60°C, a temperature close to the T_m of the primers. Using this optimisation strategy, the primer set As1/As2 only amplified DNA from *A. salmonicida*, *A. bestiarum* or those strains of *A. hydrophila* that had sequence homology with *A. salmonicida*.

The need to use stringent PCR conditions for the As1/As2 primers may not have been necessary if the length of primer As1 was increased. This primer is only 17 nucleotides long, a short oligonucleotide, particularly for a locus with a low amount of sequence variation. A four-fold increase in specificity can be achieved by the addition of only one nucleotide and it is reasonable to assume that the functionality of the primer pair may have been improved by increasing the length of primer As1. Whether such a modification would have improved specificity is not known, but it should be noted that the primer set developed by Høie *et al.* (1996) were 3 and 4 nucleotides longer than As1 and As2 respectively and did not appear to improve specificity. No cross-reaction was reported when tested with a limited range of aeromonads even with less stringent conditions using assay conditions of 3 mM $MgCl_2$ and 40 amplification cycles. Specificity could be achieved with primers As1/As2 using the PCR conditions used for the other fish pathogens but only with a nested PCR format when a 785 bp fragment of the 16S rRNA fragment was first amplified. This technique, although improving the detection limit, requires an extra amplification step, it is not warranted for rapid identification of cultures since the single step stringent PCR assay is specific for *A. salmonicida*.

PCR lends itself to rapid identification. The overall time to run a 35 cycle amplification is close to 2½ hours and allowing for sample preparation and the time to detect amplicon, if present, by gel electrophoresis, an identification can be achieved in a little over four hours. The amplification time was optimised to maximise amplicon yield, but in one trial with *A. salmonicida* (data not presented), it was possible to reduce the amplification time to 1½ hours by reducing the PCR extension time from 2 minutes to 30 seconds, without any apparent loss of sensitivity. This reduction in amplification time stems from the small length of DNA that needs to be amplified. The largest fragment is only 285 bp and since *Taq* polymerase can assemble at the rate of 30 bp sec^{-1}, (Powell 1995) a short extension time is all that is required. A fast protocol has considerable merit and further optimization of a 30s extension cycle or even the use of a two step cycle of denaturation and annealing/extension (Cha & Thilly 1995) should be undertaken.

Functionality of the PCR assays was assessed in terms of specificity, both intra- and inter-species as well as inter-genus, together with determining the detection limit of template DNA. The primer sets for *A. salmonicida* *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* appeared to be highly specific for

Species	Primer set	Source of isolates
A. salmonicida	As1/As2	Australia (Tas, Vic, NSW), UK[1]
E. seriolicida	Es9/Es6	Australia (Tas, Vic), South Africa, Japan
Fx. maritimus	rFlex1/rFlex3	Australia (Tas), Japan, France, UK
Y. ruckeri	Yr2/Yr1	Australia (Tas, Vic), USA[1]

Table 4.6 Origin of isolates testing positive by PCR
[1]Type strain, DNA only used for testing in Australia

their respective species and predictive as measured by their ability to detect different strains and serotypes as well as enzootic and exotic isolates (Table 4.6). All isolates, previously identified by phenotypic methods, produced an amplicon of expected size with their respective primer sets. The uniformity of the inter-species PCR reactions confirms the predictive nature of the probe design and indicates the high probability of detecting by PCR all prospective strains of the four species.

Based on sequence data, it was predicted that inter-genus specificity of the primer sets would be high for *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri*. The suitability of the probes for identification was further confirmed through extensive testing with phenotypically and genotypically related species and genera to demonstrate that amplification by PCR would not occur. Species for testing were chosen based on either close sequence homology to the target species, or similar phenotype or likely presence in fish tissues. An important group of bacteria that did not react were those which were identified as having a partially shared sequence homology with the chosen primer sets; none of these bacteria reacted by PCR. It was anticipated that the primers maybe used in a nested format in some circumstances to improve the sensitivity of the test. Using species with partial sequence homology, only *Y. ruckeri* and *Fx. maritimus* were amplified in this sensitive format. This was not the case with *E. seriolicida* primers which in nested format produced a weak band with the anaerobe *Clostridium putrificum* which has a 5 base pair difference to primer

Es6 and no sequence homology to the other primer, Es9. Amplification only occurred in the nested format and only at a high template concentration of 100 ng DNA $20\mu l^{-1}$. The basis of this observation is not clear but in practical terms, it was not considered a defect that limited the use of the *E. seriolicida* primers. The primers may give rise to non-specific priming in the presence of highly amplified DNA of *Cl. putrificum* but the circumstances that would give rise to such a situation are unlikely to occur since *Cl. putrificum* is an obligate anaerobe associated with soil (Cato *et al.* 1986), is not known to be a pathogen of fish and is unlikely to be present in an amount sufficient to be amplified to a detectable level. In the nested PCR, the annealing temperature of the first round of amplification is only 37°C and the lack of specificity may occur from mis-priming, the result of low stringency during primer-template hybridization. No amplification occurred with *Cl. putrificum* in the un-nested PCR format where primer annealing takes place at 65°C and is evidence of the specificity of the primers under appropriate conditions. The nested PCR cross reaction with *Cl. putrificum* should be considered erroneous but requires further investigation to confirm this opinion.

The intra-species specificity of the *A. salmonicida* primer set As1/As2 appears to be high. All isolates representing two distinct biovars of *A. salmonicida* from goldfish and greenback flounder produced a 260 bp amplicon as well as the type strain *A. salmonicida* ssp. *salmonicida*. Although not tested with *A. salmonicida* ssp. *masoucida* or *A. salmonicida* ssp. *achromogenes* it is likely that these two species will be amplified by As1/As2 since the two sub-species have complete 16S rRNA sequence homology with the greenback flounder biovar (section 3.1.1) DPIF 93/1061-1. The likelihood of the primers amplifying the two sub-species is supported by evidence from Høie *et al.* (1996) whose primer pair have overlapping sequences with As1/As2, amplified these two subs-species of *A. salmonicida*. By stringent un-nested PCR and by nested PCR, the As1/As2 primers showed a high level of inter-species and inter-genus specificity. Apart from *A. bestiarum* and some strains of *A. hydrophila* which were amplified by the primer set as discussed (section 4.1.2), species with close sequence homology such as *A. hydrophila* or

A. sobria which has an identical sequence homology to primer As1, were not amplified.

Using purified DNA, in un-nested as well as nested formats, it was possible to achieve detection limits of as little as 10 fg of DNA, approximately equivalent to 2 bacterial cells. This detection limit was achieved in a nested format for *A. salmonicida* and was the best detection level obtained in this study. For the other bacteria the smallest quantities of DNA which were detected at any time by un-nested PCR ranged from 1 ng DNA (300,000 genome equivalents) for *E. seriolicida* to 5 pg DNA (1,250 GE) for *Y. ruckeri*. These values represent the best detection levels obtained in this study, but a reliable detection limit of 1 ng DNA could be consistently achieved and was taken as the conservative lower limit of detection. By nested PCR, smaller quantities of DNA could be detected from as little as 10 fg DNA for *A. salmonicida* to 10 pg DNA for *Y. ruckeri*. Detection limits by PCR for these pathogens are hard to compare with published data because of differences in PCR conditions and because repeatable detection limit values are not stated. Data for several fish

Species	Detection limit[†]	GE[§]	Target	Reference
A. salmonicida	5 fg	1	*vapA*	Gustafson *et al.* 1992
A. salmonicida	100 fg	250	Asal-3	Miyata *et al.* 1996
A. salmonicida	1,000 fg	2,500	PAAS	O'Brien *et al.* 1994
E. seriolicida	16 fg	4	16S rRNA	Zlotkin *et al.* 1998
V. trachuri	1,000 fg	2,500	*Pst*-I	Iwamoto *et al.* 1996
V. penaeicida	1,000 fg	2,500	16S rRNA	Genmoto *et al.* 1996
R. salmoninarum	50 fg	12	57-kDa MSA	McIntosh *et al.* 1996
R. salmoninarum	900 fg	225	pMAM29	León *et al.* 1994b

Table 4.7 PCR detection limits with purified DNA for some bacterial fish pathogens
[†]expressed as purified DNA
[§]genome equivalents based on 4 fg DNA ~ 1 cell

pathogens (Table 4.7), expressed as femtograms of DNA per PCR reaction volume, show that the detection levels for other PCR systems are considerably lower than those found consistently with the PCR formats developed in this study. It should be noted however that in some circumstances, detection levels by PCR were in a range similar to that reported by other workers indicating the potential ability of the system to amplify in some circumstances when template DNA is less than 1ng. It is not clear why there is such a marked difference in

detection limits for the PCR systems developed here and those reported for similar assays. Consideration was given to annealing temperatures, number of cycles, $MgCl_2$ concentration and primer concentration to optimise the systems. A priority was allocated to ensuring specificity of the PCR reactions and it is possible that this has been at the expense of reduced sensitivity. Inhibition of amplification can be caused by many factors (Wilson 1997) which can be classed as either endogenous or exogenous in origin. It is likely that the low detection levels determined for the four fish pathogens may be endogenous in origin and relate to factors such as secondary structure of the template, the type of polymerase or purity of the template DNA . Improved PCR yields have been achieved by the addition of various reagents to reaction mixtures such as co-solvents (dimethyl sulphoxide) (Persing 1993b) or additives such as PEG 6000 (Roux 1995). Relief of amplification inhibition has been achieved through the addition of bovine serum albumin (BSA) or T4 gene 32 protein to PCR reaction mixes (Kreader 1996). In one trial with purified DNA from *Fx. maritimus* and BSA, a detection limit of 1 pg of DNA (250 GE) was achieved and represented a 10 fold increase in detection level. The evident improvement in the performance of the PCR assay where BSA was included suggests that further optimisation is possible with the four pathogens and that enhancement of detection levels can be achieved without necessarily having to sacrifice specificity.

Objective 2: Develop methodologies using molecular probes for the rapid identification of bacterial fish pathogens recovered by conventional culture techniques

Summary

- A rapid identification technique for *A. salmonicida*, *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* was developed using an un-nested PCR format.
- The PCR format enables identification to be achieved within 4 hours using as little as one bacterial colony.
- The primer sets for *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* were found highly specific as predicted by 16S rRNA design.
- To achieve specificity for *A. salmonicida*, a stringent un-nested PCR format or nested PCR was required; in either format, cross reactions occurred with *A. bestiarum* and some, but not all, strains of *A. hydrophila*.
- All enzootic and exotic strains and isolates of the target bacteria reacted in the PCR formats with their respective primers.
- It was possible to consistently amplify 1 *n*g of purified DNA, which approximates to 300,000 genome equivalents
- In un-nested formats 5pg of DNA (1,250 GE) could be achieved on occasion and in nested formats 10fg of DNA (2 GE) was detected.
- Improvements in levels of reliable detection are foreshadowed using PCR enhancers such as bovine serum albumin or co-solvents such as dimethyl sulphoxide

4.3 Develop procedures for testing fish by molecular probes for the presence of bacterial fish pathogens

4.3.1 Direct detection

Development of tools that can detect bacterial pathogens in fish tissues, preferably at low levels, is an important goal in achieving a capacity to effectively monitor fish for signs of infection before overt signs of disease emerge. The ability to amplify low copy numbers of target DNA sequences through the polymerase chain reaction makes it an attractive technology particularly in situations where the target pathogen may only be present in tissue in small numbers.

The usefulness and validity of direct detection PCR in fish disease diagnosis has been reviewed by Crane & Bernoth (1996) who concluded that while the tool has potential more research is required before it is used routinely. Perceived constraints to using direct detection PCR in fish disease diagnosis or monitoring are founded on concerns regarding the apparent lack of sensitivity, insufficient testing of primers to establish their specificity and no basis for interpreting the significance of positive findings established by direct detection PCR (Hiney & Smith 1995; Crane & Bernoth 1996; Bernoth 1997; Hiney 1997).

The utility of direct PCR for bacterial fish pathogen detection has been investigated principally for the slow growing fastidious pathogen *Renibacterium salmoninarum* and the almost equally fastidious pathogen *A. salmonicida*. The novelty of the technology has confined the application of direct detection PCR to developing and validating methodologies (McIntosh *et al.* 1996) or undertaking limited prospective surveys of fish populations (O'Brien *et al.* 1994).

4.3.2 Performance of direct detection PCR

Using deliberately seeded tissues, target bacterial DNA was readily extracted from kidney, brain, skin and gut and successfully amplified by PCR. Several extraction processes were considered but the best yields and most convenient method was the QIAamp® spin column system for tissues. The detection levels for the four bacterial pathogens in seeded fish tissues reflect in part the levels obtained with purified DNA. In the un-nested assays used for *E. seriolicida*,

Fx. maritimus and *Y. ruckeri*, detection levels were $1x10^7$ (kidney), $1x10^5$ (skin) and $1x10^4$ (kidney) cfu g^{-1} respectively. Comparative data is not available for these bacterial species but in un-nested PCR assays for *A. salmonicida* a detection level of $1x10^4$ cfu g^{-1} of kidney (Høie *et al.* 1997) was achieved using 16S rRNA primers and for *R. salmoninarum*, $5x10^3$ cfu ml^{-1} kidney homogenate could be detected with primers for the p57 major surface antigen (McIntosh *et al.* 1996). On the basis of these observations it would appear that the assays for *Fx. maritimus* and *Y. ruckeri* are similar to the levels of bacteria detected for seeded samples containing *A. salmonicida* and *R. salmoninarum*.

By nested PCR *A. salmonicida* and *Y. ruckeri* were detected at a level of $1x10^1$ cfu g^{-1} of either kidney, skin or gut. This level of detection, while highly satisfactory, must be considered invalid or at least questionable in so far that the detection level achieved is lower than that obtained with purified DNA. The most probable explanation for this remarkable level of detection is the presence of naturally occurring *Y. ruckeri* and *A. hydrophila* in the fish tissues. By un-nested PCR, all unseeded tissues were negative, as were the PCR negative controls. In the nested PCR however, unseeded tissues were positive for both *Y. ruckeri* and *A. salmonicida* but not for *E. seriolicida* or *Fx. maritimus*; there was no evidence of sample contamination as confirmed by the PCR negative controls. The presence of a single weak band at an appropriate band size in un-seeded tissue strongly suggests the presence of pre-existing *Y. ruckeri* and *A. salmonicida* or *A. hydrophila*, in the samples. The assumption of pre-existing cells of *Y. ruckeri* in fish tissues is based on the knowledge that yersiniosis is enzootic in Tasmania (Carson 1990) and all hatchery populations have a level of asymptomatic carriage of *Y. ruckeri* which can be as high as 18% (J. Carson & T. Wilson, unpublished findings). The development of a 260 bp band in the *A. salmonicida* PCR is most likely the result of cross reaction with *A. hydrophila*, a commonly occurring component of the flora of fish in freshwater or *A. bestiarum* found in association with salmon (Kokka *et al.* 1991). It has been established that the 16S rRNA primers As1/As2 cross react with strains of *A. hydrophila* as well as *A. bestiarum*. The PCR reaction must be considered a false-positive result, particularly since there has been no clinical evidence of

furunculosis in Tasmania (or Australia) nor has *A. salmonicida* been isolated from farmed salmonids monitored as part of a comprehensive health surveillance programme (Percival *et al.* 1995). Høie *et al.* (1997) in a study of feral Atlantic salmon in rivers, found that 5/16 fish gave positive reactions with their 16S rRNA *A. salmonicida* PCR. They considered these false positive reactions because fish from that river system had no previous history of furunculosis. It should be noted that the Norwegian *A. salmonicida* primers cross react with *A. hydrophila* (Høie *et al.* 1997) and that even using an *A. salmonicida* specific internal probe it was not possible to distinguish *A. salmonicida* from *A. hydrophila* (S. Høie pers. comm.). It is likely therefore that the positive PCR reactions observed in these furunculosis free fish was due to cross reactions with *A. hydrophila* or *A. bestiarum*.

Direct detection of the target pathogens in fish tissues is achievable by PCR for all four of the pathogens. The value of such a procedure in disease diagnosis and monitoring is circumscribed by two important factors: specificity and detection level. In regard to specificity, the primers appear to be highly specific for the target pathogens except for *A. salmonicida* where there is a risk of false positives due to cross reaction with *A. hydrophila*. The second factor, relates to the efficiency of detection, that is, the number of bacteria which can be detected by the test. While the detection levels are similar to those achieved by other PCR tests, detection levels of even $1x10^4$ cfu g^{-1} are of doubtful value when monitoring fish for asymptomatic carriage of a pathogen. The detection levels achievable by PCR are readily obtained by direct culture and so it is difficult to justify the use of PCR solely on the grounds of sensitivity. PCR does have an advantage over culture however in regard to specificity and speed and in this respect its use can be justified, particularly for slow growing bacteria such as *R. salmoninarum*, *Fx. maritimus* and the greenback flounder biovar of *A. salmonicida*. As currently formatted, the direct detection PCR, could be used for screening overtly infected fish during a disease outbreak where the likelihood is high that large numbers of the pathogen will be distributed through host tissues and so be readily detected in samples by PCR.

4.3.3 Limitations of direct detection PCR

A characteristic of PCR is its ability to hugely amplify DNA from as little as a single copy of the target sequence. It would be erroneous however to equate this power of amplification with an ability to detect very small numbers of bacteria in covertly infected fish. PCR sensitivity *sensu stricto*, relates specifically to events and conditions within the PCR reaction tube but for direct detection PCR, it is a measure of PCR sensitivity *sensu stricto*, and sampling factors, especially sample size and density of the target pathogen in fish tissue.

The sensitivity of direct detection PCR is constrained by four inter-related but independent factors (Figure 4.1); these are:

- Endogenous amplification conditions
- Exogenous PCR inhibitors
- Sampling factors
- Density of target pathogen in host tissue

The sensitivity of the PCR reaction *sensu stricto*, is largely controlled by endogenous amplification conditions such as $MgCl_2$ concentrations, primer design and annealing conditions, factors which all influence stringency and hence amplicon yield.

Efficiency of amplification is also greatly influenced by external factors, which can inhibit or limit the PCR process by as much as 1000x (Wilson 1997). A wide

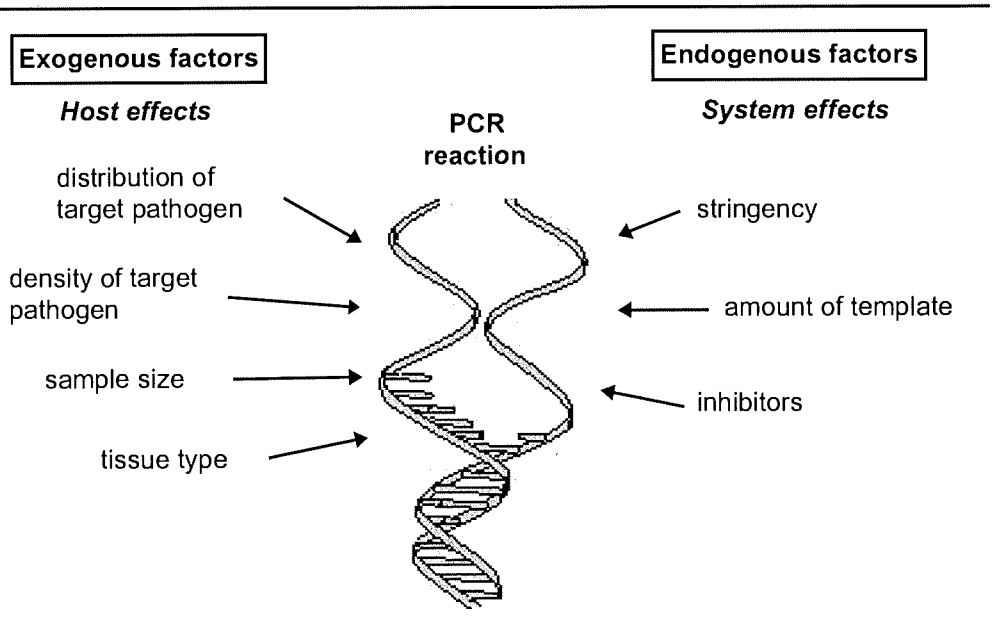


Figure 4.1 Factors affecting overall sensitivity of direct detection PCR

range of inhibitors have been described and include factors such as haemoglobin, polysaccharides, humic acids as well as high concentrations of eukaryotic DNA itself (Høie *et al.* 1996, 1997). Inhibition of direct detection PCR by fish tissues, mainly kidney, has been reported by several authors and is considered a major limitation to achieving useful detection levels for the fish pathogens *A. salmonicida* (Høie *et al.* 1997), *R. salmoninarum* (Magnússon *et al.* 1994; McIntosh *et al.* 1996) and *Y. ruckeri* (Argenton *et al.* 1996). Relief from some types of PCR inhibitors has been achieved through the use of BSA (Forbes & Hicks 1996), Chelex 100 resin (Léon *et al.* 1994b) or enrichment culture (Gustafson *et al.* 1992; Høie *et al.* 1997) and has contributed to achieving improved detection levels.

Much importance has been given to the performance of PCR reactions using purified DNA extracted from bacterial cultures or from deliberately seeded fish tissue. This approach in effect considers only endogenous or 'system' factors and fails to recognise that exogenous factors, particularly those relating to the spatial distribution of the target pathogen in the host tissue are as important, if not more so, in their effect on the sensitivity of direct detection PCR.

It was apparent that the system for extracting DNA from fish tissue developed in this study placed a significant limit on the size of sample that can realistically be processed. The largest amount of tissue that could be handled by the QIAamp® system was 50 mg, yielding 100 µl of filtrate containing both host and bacterial DNA. Inherently, PCR reactions are performed at a micro level and in the 20 µl reaction volume used in this study, only 1 µl of sample is required as the source of putative template DNA. The theoretical detection level for this system assuming that (i) there are no PCR inhibitors, (ii) that the PCR reaction can amplify a single DNA template and (iii) that there is a normal distribution of template molecules in the volume of filtrate containing extracted DNA, is calculated to be $2x10^3$ cells g^{-1} of fish tissue. This detection level represents the smallest number of bacteria that can theoretically be detected in fish tissue under optimum conditions using the system as described. This empirical deduction assumes that the target DNA molecules have a normal distribution in the filtrate containing extracted DNA, but since the number of

target DNA molecules in the filtrate will be very small, they will have a Poisson distribution (Greenfield & White 1993). Using the equation of Saiki *et al.* (1988):

$$1\text{-}e^{-n}$$

where *e* is the exponential function and n = the arithmetic mean number of target molecules of DNA μl^{-1} of filtrate, the fraction of filtrate samples containing at least one copy of target DNA can be calculated for various densities of bacteria in fish tissue (Table 4.8). As bacterial cell number in fish tissue

§Bacteria g^{-1}	†Genomes 100 μl^{-1} of filtrate	‡No. of filtrate samples required for PCR testing
$1x10^4$	500	1
$1x10^3$	50	3
$1x10^2$	5	21
$2x10^1$	1	100

Table 4.8 The likelihood of PCR detection at different concentrations of target bacteria in fish tissue

§Bacterial cell number g^{-1} fish tissue
†No. of genomes (target DNA) 100 μl^{-1} of filtrate obtained from 50 mg of tissue
‡No. of 1 µl samples to be tested to detect at least one genome equivalent of target DNA.

decreases, the likelihood of detecting the target DNA in the filtrate declines exponentially. To ensure that at least one genome equivalent of DNA is encountered, multiple samples of the filtrate must be tested by PCR. The absolute limit for the system, again assuming optimal PCR conditions, is a cell density of $2x10^1$ g^{-1} which would require all 100µl of the filtrate to be tested to ensure that the one target molecule of DNA present in 100 µl of filtrate will be detected by PCR.

On the basis of this type of analysis, it is evident that the sensitivity of direct detection PCR is dependent on, not only the density of the target pathogen in host tissue, but also on the sample size and the number of samples to be tested by PCR. It is evident that reliable detection of small numbers of bacteria in fish tissue is unlikely to be achieved when the density of bacteria in fish tissue is less than $1x10^4$ cells g^{-1}. In this study, the detection limit for *Y. ruckeri* in kidney ($1x10^4$ cells g^{-1}) and *Fx. maritimus* from skin ($1x10^5$ cells g^{-1}) is close to

the computed minimum detection level and significant improvements in sensitivity are unlikely to be achieved with the existing protocol. Although direct comparisons can not be made because of different sample and treatment protocols, these levels of detection are similar to those achieved for *A. salmonicida* (Høie *et al.* 1997) and *R. salmoninarum* (McIntosh *et al.* 1996) and is further evidence of the difficulty of reliably detecting small numbers of bacteria in fish tissue by direct detection PCR.

The samples that can be used for DNA extraction are small and is one factor in limiting the sensitivity of direct detection PCR. Paradoxically, increasing the size of sample does not lead to an increase in yield of target DNA. The filtrate volume will be larger than for a small sample, but the concentration of target DNA in the filtrate volume for the large sample will be the same as that obtained from the small sample. Improved sensitivity will only occur if a concentration step is introduced to the extraction process. The most successful procedure has relied on enrichment culture to amplify the target bacterium prior to DNA extraction. This method offers several advantages the most important being that large samples, several grams in size, can be used and the culture process also dilutes any inhibitory factors associated with the sample (Wilson 1997). Enrichment culture prior to PCR has been used successfully to detect several species of bacteria including *Erwinia carotovora* in potatoes (Hyman *et al.* 1997), *Yersinia enterocolitica* in water (Sandery *et al.* 1996), *Salmonella* spp. in oysters (Bej *et al.* 1994) and *E. coli* in ground beef (Witham *et al.* 1996). By including a preliminary amplification step this form of PCR can detect as few as 1 cfu of *Vibrio cholerae* 10 g^{-1} of lettuce leaves (Koch *et al.* 1993). Application of enrichment culture to improve the detection level of bacteria in tissue by PCR has not been reported although the concept has been canvassed for *A. salmonicida*. Kidney samples from carrier fish were enriched in tryptone soy broth for 24 hr and *A. salmonicida* detected by PCR using the *vap*A primer set (Gustafson *et al.* 1992). In this small trial, by direct detection PCR *A. salmonicida* could be detected in 20/25 fish but with a preliminary enrichment step *A. salmonicida* was detected in all 25 fish. The evidence from this small trial suggests that improvements in detection level are achievable for fish

pathogens if a preliminary enrichment step is used; this strategy may be of significant value in the detection of small numbers of bacterial pathogen which occur in carrier fish.

4.3.4 Application of direct detection PCR

The inability to reliably and conveniently detect less than $1x10^4$ cells g^{-1} of kidney tissue, is as discussed, partly an inherent problem of the technique associated with sample size and partly the effect of inhibitory PCR factors that co-resolve with DNA extracted from fish tissues. Improved detection level may be achieved by further optimisation of the PCR reaction *sensu stricto*, but such a strategy is unlikely to result in significant lowering of the existing detection levels.

Application of direct detection PCR in disease investigation must be considered in regard to test sensitivity to determine an appropriate use of the technique. Since the detection level of the assay is quite high, direct detection PCR could be used where there are overt signs of disease and a likelihood that the pathogen is present in reasonably high numbers. An advantage of PCR as a diagnostic technique compared to capture ELISA or indirect fluorescent antibody tests is the specificity of the assay if the primers have been properly designed and evaluated. In comparison to culture, a PCR finding can be obtained in only a few hours, while for culture it may take up to 3 days for colonies to appear for some strains of *A. salmonicida* and *Fx. maritimus*.

Use of direct detection PCR, as currently configured, can not be considered appropriate for the detection of covertly infected fish because the detection level of the procedure is too high. The inability of the assay to detect fewer than $1x10^4$ cells g^{-1} is largely the result of the small sample size that can be accommodated by PCR. The number of bacteria likely to be present in covertly infected fish has been established by conventional culture techniques for *A. salmonicida*. In clinically normal, but covertly infected Atlantic salmon and rainbow trout, cell numbers of *A. salmonicida* can be as few as $1x10^3$ cfu g^{-1} of skin mucus (Cipriano *et al.* 1992; 1996). In covertly infected pink salmon (*Oncorhynchus gorbuscha*) cell density can be as little as low as $1x10^2$ cfu g^{-1} while in chum salmon (*Oncorhynchus keta*), cell density can be as little as

$1x10^1$ cfu g^{-1} kidney (Nomura *et al.* 1993). The detection limit by culture for *A. salmonicida* in skin mucus is close to $1x10^3$ cfu g^{-1} (Cipriano *et al.* 1992) and is more sensitive than the detection limit of $1x10^4$ cfu g^{-1} for capture ELISA (Hiney *et al.* 1994) or PCR (Gustafson *et al.* 1992; Høie *et al.* 1997). Clearly the number of target bacteria which might be carried by covertly infected fish can vary but it is apparent that the number of bacteria is likely to be less than the detection level for PCR. As currently formatted, direct detection PCR would not be a reliable tool for identifying fish asymptomatically carrying *A. salmonicida*.

Summary

- A protocol was developed to detect *A. salmonicida*, *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* in fish tissues.
- The pathogens could be detected in kidney, skin, gut or brain of fish tissue.
- The reliable theoretical detection limit for this tissue PCR assay was calculated to be $1x10^4$ cfu g^{-1} of tissue.
- In un-nested PCR, the detection limit for *E. seriolicida* in brain tissue was $1x10^5$ cfu g^{-1}, for *Fx. maritimus* it was $1x10^5$ cfu g^{-1} of skin and for *Y. ruckeri* the detection limit was $1x10^4$ cfu g^{-1} of kidney.
- In nested PCR detection levels of $1x10^1$ cfu g^{-1} of kidney were achieved for *A. salmonicida*. The apparent sensitivity of the *A. salmonicida* PCR is probably due to cross reaction with pre-existing cells of *A. hydrophila* in the tested fish tissues.
- The size of tissue sample that can be accommodated by PCR was identified as a significant limiting factor in detecting small numbers of bacteria in fish tissue.
- Direct detection PCR can be used for the rapid identification of specific pathogens in overtly infected fish but as currently formatted is not sufficiently sensitive to identify carrier fish.

4.4 Develop a secondary confirmation system for PCR assays using internal probes and hybridization

Specificity of the PCR primers is predicated on the conserved nature of the 16S rRNA gene and is the basis for a high level of confidence in achieving a correct identification of a target bacterium. The predicted specificity was verified firstly by sequence searches of ribosomal DNA, non-ribosomal bacterial DNA, bacteriophage DNA and structural RNA databases and secondly by testing the primers with bacteria identified as having some sequence similarity or likely to be encountered as normal flora associated with fish. Apart from *A. salmonicida*, it was established that the primer sets for *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* were all highly specific and no cross reactions were detected by un-nested or nested PCR.

Reliability of the primers was established by testing with known strains or isolates of the target bacteria and confirming that amplicons of expected size were produced. Visualization of the amplicon in ethidium bromide stained agarose gels does not however provide any data regarding the nature of the sequence of the amplified DNA. Where PCR is used to identify a bacterium in culture, then size of the PCR amplicon, together with any phenotypic data, may be sufficient to confirm identity. When the PCR is used for direct detection however and the primers have to discriminate between DNA sequences of the target bacterium amongst a heterologous population of DNA molecules derived from the host and other bacteria, it may not be sufficient to rely solely on size of the amplicon to identify the target bacterium. It is possible that mis-priming or priming at homologous sites on non-target bacteria may occur, resulting in an amplicon of approximately the same size as the expected amplicon, but having a different DNA sequence.

Confirmation that an amplicon has an expected sequence can be achieved in several ways. The simplest method is to use a restriction endonuclease that cuts the amplicon at a recognition site characteristic of the target bacterium. This technique has been used to confirm amplicon sequence integrity generated by the G6840/G6841 primers for the *R. salmoninarum* p57 kDa major soluble antigen (McIntosh *et al.* 1996). Alternatively, amplicon can be

sequenced to establish its integrity, a strategy used to confirm the identity of amplicon produced by primers for the p57 kDa major soluble antigen of *R. salmoninarum* (Brown *et al.* 1994). Restriction digests and amplicon sequencing were not selected as confirmation strategies in this project because both systems had some inherent limitations. Restriction digests were not used because characteristic restriction sites could not be readily identified in the amplicons for any of the four fish pathogens. Sequencing of amplicon, although the definitive method, was rejected as a routine method of confirmation because of the relative complexity of the procedure and the need for dedicated sequencing equipment. Hybridisation however, the method chosen for confirming the identity of PCR amplified DNA is an accessible technology. The method is flexible in format and can be used for Southern blot analysis of agarose gels (León *et al.* 1994b; Høie *et al.* 1997) or in a dot-blot format using PCR product (O'Brien *et al.* 1994).

Internal probes for hybridization were developed for *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* since the PCR primers for these three pathogens appeared to be suitable already for diagnostic use because of their high level of specificity established empirically and by extensive testing. It was decided not to develop a probe for *A. salmonicida* because there is known total sequence homology between *A. salmonicida* and *A. bestiarum* as well as some strains of *A. hydrophila* from primer site A at the begining of the 16S rRNA gene through to primer site As2. The absence of any differential sequences between these aeromonads across several variable regions of the 16S rRNA gene means that it is not possible to construct a probe that is unique to *A. salmonicida*. Høie *et al.* (1997) identified a sequence for this region of the 16S rRNA gene but found that it hybridised to *A. hydrophila* as well as *A. salmonicida* and could not be used to differentiate between these two species (S. Høie pers. comm.).

The probe sequences identified for the three bacterial fish pathogens were found to be specific for their respective target bacteria. Specificity was established firstly by comparing the sequences for homology with the ribosomal database held by ANGIS and secondly by ensuring that the probes hybridized only with amplicons from the homologous species and not with amplicons from

the other target bacteria. Testing of the probes was limited and their overall reliability awaits further assessment. Main areas of investigation that should be undertaken include further optimisation of the dot blot protocol to increase the strength of the hybridization signal and verification of the specificity of the hybridization by testing the probes with amplicons produced under less stringent PCR conditions. Based on the data obtained so far, the probes appear to be specific and will have an essential role in verifying the sequence of appropriately sized amplicons.

Different tagging systems for probes have been used, including γ-P^{32} radioactive marker or biotin in conjunction with streptavidin-alkaline phosphatase conjugate. A characteristic of these markers is to produce an amplified signal when the probe has hybridized with its target DNA and in this way it is possible to detect smaller amounts of DNA than by staining agarose gels with ethidium bromide. Using slot-blot hybridization and the internal probe AS15 for the PAAS amplicon for *A. salmonicida*, detection levels could be increased by 10 to 100 times (O'Brien *et al.* 1994; Hiney *et al.* 1992) making it possible to detect by PCR as little as 10 fg of DNA, equivalent to approximately 2 bacteria.

Significant improvements in detection level were not achieved with the *Fxmprb* probe for *Fx. maritimus* or the *Yrprb* probe for *Y. ruckeri* although testing in this respect was limited. In one assay for *E. seriolicida* however, a marked increase in detection level was obtained when the PCR amplification was sub-optimal. In ethidium bromide stained agarose gels, a 143 bp band was only visible when the template concentration of target DNA was 100 ng but not at a DNA template concentration of 10 or 1 ng. Following dot-blot hybridzation, the *Esprb* probe was able to detect PCR amplified DNA not only at a template concentration of 100 ng but also at the lower concentrations of 10 and 1ng which by ethidium bromide staining were not visible. The increase in detection level from 100 ng to 1 ng represents a 100 fold increase in sensitivity and is an indication that dot-blot hybridization, once fully optimised, may further improve PCR detection limits from their existing levels.

Further work is required to refine the technique, particularly to achieve reliable hybridization and to obtain improved detection levels. Based on the data obtained so far, the probes appear to be specific for their target bacteria and the dot-blot procedure appears to be a convenient format for verifying large numbers of PCR reactions. Development of these specific internal probes provides an opportunity to adopt faster more reliable methods to detect DNA amplified by PCR. A promising detection system, receiving increasing attention is hybridization-ELISA (Kawai *et al.* 1993; Kessler *et al.* 1997). With this procedure, amplicon if produced, can be detected and verified in the one assay and all in a convenient microtitre tray system. Establishment of this assay system, which relies on the use of internal probes, will greatly assist the adoption of PCR as a tool for monitoring or screening populations of fish for the presence of specific pathogens.

Summary

- Internal DNA probes were developed for *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri.*
- The probes were developed as a means of verifying the identity of PCR products.
- The probes can be used by dot-blot hybridization, a format convenient for testing large numbers of PCR samples.
- In limited assessments, the probes are specific for their target bacteria.
- Further work is required to optimise the dot-blot system, particularly in regard to reproducibility.
- In limited assessments, it was shown that dot-blot hybridization could increase DNA detection levels by a 100 times.

4.5 Formulate the developed test procedures for transfer to, and adoption by, veterinary diagnostic and research laboratories

4.5.1 Participants

Transfer of the technology was considered an important aspect of the project and was deliberately built into the programme plan. Consideration was given to the method of technology transfer and it was determined that the most effective means would be by a laboratory workshop. This technique was chosen because it was considered the most appropriate method in dealing with these issues:

- relative complexity of the technique for microbiologists without experience in PCR assays
- opportunity to provide advice and commentary on the technique to microbiologists with PCR experience
- provide an opportunity for microbiologists to use the techniques and trouble shoot any problems that may arise
- ensure that the protocols were provided in a format that could be readily adopted and used

The primary target for the technology was diagnostic veterinary laboratories. As key providers of diagnostic services, this group is important because they undertake disease diagnosis and identification of fish pathogens for aquaculture and wild fisheries industries. Microbiologists engaged in research activities relating directly or indirectly to fish disease agents were identified as a secondary group which might benefit from the technology developed during this project.

Microbiologists were provided details of the course through direct marketing or selected marketing. This strategy proved successful since every state was represented at the workshop; there were no representatives from either the Northern Territory or the Australian Capital Territory. The principal state veterinary laboratories providing fish disease diagnostic services attended the workshop as well as staff from the Fish Disease Laboratory at the Australian Animal Health Laboratory who provide exotic disease diagnostic services for Australia. The high level of interest and good attendance by state and federal

laboratories has meant that the techniques have been transferred to the major fish disease laboratories in Australia.

In addition to the diagnostic laboratories, staff from CSIRO Fisheries, Hobart and postgraduates and staff from the Department of Aquaculture, University of Tasmania also attended the workshop.

4.5.2 Development of the workshop manual

The manual was developed in two stages. A first version was written as a means of transferring the PCR technology from the University of Tasmania to the Fish Health Unit of the Department of Primary Industry and Fisheries, Tasmania so that species specificity testing could be undertaken. Fish Health Unit staff and Fish Disease Laboratory, Australian Animal Health Laboratory staff were given training with the technique using this version of the manual. The final version of the manual (see Section 6) was developed incorporating improvements coming from this first training session together with new information that arose from subsequent research developments.

4.5.3 Workshop

The workshop consisted of lectures and laboratory practicals. The purpose of the lectures was to provide some context to the techniques and to provide background information for participants unfamiliar with PCR. All participants received a copy of the PCR manual which gave detailed information regarding protocols and reagents. All participants had the opportunity to undertake rapid identification of bacterial cultures by nested and un-nested PCR and also to perform direct detection PCR with fish tissues. Following the practical sessions, the capabilities and limitations of the tests was discussed with participants.

4.5.4 Assessment

There was a high approval rating for the workshop amongst participants; the overall approval rating was 90%. Those attending the workshop indicated that that the course content, instruction and organisation were highly satisfactory and that the aim of the workshop to transfer the technology had been achieved.

Acceptance of the technology was high, 80% of participants indicating that they would use the PCR techniques for fish disease diagnosis. Over half the participants (60%) planned to use the techniques either for disease diagnosis

or rapid identification of bacterial isolates, a view that reflects the high level of acceptance of PCR technology by the participants. Interestingly, participants did not feel that the technology presented any particular obstacles to it being adopted as a diagnostic technique and is further evidence that the selection of PCR as rapid technique for bacterial identification was justified.

Summary

- Transfer of the PCR technology to diagnostic laboratories was accomplished by conducting a laboratory workshop
- The major veterinary diagnostic laboratories responsible for fish disease diagnosis in each state, as well as the national Fish Disease Laboratory of the Australian Animal Health Laboratory attended the workshop
- Their was a high level of approval for the workshop and participants indicated that the technology transfer was highly satisfactory
- Most of the participants indicated that they would use the techniques for disease diagnosis in fish
- There was a high level of acceptance of PCR as a diagnostic technology

4.6 Conclusions

The aim of this project was to create a suite of gene probes that could be used for the diagnosis of bacterial diseases in fish. This primary objective has been achieved with the development and testing of a set of DNA probes that can be used in PCR assays for the detection of the bacterial fish pathogens *Aeromonas salmonicida, Enterococcus seriolicida, Flexibacter maritimus* and *Yersinia ruckeri.*

4.6.1 Specificity of the probes

A directed probe strategy using the 16S rRNA gene as the target sequence was used to develop the PCR primers. The probes have a high level of inherent specificity because of the conserved nature of the 16S rRNA gene. The specificity of the probes was evaluated by extensive testing firstly using bacterial species which have some known sequence homology and secondly with near related species to the target bacteria as well as species that form part of the normal flora of fish. Considerable attention has been given to the issue of specificity testing, more than has been reported in the development of other PCR probes for fish pathogens.

The primers for *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* when used in the PCR format developed in this study appear to be highly specific and no cross reactions were detected with other bacteria. Development of a primer pair for *A. salmonicida* was more difficult to achieve because of the high sequence homology among the aeromonads. The probe for *A. salmonicida* has some limitations in that it cross reacts with *A. bestiarum* and some, but not all strains of *A. hydrophila*. This deficiency restricts its application, but importantly there is a clear understanding of the limitations attached to the probe. Since there is insufficient sequence variation in the 16S rRNA gene to construct a probe specific for *A. salmonicida*, it is important that further work is undertaken to develop or identify probes of known performance that can be used to reliably identify *A. salmonicida*. The work reported here provides a foundation for this line of investigation and compliments the FRDC programme 95/060 "Development of methodology for the detection of covert

Aeromonas salmonicida infections in carrier salmonid populations" (Fish Disease Laboratory, AAHL, CSIRO).

4.6.2 PCR tests for the identification of bacterial fish pathogens

The PCR systems that have been developed in this study represent a significant advancement in Australia's capability to detect disease agents of major importance to farmed and wild fish. Previously, diagnostic technologies that were widely available to state veterinary and federal laboratories were limited to culture and immunofluorescent techniques. These methodologies are still important for disease diagnosis but it is becoming increasingly apparent that their effectiveness is limited to certain types of investigation. The PCR techniques that have been developed in this study must be seen as complimentary to established techniques and are a means of strengthening the armamentarium available for detecting and assessing the impact of infectious disease in fish.

The major strengths of PCR technology coupled with appropriate primer design, are: speed, sensitivity and specificity. The probes and PCR format developed in this study can be used for the rapid identification of bacteria isolated in culture and are sufficiently reliable that identifications of high probability can be obtained in a little over four hours. This represents a significant advantage over conventional identification methods that can take as long as two weeks to identify slow growing bacteria such as *Fx. maritimus*. The high level of specificity associated with the 16S rRNA probes means that as a presumptive means of identifying bacteria PCR compares well against fluorescent antibody or slide agglutination tests. Currently there are no fluorescent antibody tests available for *Fx. maritimus* or *E. seriolicida* and the PCR tests developed here represent the only evaluated rapid identification technique for these species. Slide agglutination tests are available for *Y. ruckeri* but there are several discrete serotypes for this species that require the use of several antibodies to ensure an adequate level of testing; the PCR test is independent of serotype and is a more convenient basis of rapidly identifying the species. Typically, *A. salmonicida* is of a higher order of complexity than the other three bacteria discussed and alternative rapid test technologies have

defined limits of reliability. Polyclonal antibodies for *A. salmonicida* suitable for fluorescent antibody tests can cross react with some strains of *A. hydrophila* while monoclonal antibodies, although offering a high level of specificity do not react with all strains of *A. salmonicida*. The PCR test with 16S rRNA primers since it is known to cross react with *A. bestiarum* as well as some strains of *A. hydrophila*. If the test is used to identity pure cultures, it can be used with greater assurance since additional phenotypic tests can be used to verify any positive findings.

4.6.3 PCR as a tool for disease monitoring and surveillance

A significant addition to disease detection capability in fish has been achieved through the development of gene probes for use in direct detection PCR. As currently formatted, the PCR techniques enable *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* to be rapidly detected in overtly diseased fish. This capability does not extend to *A. salmonicida* using the 16S rRNA primers because of the known cross reaction with *A. hydrophila* and *A. bestiarum*, species both found commonly associated with fish in freshwater. A limitation of direct detection PCR however, is the small sample that must be used. Currently, this restricts the detection limit to approximately $1x10^4$ cfu g^{-1} of fish tissue with the PCR format developed in this study. This detection level is adequate for testing overtly diseased fish and even some covertly infected fish, but as a sensitive surveillance tool it has limited application as currently formatted. Nevertheless, the PCR and its primers developed in this study form the basis of a reliable and versatile technology. With improvements to sample pre-treatments to concentrate or extract the target pathogens from fish tissue, direct detection PCR has the potential to be a powerful and useful tool for the monitoring of fish populations for early signs of disease.

4.6.4 Further work

The PCR techniques developed during this study are well advanced. A good understanding has been achieved regarding the specificity of the primer sets and their respective PCR protocols. Beyond perhaps extending the range of bacterial flora which could be tested to add further confidence in the specificity of the test, there are no immediate requirements for additional work in this area.

The protocol for the identification of bacterial colonies is well defined and there are no apparent restrictions to its use.

Some further work is probably required to define and perhaps improve the detection level of the PCR formats when using purified DNA. This optimisation will ensure that the PCR conditions will allow the maximum yield of amplicon from the smallest quantity of template. Initial experimentation with BSA has indicated that improvements can be achieved and this line of experimentation should be pursued.

Although a PCR format was developed for direct detection of bacteria in fish tissue, realistically the level of detection makes this technique of marginal value if it is to be used for detecting carrier fish. As indicated, the main limitation appears to be a problem associated with sample preparation and less to do with the PCR test, *sensu stricto*. A number of possibilities could be considered as a means of concentrating the target bacterium prior to PCR and include:

- enrichment culture
- immunomagnetic entrapment (Nilsson *et al.* 1996)
- reverse transcriptase amplification of rRNA (Klein & Juneja 1997)
- sequence capture PCR (Mangiapan *et al.* 1996)

All of these strategies have been used with PCR for bacteria other than fish pathogens and have been found to improve detection level significantly.

An important aspect of the technology is to ensure that it is adopted by diagnostic laboratories, a philosophy neatly summed up as "From the benches to the trenches" (Persing 1991). A limitation of PCR is the real difficulty of scaling up the technology so that several hundred PCR reactions can be set up and analysed simultaneously. The use of microtitre trays and multi-channel pipettes greatly increases productivity but also introduces problems of its own, particularly cross-contamination and the generation of false positive results. Although useful decontamination protocols based on uracil glycosylase have been developed (Hartley & Rashtchian 1995) the PCR systems will require additional optimisation to accommodate this procedure. Detection of amplicons in a few PCR reactions is most simply undertaken by agarose electrophoresis but where 200 or 300 samples need to be assayed this becomes a

cumbersome procedure. A practical solution to this problem is the use of hybridization-ELISA which offers all the convenience of microtitre technology combined with a verification system to confirm that the amplicon has the sequence signature of the target bacterium. The verification probes for *E. seriolicida*, *Fx. maritimus* and *Y. ruckeri* have been identified already but require integration into such a system.

Section 5

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Section 6

Appendix

6.1 Culture Media

6.1.1 YEPP medium (Atlas 1993)

Proteose peptone	10g
NaCl	5g
Yeast extract	3g
Distilled water	1000ml
pH	7.2

6.1.2 Sheep's blood agar

Blood agar base no.2 (Oxoid, CM 271)	1000ml
Defibrinated sheep's blood	70ml

6.1.3 Cooked meat medium (Oxoid, CM 81)

6.1.4 Marine Ordal's medium (L. Schmidtke & J. Carson, DPIF)

Bacto-peptone (Difco)	0.5g
Yeast extract	0.5g
Sodium acetate	0.2g
Beef extract (Oxoid, Lab Lemco)	0.2g
Sodium pyruvate	0.1g
Agar	11.0g
"Aged" seawater	900ml
Distilled water	100ml
pH	7.2

6.1.5 Freshwater Ordal's medium (Anacker & Ordal 1959)

Tryptone	0.5g
Yeast extract	0.5g
Sodium acetate	0.2g
Beef extract (Oxoid, Lab Lemco)	0.2g
Agar	11.0g
Distilled water	1000mL
pH	7.2

6.1.6 Chocolate blood agar

Blood agar base no. 2 with 10% v/v defibrinated sheep's blood, heated to 80°C for 10 min to coagulate the blood.

6.1.7 *Vibrio* nutrient agar (J. Carson, DPIFT)

Lab-lemco (Oxoid)	5.0 g
Yeast extract	3.0
Peptone, (Oxoid L37)	10.0
NaCl	10.0
Agar	15.0
Distilled water	1000.0 mL
pH	7.4

6.2 Workshop Manual

Detection of Fish Pathogens by Polymerase Chain Reaction (PCR)

The University of Tasmania and the
Department of Primary Industries & Fisheries, Tasmania

A Fisheries Research and Development Corporation funded program

This workshop is part of an FRDC project entitled: 'Development of molecular probes for use in bacterial disease diagnosis and health monitoring of farmed and wild fin fish in Australia'.

The project is a collaborative research program between the Department of Agricultural Science, University of Tasmania and the Fish Health Unit of the Department of Primary Industries & Fisheries, Tasmania. The project objectives were developed by Jeremy Carson and Peter Franzmann; the research program itself was undertaken at the University of Tasmania.

A number of people have contributed to the project over the past three years. These contributors, in approximate chronological order, were:

University of Tasmania

Peter Franzmann	1993-94
Janine Miller	1993-94
Sue Dobson	1994-96
Sharee McCammon	1994-95
Michelle Williams	1995-96
John Bowman	1996

Department of Primary Industry and Fisheries

Jeremy Carson	1993-96

The operational costs of the project were funded by the Fisheries Research and Development Corporation and indirectly by the University of Tasmania and the Department of Primary Industry and Fisheries, Tasmania. The support of Salmon Enterprises of Tasmania P/L is acknowledged.

Detection of Fish Pathogens by Polymerase Chain Reaction (PCR)

A Laboratory Workshop 17-19 June 1996
Conducted by:
The University of Tasmania and the
Department of Primary Industries & Fisheries, Tasmania

A Fisheries Research and Development Corporation funded program

Course Outline

Monday 17 June

10.00	Introduction	Jeremy Carson
10.10	Molecular Probes and PCR	John Bowman
11.00	Coffee	
11.15	PCR of bacterial colonies - DNA extraction - Set up PCR, nested and un-nested	Michelle Williams
1.00	Lunch	
2.00	PCR of seeded fish tissue - first stage DNA extraction	Michelle Williams
3.30	Tea	
3.45	PCR of bacterial colonies - set second round of nested PCR	
5.00	Summary of days work	

Tuesday 18 June

10.00	PCR for fish disease detection	Jeremy Carson
10.30	Practical aspects of PCR	Michelle Williams
11.00	Coffee	
11.15	PCR of bacterial colonies - gel electrophoresis of PCR product PCR of seeded fish tissue - continue DNA extraction	Michelle Williams
1.00	Lunch	
2.00	PCR of seeded fish tissue - set up PCR	Michelle Williams
3.30	Tea	
3.45	PCR of bacterial colonies - Determine concentration of extracted DNA - Gel electrophoresis of extracted DNA	
5.00	Summary of days work	

Wednesday 19 June

10.00	PCR of seeded fish tissue Gel electrophoresis of PCR products	Michelle Williams
11.00	Coffee	
11.15	Discussion of results	
12.00	Finish	

1. PCR of Bacteria Colonies

Monday 17 June

We will be working with four fish pathogens: *Aeromonas salmonicida*, *Enterococcus seriolicida*, *Flexibacter maritimus* and *Yersinia ruckeri*. Each participant will be provided with one bacterial culture growing on an agar plate. A crude DNA sample will be extracted from a bacterial colony and PCR performed to confirm the identity of the culture. For *A. salmonicida* nested PCR is necessary. This will require 2 rounds of PCR, the initial round using the Universal primers A and 785r and the second round using the species specific primers As1 and As2. Initially one quarter of you will be allocated *A. salmonicida* and will perform the first round. After this, however, everyone will be required to set up the second round of the *A. salmonicida*. Those who aren't allocated *A. salmonicida* initially will be allocated one of the other 3 pathogens.

11:15

1a) DNA extraction

From the agar plate, scrape off a bacterial colony using a sterile loop or toothpick. Suspend the cells in 100µl sterile water. Heat the cell suspension at 100°C in the heating block for 10 min. and then cool on ice for 5 min. Centrifuge the tubes at full speed for 1 min. to pellet the cell debris. DNA is now ready for PCR. Ensure that your tubes are properly labelled (with your initials, date and the initials of the bacterial species) as we will be using these samples again on Tuesday.

1b) PCR of cell lysates

PCR is to be performed using species specific primers. Samples will include

1) cell lysate
2) positive control (purified genomic DNA, supplied)[1]
3) negative control (water)

Conditions for PCR

2.5 mM $MgCl_2$
200 µM each $dNTPs_2$
50 pmol each $primer_3$
0.2 U Taq polymerase (Advanced Biotechnologies)

PCR of lysates from *E. seriolicida*, *F. maritimus* and *Y. ruckeri* will be performed under the following cycle conditions

Cycle 1.	Step 1.	94°C	3:00 min.
(x 1)	Step 2.	65°C	0:30 min.
	Step 3.	72°C	2:00 min.
Cycle 2.	Step 1.	94°C	0:30 min.
(x 36)	Step 2.	65°C	0:30 min.
	Step 3.	72°C	2:00 min.
Cycle 38.	Step 1.	72°C	4:00 min.
(x 1)	Step 2.	4°C	1:00 min.

For *A. salmonicida* nested PCR is necessary. The first round of this is performed using primers A_4 and 785r[4] and the second round with the species specific primers As1 and As2. Both rounds are performed under the following cycle conditions

Cycle 1.	Step 1.	94°C	3:00 min.
(x 1)	Step 2.	37°C	0:30 min.
	Step 3.	72°C	2:00 min.
Cycle 2.	Step 1.	94°C	0:30 min.
(x 36)	Step 2.	37°C	0:30 min.
	Step 3.	72°C	2:00 min.
Cycle 38.	Step 1.	72°C	4:00 min.
(x 1)	Step 2.	4°C	1:00 min.

1 PCR reaction

	1
10x Buffer	2µl
MgCl2	2µl
dNTPs	1.6µl
Primer 1	5µl
Primer 2	5µl
MQ H2O	3.2µl
Taq	0.2µl
sample	1µl

Reaction mixes for the 3 reactions to be set up are shown over the page.

Reaction Mixes

	Round 1
	As Primers
10x Buffer	8µl
MgCl2	8µl
dNTPs	6.4µl
Primer 1	20µl A
Primer 2	20µl 785r
MQ H2O	12.8µl
Taq	0.8µl

	Es Primers	Fm Primers	Yr Primers
10x Buffer	8µl	8µl	8µl
MgCl2	8µl	8µl	8µl
dNTPs	6.4µl	6.4µl	6.4µl
Primer 1	20µl Es6	20µl rF1	20µl Yr1
Primer 2	20µl Es9	20µl rF3	20µl Yr2
MQ H2O	12.8µl	12.8µl	12.8µl
Taq	0.8µl	0.8µl	0.8µl

NB. Always make up excess Reaction mix (at least one extra reaction each time).

Aliquot out 19µl lots of reaction mix into labelled 0.2ml PCR tubes. To each tube add 1µl of the appropriate DNA sample or MQ H_2O. Mix the samples by gently flicking the tubes. Preheat the thermo-cycler to 94°C, place samples in and run on the appropriate program.

3:30 Afternoon Tea

3:45

1c) Round 2 of nested PCR (A. *salmonicida*)

Everyone will be required to set up a set of the Round 2 reactions even if you weren't allocated *A. salmonicida* originally. The PCR conditions and program are identical to the first round. Reaction mix will be made up as for Round 1, however we will be using the species specific primers As1 and As2 (instead of A and 785r). Template for this PCR will be the PCR product from the Round 1 As reactions, so you will be again setting up 3 PCR reactions. Reaction mixes are again shown over the page.

	Round 2
	As Primers
10x Buffer	8µl
MgCl2	8µl
dNTPs	6.4µl
Primer 1	20µl As1
Primer 2	20µl As2
MQ H2O	12.8µl
Taq	0.8µl

Again aliquot 19µl of the reaction mix out into 0.2ml tubes. To the corresponding tube add 1µl of Round 1 product. Please note whose Round 1 product you used.

Tuesday 18 June

11:15

1d) Gel electrophoresis of PCR products.

Agarose gels[5] will be prepared for you. A 2% gel is used for E. seriolicida to give better resolution of the smaller PCR product. For the other PCR products either a 1% or 2% is acceptable. To the PCR samples add 4µl of 6 x GLB[6] and gently flick tubes to mix. Load 8µl of sample into each well and load 6µl of markers[7] along side.

Expected band sizes:[4]

A. salmonicida	260 base pairs
E. seriolicida	143 base pairs
F. maritimus	285 base pairs
Y. ruckeri	223 base pairs

Run the small gel at 70V for about 40 min and the larger gel at 130V for 1hr. When the gel has finished, remove and photograph under UV light.

3:45

1e) Quantitation and Visualisation of DNA (if time allows)

To 95µl of H_2O in the cuvette add 5µl of DNA sample from lysed cells and measure the A_{260} and A_{280} in the GeneQuant. Calculate concentrations (assuming that one A_{260} unit equals 50µg of double stranded DNA per ml)[8] and purity. Also take 10µl of the sample and add 2µl of 6 x GLB. Run on a 1% agarose gel along with SPP1 markers[9] to visualise the DNA.

2. PCR of Seeded Fish Tissue

Monday 17 June

For this section you will be working in pairs. Each pair will be given a piece of fish tissue which has been seeded with a bacterial culture and a piece of tissue which has not been seeded. DNA will be extracted from these tissues and PCR performed with different primer sets in order to determine which bacteria the tissue was seeded with. This afternoon we will begin DNA extractions which we will complete tomorrow. We will be using the QIAamp Tissue Kit which is available through QIAGEN. This kit is designed specifically to purify total DNA for reliable PCR. All tissue samples will be given a pre-treatment with lysozyme (this is usually only necessary for samples containing *E. seriolicida*).

2:00

2a) Lysis of seeded fish tissue

- Add 40µl of lysozyme (50mg/ml) to each tissue sample and incubate at 37°C for 30 min.
- Add 320µl of ATL buffer and 40µl of Proteinase K (18mg/ml). Incubate over night at 55°C in the Hybaid oven.

Tuesday 18 June

11:45

2b) Continuation of DNA extractions from fish tissue.

- Add 400µl Buffer AL to the sample, mix thoroughly by vortexing, and incubate at 70°C for 10 min.
- Add 420µl of ethanol to each sample, and mix thoroughly by vortexing.
- Place the QIAamp spin columns in the 2ml collection tubes provided (tubes without lids). Carefully apply the ethanol mixture (including the precipitate) to the column without moistening the rim, close the cap, and centrifuge at 6000 x g (~8000rpm) for 1 min.
- Place the column in a clean 2ml collection tube and discard the tube containing the filtrate.
- Carefully open the column and add 500µl of Buffer AW. Centrifuge at 6000 x g for 1 min. Place the column in a clean 2ml collection tube and discard the tube containing the filtrate.
- Carefully open the column and add another 500µl of Buffer AW. Centrifuge at 6000 x g for 1 min and at full speed for a further 2 min (or until Buffer AW has passed through the column).
- Place the column in a sterile 1.5ml eppendorf tube and discard the collection tube containing the filtrate.

- Carefully open the column. Elute the DNA with 100µl of Buffer AE preheated to 70°C. Incubate at 70°C for 5 min. then centrifuge at 6000 x g for 1 min.
- Elute the DNA again with the same 100µl containing the DNA. Incubate at 70°C for 5 min. then centrifuge at 6000 x g for 1 min.

1:00 Lunch

2:00
2c) PCR of seeded fish tissue.

Each pair should now have 2 DNA samples (from seeded and unseeded tissue). Both of these will be used in the PCR along with a positive and a negative control (supplied) to give a total set of four samples. Each pair will be required to set up 3 sets of PCR reactions each using a different primer pair for each set.

PCR Es

Samples: Es 1) Fish tissue + bacteria
Es 2) Fish tissue
Es 3) Es DNA
Es 4) H_2O

Reaction Mix:

	Es Primers
10x Buffer	10µl
MgCl2	10µl
dNTPs	8µl
Primer 1	25µl Es6
Primer 2	25µl Es9
MQ H2O	16µl
Taq	1µl

PCR Fm

Samples: Fm 1) Fish tissue + bacteria
Fm 2) Fish tissue
Fm 3) Fm DNA
Fm 4) H_2O

Reaction Mix:

	Fm Primers
10x Buffer	10µl
MgCl2	10µl
dNTPs	8µl
Primer 1	25µl rF1
Primer 2	25µl rF3
MQ H2O	16µl
Taq	1µl

PCR Yr

Samples: Yr 1) Fish tissue + bacteria
Yr 2) Fish tissue
Yr 3) Yr DNA
Yr 4) H_2O

Reaction Mix:

	Yr Primers
10x Buffer	10µl
MgCl2	10µl
dNTPs	8µl
Primer 1	25µl Yr1
Primer 2	25µl Yr2
MQ H2O	16µl
Taq	1µl

Aliquot out 19µl of reaction mix into labelled PCR tubes. Add 1µl of the appropriate sample to each. Place in the pre-heated thermo-cycler and run on the appropriate program.

Wednesday 19 June

10:00
2d) Gel electrophoresis of PCR product

Again gels will be prepared for you. To the PCR samples add 4µl of 6 x GLB and gently flick tubes to mix. Load 8µl of sample into each well and load 6µl of marker along side.

Load the gels in the order:

1) Es 1	7) Fm 3
2) Es 2	8) Fm 4
3) Es 3	9) Yr 1
4) Es 4	10) Yr 2
5) Fm 1	11) Yr 3
6) Fm 2	12) Yr 4

Expected band sizes:

E. seriolicida	143 base pairs
F. maritimus	285 base pairs
Y. ruckeri	223 base pairs

Run the small gel at 70V for about 40 min and the larger gel at 130V for 1hr. When the gel has finished, remove and photograph under UV light.

Appendices

A1. Genomic DNA

Genomic DNA will be supplied at a concentration of 100ng/µl for all positive controls.

A2. Dilution of dNTPs

dNTPs are supplied in individual tubes at 100mM each:

- dGTP
- dTTP
- dATP
- dCTP

I use a 1:40 dilution of each, so I take 10µl of each dNTP and add 360µl of MQ H_2O, to give a concentration of 2.5mM in the stock dNTP mix. I then use 1.6µl of this per 20µl PCR reaction to give a concentration of 200µM each.

A3. Dilution of primers.

Depending on who supplies the primers the amount is either given as nmoles or µg of DNA (the primers are supplied as a dried pellet). For PCR we need to know the molar concentrations of the primers.

If only the dry weight of the primer has been given, this can be converted to n.moles by calculating the extinction coefficient for each primer. To do this we need to know the extinction coefficient of each dNTP and the sequence of the primer.

Nucleotide	Extinction coefficient
dGTP	11.7 µl/µmol
dCTP	7.3µl/µmol
dATP	15.4µl/µmol
dTTP	8.8µl/µmol

Multiply the number of times each base appears in the primer sequence by its extinction coefficient to give a total (*E*).

Concentration (µmol/ml) = A_{260}/E

eg. for primer A: AGA GTT TGA TCC TGG CTC AG

dGTP = 6 x 11.7 = 70.2
dCTP = 4 x 7.3 = 29.2
dATP = 4 x 15.4 = 61.2
dTTP = 6 x 8.8 = 52.8
E = 213.8

Primers were made up to a concentration of 1µg/µl. So if we take 5µl of this, add 195µl of H_2O and measure the A_{260}:

A_{260} =1.141
[ ] = 1.141÷213.8 x 0.2÷5
= 0.213 nmol/µl

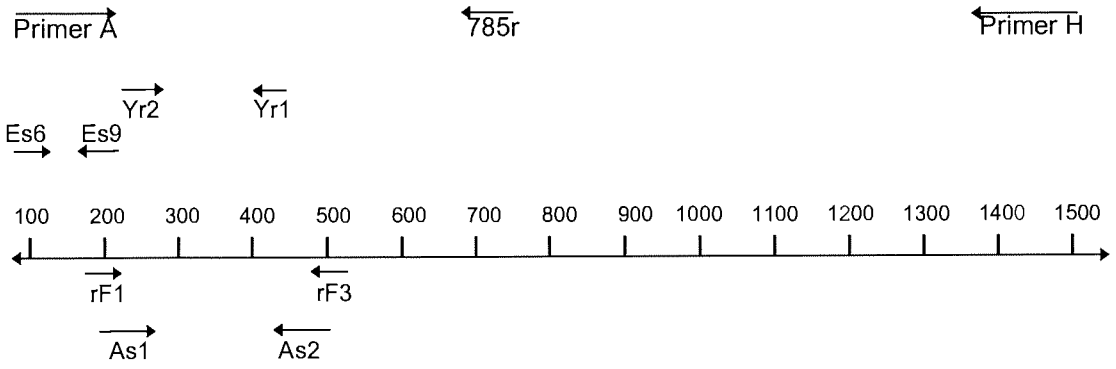
So to get a 10 pmol/µl solution:
= 4.7µl primer stock + 95.3µl H_2O

Primer	Sequence 5' to 3'	Direction	*E*
A	AGA GTT TGA TCC TGG CTC AG	forward	213.8
785r	GTG GAC TAC CAG GGT ATC TAA TCC	reverse	238.7
As1	TTT CGC GAT TGG ATG AA	forward	187.5
As2	TTG ACA CGT ATT AGG CGC CA	reverse	216
Es9	CGA GCG ATG ATT AAA GAT AGC TTG CTA	forward	311.3
Es6	ATA AGA ATC ATG CGA TTC TCA	reverse	240.3
rFlex1	GGA ATG GCA TCG TTT TAA AG	forward	230
rFlex3	AAT ACC TAC TCG TAG GTA CG	reverse	219.7
Yr2	AAC CCA GAT GGG ATT AGC TAG TAA	forward	259.3
Yr1	GTT CAG TGC TAT TAA CAC TTA ACC C	reverse	264.4

Where the amount of the primer is given in n.moles the following procedure can be used. Spin the tube to collect the powder at the bottom of the tube. Add 100µl of MQ water and stand for 2 min. Vortex for 15 sec. and pulse again for 10 sec. Dilute as required to 10pmol/µl.

For example: supplied amount of primer 785r = 76nmol. Dissolved in 100µl of water gives a stock of 760 pmol/µl. A 10pmol/µl solution is made by adding 10µl of the stock to 750µl of water.

A4. Position of primers on the 16S gene



16S sequences of the fish pathogens and *E.coli*

```
[          1                    Primer A                          50 ]
Arm.salmo3  .......... ..GAGTTTGA TCATGGCTCA GATTGAACGC TGGCGGCAGG
E.coli      ~~~~AAATTG AAGAGTTTGA TCATGGCTCA GATTGAACGC TGGCGGCAGG
Eco.seriol  .......... .......... .......... .....AACGC TGGCGGCGTG
Flx.marit2  ~NATACAATG AAGAGTTTGA TCCTGGCTCA GGATGAACGC TAGCGGCAGG
Yer.rucker  .......... .......... ....GGCTCA GATTGAACGC TGGCGGCAGG

[         51                                                   100 ]
Arm.salmo3  CCTAACACAT GCAAGTCGAG CGGCAGCGG- ---GAAAGTA GCTTGCTACT
E.coli      CCTAACACAT GCAAGTCGAA CGGTAACAG- ----GAAGAA GCTTGCTTCT
                                          Primer Es9
Eco.seriol  CCTAATACAT GCAAGTCGAG CGAT-----G ATTAAAGATA GCTTGCTATT
Flx.marit2  CTTAACACAT GCAAGTCGAG GGGTAACAT- ------TGTA GCTTGCTACA
Yer.rucker  CCTAACACAT GCAAGTCGAG CGGCAGCGG- ----AAAGTA GCTTGCTACT

[        101                                                   150 ]
Arm.salmo3  TTT----GCC GGCGAGCGGC GGACGGGTGA GTAATGCCTG GGG-ATCTGC
E.coli      TT-----GCT GACGAGTGGC GGACGGGTGA GTAATGTCTG GGA-AACTGC
Eco.seriol  TTTATGA--- -AG-AGCGGC GAACGGGTGA GTAACGCGTG GGAAATCTGC
Flx.marit2  -------GAT GACGACCGGC GCACGGGTGC GTAACGCGTA TAGAATCTGC
Yer.rucker  TT-----GCC GGCGAGCGGC GGACGGGTGA GTAATGTCTG GGG-ATCTGC

[        151                                                   200 ]
Arm.salmo3  CCAGTCGAGG GGGATAACAG TTGGAAACGA CTGCTAATAC CGCATACG--
E.coli      CTGATGGAGG GGGATAACTA CTGGAAACGG TAGCTAATAC CGCATAAC--
Eco.seriol  CGAGTAGCGG GGGACAACGT TTGGAAACGA ACGCTAATAC CGCATAACAA
Flx.marit2  CTTCTACAGA GGGATAGCCT TTAGAAATGA AGATTAATAC CTCATAACAC
Yer.rucker  CTGATGGAGG GGGATAACTA CTGGAAACGG TAGCTAATAC CGCATAAC--

[        201                                                   250 ]
Arm.salmo3  ------CC-T ACGGGG---- ----GAAAGG AGGGGACCTT CGGGCCTTTC
E.coli      ------GTCG CAAGAC---- ----CAAAGA GGGGGACCTT CGGGCCTCTT
                  Primer Es 6
Eco.seriol  TGAG-AATCG CATGATTCTT ATTTAAAAGA AGC-----AA TT---GCTTC
                Primer revFlex 1
Flx.marit2  TTTGGAATGG CATCGTTTTA AAGTTAAAGA --------TT TA------TC
Yer.rucker  ------CTCG CAAGAG---- ----CAAAGT GGGGGACCTT CGGGCCTCAC

[        251                                                   300 ]
              Primer As 1
Arm.salmo3  GCGATTGGAT GAACCCAGGT GGGATTAGCT AGTTGGTGGG GTAATGGCTC
E.coli      GCCATCGGAT GTGCCCAGAT GGGATTAGCT AGTAGGTGGG GTAACGGCTC
Eco.seriol  ACTACTTGAT GATCCCGCGT TGTATTAGCT AGTTGGTAGT GTAAAGGNCN
Flx.marit2  GGTAGAAGAT GACTATGCGT CCTATTAGCT AGATGGTAAG GTAACGGCTT
                                Primer Yr 2
Yer.rucker  GCCATCGGAT GAACCCAGAT GGGATTAGCT AGTAAGTGGG GTAATGGCTC
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[         301                                                    350 ]
Arm.salmo3  ACCAAGGCGA CGATCCCTAG CTGGTCTGAG AGGATGATCA GCCACACTGG
E.coli      ACCTAGGCGA CGATCCCTAG CTGGTCTGAG AGGATGACCA GCCACACTGG
Eco.seriol  ACCAAGGCGA TGATACATAG CCNNCCTGAG AGGGTGATCG GCCACACTGG
Flx.marit2  ACCATGGCAA CGATAGGTAG GGGTCCTGAG AGGGAGATCC CCCACACTGG
Yer.rucker  ACCTAGGCGA CGATCCCTAG CTGGTCTGAG AGGATGACCA GCCACACTGG

[         351                                                    400 ]
Arm.salmo3  AACTGAGACA CGGTCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG
E.coli      AACTGAGACA CGGTCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG
Eco.seriol  GACTGAGACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTA GGGAATCTTC
Flx.marit2  TACTGAGACA CGGACCAGAC TCCTACGGGA GGCAGCAGTG AGGAATATTG
Yer.rucker  AACTGAGACA CGGTCCAGAC TCCTACGGGA GGCAGCAGTG GGGAATATTG

[         401                                                    450 ]
Arm.salmo3  CACAATGGGG GAAACCCTGA TGCAGCCATG CCGCGTGTGT GAAGAAGGCC
E.coli      CACAATGGGC GCAAGCCTGA TGCAGCCATG CCGCGTGTAT GAAGAAGGCC
Eco.seriol  GGCAATGGGG GCAACCCTGA CCGAGCAACG CCGCGTGAGT GAAGAAGGTT
Flx.marit2  GGCAATGGAG GCAACTCTGA CCCAGCCATG CCGCGTGCAG GAAGACTGCC
Yer.rucker  CACAATGGGC GCAAGCCTGA TGCAGCCATG CCGCGTGTGT GAAGAAGGCC

[         451                                                    500 ]
                                                        Primer As 2
Arm.salmo3  -TTCG-GGTT GTAAAGCACT TTCAGCGAGG AGGAAAGGTT GGCGCC-TAA
E.coli      -TTCG-GGTT GTAAAGTACT TTCAGCGGGG AGGAA-GGGA GTAAAGTTAA
Eco.seriol  -TTCG-GATC GTAAAACTCT GTTGTTAGAG AAGAACGTTA AGTAGAGTGG
                                                  Primer revFlex 3
Flx.marit2  CTAT-GGGTT GTAAACTGCT TTTATACAGG AAGAAACGTA CCTA---CGA
                                                        Primer Yr 1
Yer.rucker  -TTCG-GGTT GTAAAGCACT TTCAGCGAGG AGGAA-GGGT TAAGTGTTAA

[         501                                                    550 ]
Arm.salmo3  TACGTGTCAA CTGTGACGTT ACTCGCAGAA GAAGCACCGG CTAACTCCGT
E.coli      TACCTTTGCT CATTGACGTT ACCCGCAGAA GAAGCACCGG CTAACTCCGT
Eco.seriol  AAAATTACTT AAGTGACGGT ATCTAACCAG AAAGGGACGG CTAACTACGT
Flx.marit2  G----TAGGT ATTTGACGGT ACTGTAAGAA TAAGGACCGG CTAACTCCGT
Yer.rucker  TAGCACTGAA CATTGACGTT ACTCGCAGAA GAAGCACCGG CTAACTCCGT

[         551                                                    600 ]
Arm.salmo3  GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG
E.coli      GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG
Eco.seriol  GCCAGCAGCC GCGGTAATAC GTAGGTCCCA AGCGTTGTCC GGATTTATTG
Flx.marit2  GCCAGCAGCC GCGGnnATAC GGAGnGTCCn AGCGTTATCC GGAATCATTG
Yer.rucker  GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG

[         601                                                    650 ]
Arm.salmo3  GGCGTAAAGC GCACGCAGGC GGTTGGATAA GTTAGATGTG AAAGCCCCGG
E.coli      GGCGTAAAGC GCACGCAGGC GGTTTGTTAA GTCAGATGTG AAATCCCCGG
Eco.seriol  GGCGTAAAGC GAGCGCAGGT GGTTTCTTAA GTCTGATGTA AAAGGCAGTG
Flx.marit2  GGTTTAAAGG GTCCGCAGGC GGTCGATTAA GTCAGAGGTG AAATCCCATA
Yer.rucker  GGCGTAAAGC GCACGCAGGC GGTTTGTTAA GTCAGATGTG AAATCCCCGA

[         651                                                    700 ]
Arm.salmo3  GCTCAACCTG GGAATTGCAT TTAAAACTGT CCAGCTAGAG TCTTGTAGAG
E.coli      GCTCAACCTG GGAACTGCAT CTGATACTGG CAAGCTTGAG TCTCGTAGAG
Eco.seriol  GCTCAACCAT TGTGT-GCAT TGGAAACTGG GAGACTTGAG TGCAGGAGAG
Flx.marit2  GCTTAACTAT GGAACTGCCT TTGATACTGG TTGACTTGAG TAATACGGAA
Yer.rucker  GCTTAACTTG GGAACTGCAT TTGAAACTGG CAAGCTAGAG TCTTGTAGAG

[         701                                                    750 ]
Arm.salmo3  GGGGGTAGAA TTCCAGGTGT AGCGGTGAAA TGCGTAGAGA TCTGGAGGAA
E.coli      GGGGGTAGAA TTCCAGGTGT AGCGGTGAAA TGCGTAGAGA TCTGGAGGAA
Eco.seriol  GAGAGTGGAA TTCCATGTGT AGCGGTGAAA TGCGTAGATA TATGGAGGAA
Flx.marit2  GTAGATAGAA TATGTAGTGT AGCGGTGAAA TGCATAGATA TTACATAGAA
Yer.rucker  GGGGGTAGAA TTCCAGGTGT AGCGGTGAAA TGCGTAGAGA TCTGGAGGAA

[         751                                                    800 ]
Arm.salmo3  TACCGGTGGC GAAGGCGGCC CCCTGGACAA AGACTGACGC TCAGGTGCGA
E.coli      TACCGGTGGC GAAGGCGGCC CCCTGGACGA AGACTGACGC TCAGGTGCGA
Eco.seriol  CACCGGAGGC GAAAGCGGCT CTCTGGCCTG TAACTGACAC TGAGGCTCGA
Flx.marit2  TACCGATTGC GAAGGCAGTC TACTACGTAT TTACTGACGC TCATGGACnA
Yer.rucker  TACCGGTGGC GAAGGCGCCC CCCTGGACAA AGACTGACGC TCAGGTGCGA
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[         801                                Primer 785r                    850 ]
Arm.salmo3  AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
E.coli      AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
Eco.seriol  AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
Flx.marit2  AAGCGTGGGG AGCGAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA
Yer.rucker  AAG-GTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCTGTAAA

[         851                                                       900 ]
Arm.salmo3  CGATGTCGAT TTGGAGGCTG TGTC-CTTGA -GACGTGGCT TCCGGAGCTA
E.coli      CGATGTCGAC TTGGAGGTTG TGCC-CTTGA -GGCGTGGCT TCCGGAGCTA
Eco.seriol  CGATGAGTGC TAGCTGTAGG GAGCT-ATA- AGTTCTCTGT AGCGCAGCTA
Flx.marit2  CGATGGACAC TAGTTGTTGG GA----AATG ---TCTCAGT GACTAAGCGA
Yer.rucker  CGATGTCGAC TTGGAGGTTG TGCC-CTTGA -GGCGTGGCT TCCGGAGCTA

[         901                                                       950 ]
Arm.salmo3  ACGCGTTAAA TCGACCGCCT GGGGAGTACG GCCGCAAGGT TAAAACTCAA
E.coli      ACGCGTTAAG TCGACCGCCT GGGGAGTACG GCCGCAAGGT TAAAACTCAA
Eco.seriol  ACGCATTAAG CACTCCGCCT GGGGAGTACG ACCGCAAGGT TGAAACTCAA
Flx.marit2  AAGTGATAAG TGTCCCACCT GGGGAGTACG ATCGCAAGAT TGAAACTCAA
Yer.rucker  ACGCGTTAAG TCGACCGCCT GGGGAGTACG GCCGCAAGGT TAAAACTCAA

[         951                                                      1000 ]
Arm.salmo3  ATGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA
E.coli      ATGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA
Eco.seriol  AGGAATTGAC GGNGGCCNGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA
Flx.marit2  AGGAATTGAC GGGGGCCnGC ACAAGCGGTG GAGCATGTGG TTTAATTCGA
Yer.rucker  ATGAATTGAC GGGGGCCCGC ACAAGCGGTG AAGCATGTGG TTTAATTCGA

[        1001                                                      1050 ]
Arm.salmo3  TGCAACGCGA AGAACCTTAC CTGGCCTTGA CATG-TCTGG -AATCCTGTA
E.coli      TGCAACGCGA AGAACCTTAC CTGGTCTTGA CATC-CACGG -AAGTTTTCA
Eco.seriol  AGCAACGCGA AGAACCTTAC CAGGTCTTGA CATA-CTCGT GCTATCCTTA
Flx.marit2  TGATACGCGA GGAACCTTAC CAGGGCTTAA ATGTGGAATG -ACAGGGCTA
Yer.rucker  TGCAACGCGA AGAACCTTAC CTACTCTTGA CATC-CACAG -AACTTGGCA

[        1051                                                      1100 ]
Arm.salmo3  GAGATACGGG AGTGCCTTCG GGAA-TCAGA ACACAGGTGC TGCATGGCTG
E.coli      GAGATGAGAA TGTGCCTTCG GGAA-CCGTG AGACAGGTGC TGCATGGCTG
Eco.seriol  GAGATNAGGA GTT-CCTTCG GG-ACACGGG ATACAGGTGG TGCATGGTTG
Flx.marit2  GAGATAGCnT TT--TCTTCG GA---CATTT CACAAGGTGC TGCATGGTTG
Yer.rucker  GAGATGCCTT GGTGCCTTCG GGAA-CTGTG AGACAGGTGC TGCATGGCTG

[        1101                                                      1150 ]
Arm.salmo3  TCGTCAGCTC GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA
E.coli      TCGTCAGCTC GTGTTGTGAA ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA
Eco.seriol  TCGTCAGCTC GTGTCGTGAG ATGTTNGGTT AAGTCCCGCA ACGAGCGCAA
Flx.marit2  TCGTCAGCTC GTGCCGTGAG GTGTCAGGTT AAGTCCTATA ACGAGCGCAA
Yer.rucker  TCGTCAGCTC GTGTTGTGAA ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA

[        1151                                                      1200 ]
Arm.salmo3  CCCCTGTCCT TTGTTGCCAG CACGTAATGG TGGGAACTCA AGGGAGACTG
E.coli      CCCTTATCCT TTGTTGCCAG CGGTCCGG-C CGGGAACTCA AAGGAGACTG
Eco.seriol  CCCTTATTAC TAGTTGCCAT CA-TTAAGT- TGGGCACTCT AGTGAGACTG
Flx.marit2  CCCCTATTGT TAGTTGCTAG CAGGTAAAGC TGAGGACTCT AGCGAGACTG
Yer.rucker  CCCTTATCCT TTGTTGCCAG CACGTAATGG TGGGAACTCA AGGGAGACTG

[        1201                                                      1250 ]
Arm.salmo3  CCGGTGATAA ACCG-GAGGA AGGTGGGGAT GACGTCAAGT CATCATGGCC
E.coli      CCAGTGATAA ACTG-GAGGA AGGTGGGGAT GACGTCAAGT CATCATGGCC
Eco.seriol  CCGGTGATNA ACCG-GAGGA AGGTGGGGAT GACGTCAAAT CATCATGCCC
Flx.marit2  CCGGTG-CAA ACCGCGAGGA AGGTGGGGAT GACGTnnAAT CATCACGGCC
Yer.rucker  CCGGTGACAA ACCG-GAGGA AGGTGGGGAT GACGTCAAGT CATCATGGCC

[        1251                                                      1300 ]
Arm.salmo3  CTTACGGCCA GGGCTACACA CGTGCTACAA TGGCGCGTAC AGAGGGCTGC
E.coli      CTTACGACCA GGGCTACACA CGTGCTACAA TGGCGCATAC AAAGAGAAGC
Eco.seriol  CTTATGACCT GGGCTACACA CGTGCTACAA TGGATGGTAC AACGAGTCGC
Flx.marit2  CnTACGTCCT GGGCTACACA CGTGCTACAA TGGTATGGAC AATGAGCAGC
Yer.rucker  CTTACGAGTA GGGCTACACA CGTGCTACAA TGGCAGATAC AAAGTGAAGC
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[        1301                                                    1350 ]
Arm.salmo3  AAGCTAGCGA TAGTGAGCGA ATCCCAAAAA GCGCGTCGTA GTCCGGATCG
E.coli      GACCTCGCGA GAGCAAGCGG ACCTCATAAA GTGCGTCGTA GTCCGGATTG
Eco.seriol  CAACCCGCGA GGGTGCGCTA ATCTCTTAAA ACCAATCTCA GTTCGGATTG
Flx.marit2  CATCTGGCAA CAGAGAGCGA ATCTAC-AAA CCATATCACA GTTCGGATCG
Yer.rucker  GAACTCGCGA GAGCAAGCGG ACCACATAAA GTCTGTCGTA GTCCGGATTG

[        1351                                                    1400 ]
Arm.salmo3  GAGTCTGCAA CTCGACTCCG TGAAGTCGGA ATCGCTAGTA ATCGCGAATC
E.coli      GAGTCTGCAA CTCGACTCCA TGAAGTCGGA ATCGCTAGTA ATCGTGGATC
Eco.seriol  CAGGCTGCAA CTNGCCTGCA TGAAGTCGGA ATCGCTAGTA ATCGCGGATC
Flx.marit2  GAGTCTGCAA CTCGACTCCG TGAAGCTGGA ATCGCTAGTA ATCGGATATC
Yer.rucker  GAGTCTGCAA CTCGACTCCA TGAAGTCGGA ATCGCTAGTA ATCGTAGATC

[        1401                                                    1450 ]
Arm.salmo3  AGA-ATGTCG CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
E.coli      AGA-ATGCCA CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
Eco.seriol  AGC-ACGCCG CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
Flx.marit2  AGCCATGATC CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
Yer.rucker  AGA-ATGCTA CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA

[        1451                                                    1500 ]
Arm.salmo3  CACCATGGGA GTGGGTTGCA CCAGAAGTAG ATAGCTTAAC CTTCGGGAGG
E.coli      CACCATGGGA GTGGGTTGCA AAAGAAGTAG GTAGCTTAAC CTTCGGGAGG
Eco.seriol  CACCACGGAA GTTGGGAGTA CCCAAAGTAG GTTGCCTAAC CGCAAGGAGG
Flx.marit2  AGCCATGGAA GCnGGTTGTA CCTGAAGTTG G-----nTAC CGCAAGGAG-
Yer.rucker  CACCATGGGA GTGGGTTGCA AAAGAAGTAG GTAGCTTAAC CTTCGGGAGG

[        1501                                                    1550 ]
Arm.salmo3  GCGTTTACCA CGGTGTGATT CATGACTGGG GTGAAGTCGT AACAAGGTAA
E.coli      GCGCTTACCA CTTTGTGATT CATGACTGGG GTGAAGTCGT AACAAGGTAA
Eco.seriol  GCGCTTCCTA AGGTAAGACC GATGACTGGG GTGAAGTCGT AACAAGGTAG
Flx.marit2  ---CCGCCTA GGGTAAAACT GGTAACTAGG .......... ..........
Yer.rucker  GCGCTTACCA CTTTGTGATT CATGACTGGG GTGAAGTCGT AACAAGG...

[        1551                  Primer H        1585 ]
Arm.salmo3  .......... .......... .......... .....
E.coli      CCGTAGGGGA ACCTGCGGTT GGATCACCTC CTTA~
Eco.seriol  CCGTATCGGA AGG....... .......... .....
Flx.marit2  .......... .......... .......... .....
Yer.rucker  .......... .......... .......... .....
```

A5. Agarose gel

Agarose gel is made up w/v in 1xTAE buffer[10]

Add agarose to buffer and dissolve it by heating in the microwave on high for a few minutes. Let it cool slightly and then add ethidium bromide to a final concentration of 0.5µg/ml (ie. to 100ml of gel add 5µl of 10mg/ml EtBr).

A6. 6 x GLB

30% sucrose
7.5 mM EDTA[11]
1.5% SDS[12]
0.3% Bromophenol Blue

For 1 ml
- 0.5ml 60% sucrose (or 0.3g sucrose)
- 15µl 0.5M EDTA
- 150µl 10% SDS
- 335µl H_2O (or make up to 1 ml with H_2O)
- 3mg Bromophenol Blue

A7. pUC19 DNA Markers (Bresatec)

Fragment no.	Size base pairs
1	501
2	489
3	404
4	331
5	242
6	190
7	147
8	111
9	110
10	67
11	34
12	34
13	26

A8. How to calculate the concentration of DNA samples using A_{260}

1 OD unit at 260nm (A_{260}) = 33µg/ml oligo DNA
= 50µg/ml double stranded DNA
= 40µg/ml RNA

[ds DNA] = A_{260} x 50 x dilution factor

For example, you put 5µl of your DNA sample into 95µl of H_2O
A_{260} = 0.5

[ds DNA] = A_{260} x 50 x dilution factor
= 0.5 x 50 x 100/5
= 500 µg/ml

A9. SPP1 DNA markers (Bresatec)

Fragment no.	Size (Kb)
1	8.51
2	7.35
3	6.11
4	4.84
5	3.59
6	2.81
7	1.95
8	1.86
9	1.51
10	1.39
11	1.16
12	0.98
13	0.72
14	0.48
15	0.36

A10. 50xTAE

For 1 lt
242g Tris base
57.1 ml glacial acetic acid
100ml 0.5M EDTA (pH 8.0)

Add all 3 together, make up to ~900ml with H_2O. Dissolve the Tris and then make up to 1 lt. The working concentration is 1xTAE (0.04M Tris acetate, 0.001M EDTA). It is not necessary to autoclave this.

A11. 0.5M EDTA (pH 8.0)

For 250ml
46.5g EDTA
add ~150ml H_2O

pH to 8.0 (NB the EDTA will not dissolve until pH 8.0 is reached). Either use 5M NaOH or add ~5g of NaOH pellets. Make up to 250ml with H_2O. Autoclave at 121°C for 15 min.

A12. 10% SDS w/v

For 500ml
50g SDS
Add 400ml H_2O and adjust the pH to 7.4 with NaOH. Make up to 500ml. DO NOT AUTOCLAVE.

Internet Resources

The Internet is a tremendous source of information, although finding useful data can sometimes be a hit and miss affair. Given here is a list of web sites which may useful.

Pharmacia
Home page, News, Information, Catalogue, Contact point
http://www.biotech.pharmacia.se

Gibco BRL Life Technologies
Products, Primers, Tech On-line, Training, What's new, Mail
http://www.lifetech.com

Promega
Products, Applications, Technical Resources, Catalogue
http://www.promega.com

Pedro's BioMolecular Research Tools
A very comprehensive list of links to molecular biology web sites
http://www.public.iastate.edu/~pedro/research_tools.html

GenBank Search
Search GenBank for bacterial genome sequences
http://ncbi.nlm.nih.gov/cgi-bin/genbank

ATCC
Search the American Type Culture Collection for bacterial strains
http://www.atcc.org/

Advanced Biotechnologies
This site is packed with information about the company's products; much of it is of general interest and application.
http://www.adbio.co.uk/

Advanced Biotechnologies has an impressive list of links for molecular biology. These sites and their URLs are given here together with a copy of the Web page itself. You will see that the page also has access to three web search engines which is a useful feature.
http://www.adbio.co.uk/hotlinks.htm

Reference

- The World Wide Web Virtual Library: Biotechnology
  http://www.webpress.net/interweb/cato/biotech/
- Primer on Molecular Genetics from the Department of Energy
  http://www.gdb.org/Dan/DOE/intro.html

- Genome Data Base
  http://gdbwww.gdb.org/

- PCR Methods & Applications
  http://www.cshl.org/journals/pcr/

- NWFSC Molecular Biology Protocols
  http://research.nwfsc.noaa.gov/protocols.html

- Nucleic Acids Research Online from Oxford Online Journals
  http://www.oup.co.uk/oup/smj/journals/ed/titles/nar/

- REBASE - Restriction Enzyme Database
  http://www.neb.com/rebase/index.html

- Dictionary of Biotechnology Terms
  http://biotech.chem.indiana.edu/pages/dictionary.html

- Human Genome Project Information
  http://www.ornl.gov/TechResources/Human_Genome/home.html

- BioTechNet WWW
  http://www.biotechniques.com/

- BioSupplyNet
  http://www.biosupplynet.com/bsn/

- Molecular Genetics Database
  http://www2.ncbi.nlm.nih.gov/medline/query_form.html

Resources

- PCR Jump Station
  http://www.apollo.co.uk/a/pcr/

- Molecular Biology Jump Station: A comprehensive collection of USEFUL links for the molecular biologist.
  http://www.ifrn.bbsrc.ac.uk/gm/lab/docs/molbiol.html

- Internet for the Molecular Biologist: A good introduction to the internet for the molecular biologist.
  http://www.ifrn.bbsrc.ac.uk/gm/lab/docs/iftmb.html

- Books for Molecular Biology. Includes full chapter abstracts, book reviews, and ordering information.
  http://www.apollo.co.uk/a/horizon

- Protocols on the WWW. An extensive directory of the sites providing protocols for molecular biology, microbiology, genetics, biochemistry, and cell biology.
  http://www.ifrn.bbsrc.ac.uk/gm/lab/docs/protocols.html

- Microbiology Jump Station: A site for microbiology containing links to microbiology institutes, organizations, journals, culture collections, newsgroups, protocols, information, and directories.
  http://www.ifrn.bbsrc.ac.uk/gm/lab/docs/micro.html

- Genetics Jump Station: A large collection of links for the geneticist.
  http://www.ifrn.bbsrc.ac.uk/gm/lab/docs/genetics.html

- BioTech - Best WWW Biotechnology Resources
  http://biotech.chem.indiana.edu/pages/best.html

- BIOSCI - NewsGroups and email lists.
  http://www.bio.net/

- Web Addresses of selected Life Sciences Companies
  http://165.123.33.33/yr1996/jan/webaddr_960108.html

- BiolRes: Biological and Chemical Internet Resources
  http://pegasus.uthct.edu/OtherUsefulSites/BiolRes.html

- Paul N. Hengen at the National Cancer Institute
  http://www-lmmb.ncifcrf.gov/~pnh/

- The Sanger Centre
  http://www.sanger.ac.uk/

Software

- PrimerDesign
  http://www.chemie.uni-marburg.de/~becker/

Others

- SCIENCE On-Line Magazine
  http://science-mag.aaas.org/science/

- Nature
  http://www.nature.com/

News Groups

- bionet.announce
- bionet.general
- bionet.genome.chromosomes
- bionet.microbiology
- bionet.molbio.proteins
- bionet.molbio.rapd
- bionet.software.www
- bionet.molbio.methds-reagents
- sci.bio.microbiology

The University of Oklahoma

The molecular biology protocols from the Department of Chemistry and Biochemistry cover a wide variety of techniques. The home site URL does not appear to be active at the moment but the protocols are carried by a number of other web sites. The protocols can be accessed at:

http://algodon.tamu.edu/homepage/lazo/methods/uo/uo.html

PCR Equipment and Reagents

Finding the right equipment or buying reagents can be a problem for the first time as there are so many suppliers and products. This list has been compiled based on one laboratories experience. It is not intended to be an exhaustive list or necessarily authoritative but it does represent a starting point.

Most importantly talk to colleagues in your own laboratory or another institution about equipment and reagents and get their opinions. Browse the news groups, they often carry opinions about products, or ask for comment.

Amresco
Astral Scientific
PO Box 232
Gymea
NSW 2227
Tel: 1800 221 280

Bresatec
PO Box 11
Rundle Mall
Adelaide
SA 5000
Tel: 1800 882 555

Epicentre Technologies
Astral Scientific
PO Box 232
Gymea
NSW 2227
Tel: 1800 221 280

Gibco BRL Life Technologies
PO Box 4296
Mulgrave
Victoria 3170
Tel: 1800 331 627

Integrated Sciences
PO Box 731
Willoughby
NSW 2068
Tel: 1800 252 204

Interpath Services
PO Box 340
West Heildelberg
Victoria 3081
Tel: 1800 626 369

Pharmacia
AMRAD Pharmacia Biotech
34 Wadhurst Drive
Boronia
Melbourne
Victoria
Tel: 008 252 265

Promega
PO Box 168
Annandale
NSW 2038
Tel: 1800 225 123
Fax: 1800 626 017

Quantum Scientific
PO Box 164
Paddington
Queensland 4064
Tel: 1800 625 547

Materials

Agarose gel electrophoresis unit
Gibco-BRL: Horizon 58, cat. no. 41060-013
Combs: 8-tooth, 0.8mm thick; cat. no. 21065-073
14-tooth, 0.8mm thick; cat. no. 21065-115

Bench top chillers
These are useful if you don't have access to crushed ice or you don't want to keep opening and shutting the door of the freezer when getting out reagents.
Nalgene
Labtop Cooler 5115-0032
Quick Chill unit 5114-0012

Disposal of ethidium bromide
Amresco ethidium bromide de-staining bags; cat. no. E732

Deoxyribonucleoside triphosphates
dNTPs can be obtained as the individual nucleotides, as a set of 4 nucleotides or pre-mixed. In whatever form make sure they are suitable for use with polymerase reactions and that they are supplied neutralised (pH 7) or in a weak buffer such as Tris.

Epicentre: 2.5mM pre-mixed, D08104
Gibco: 10mM pre-mixed, 18427-013
Promega: set of 4 separate nucleotides, 40μmoles ea, U1240

DNA markers
Bresatec
pUC19: base pair range 26-501; cat no. DMW-P1
SPP-1: base pair range 0.36-8.51 kb; cat. no. DMW-S1

Ethidium bromide
Sigma cat no. E1510; 10ml, 10mg/ml solution

Filter set for photography
Polaroid filter no. 15, orange for ethidium bromide stain.

Microcentrifuge tubes and PCR tubes
Quantum Scientific
1.5 ml flat top cap; cat. no. 502-PLN
Locking cap type: L-510 GRD
Screw cap with O-ring: 500μL; cat. no. 512
Screw cap with O-ring: 1500μL; cat. no. 513

Interpath
1.5 ml tube; HT-15-N
Locking cap type 'Twistlock'; LT-15-NG
Cap opener; TO-XX-A
Cap locks; CH-05-A
UltraFlux PCR Microtubes, 0.2ml strip tubes with attached caps; TC-028-N

Bresatec
Robbins 0.2ml PCR tubes; RS1045-20-0

Mini microcentrifuge
6-place microcentrifuge for pulsing microcentrifuge tubes.
Tomy Capsulefuge HF 120, Quantum Scientific

Oligonucleotides
Available from several suppliers, eg. Bresatec or Gibco. Specify de-salted and de-protected. Typically 50 nmoles is sufficient. Manufacturers will supply special order forms for sequences.

Pipetters
Aim for autoclavable standard in case of contamination.
Volume ranges: 0.5-10µl; 2-20µl; 10-100µl; 100-1000µl.
A separate pipetter, 2-20µl, would be prudent for loading gels.

Pipette Tips
Quantum Scientific - QSP
Barrier filter tips, non-sterile, autoclavable, 0.5µl to 1000µl
Racked or loose for autoclaving.

Polaroid film
Type 667, black and white, coaterless, high speed

Polymerases
Polymerases are supplied with reaction buffer and magnesium chloride
Advanced Biotechnologies (Integrated Sciences)
Thermostable DNA polymerase AB 0192
Red-Hot Thermostable DNA polymerase AB 0406

Reagents
Choose molecular biology grade. Available from Amresco, Sigma, Gibco and Promega

Tube containers
Use glass beakers with foil tops or wide mouth polypropylene jars with lids, Nalgene cat. no. 2118-0032

Thermal Cyclers
The information given here is based on personal experience. Several brands of thermal cycler exist and all have their supporters and detractors. When talking to users of different machines in Australia however, everyone expressed satisfaction with what they had and several institutions were on the point of ordering further machines of the same type. One conclusion is that there may not be much to choose between brands.

Most manufacturers and suppliers will let you try a demonstration machine for a week or two so take advantage of this offer. When choosing a thermal cycler consider the following: precision of temperature control; similarity of temperatures in different wells; ramp speed; ability to adjust ramp speed; ease of programming; number of tubes that can be used; ability to use a microtitre style tray; availability of a heated lidded.

Water
Use 18 MΩ water for all solutions to be used for PCR. If a reliable supply is not available use Sigma water, cat. no. W-4502. This has been tested to be DNase and RNase free.