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PCR-based assays for the fish pathogen *Aeromonas salmonicida*. I. Evaluation of three PCR primer sets for detection and identification

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ABSTRACT: In an effort to develop a rapid diagnostic test for the fish pathogen *Aeromonas salmonicida*, the performance of 2 polymerase chain reaction (PCR) primer sets (AP and PAAS) targeting the fish pathogen *A. salmonicida* and 1 PCR primer set (MIY) targeting *A. salmonicida* subsp. *salmonicida* were evaluated. Initially, the PCR assays were used to screen purified DNA extracted from 308 *A. salmonicida* isolates. The AP and PAAS PCR tests were demonstrated to be 100% specific for the species *A. salmonicida* and did not cross-react with any of the non-target organisms (bacterial species other than *A. salmonicida*) used in this study. The combined sensitivity of the AP and PAAS tests was 99.4% and offered the best coverage in terms of identifying the target organism. The MIY PCR appeared to be 100% sensitive and specific for *A. salmonicida* subsp. *salmonicida*. Studies with tissues, spiked with known quantities of bacteria, were conducted to determine the lower detection limit of the PCR tests, and then the ability of these PCR tests to detect *A. salmonicida* in experimentally infected salmonids was assessed.

KEY WORDS: *Aeromonas salmonicida* · Furunculosis · Detection · Identification · Experimental infections · Polymerase chain reaction

INTRODUCTION

Aeromonas salmonicida is a complex group of bacterial pathogens, many strains of which cause a range of diseases in a wide variety of fish species (Austin & Austin 1999). *A. salmonicida* subsp. *salmonicida*, also known as 'typical *A. salmonicida*', is the causative agent of furunculosis, an often devastating disease of salmonids (Hiney & Olivier 1999).

Furunculosis poses a very serious exotic disease threat to the growing salmonid aquaculture industries in Australia. This threat is further complicated by the fact that the bacterium can be present as clinically inapparent or 'covert' infections in 'carrier' fish (Hiney et al. 1997). Carriers can be extremely difficult to detect, due to the fact that *Aeromonas salmonicida* is likely to be present only in low numbers, and its primary location within the carrier host remains unknown. All of these factors lead to the possibility that *A. salmonicida* subsp. *salmonicida* could enter Australia undetected, establish covert infections in fish and become widely disseminated before the first clinical case of furunculosis is reported.

Some atypical strains of *Aeromonas salmonicida* are already considered enzootic to Australia (Whittington et al. 1995, Trust et al. 1996). The incidence of disease outbreaks caused by atypical strains, not only in Australia but also world-wide, appears to be increasing and could potentially affect both aquaculture and natural fisheries over a wide geographic range within southern Australia. It is essential that the technology to differentiate between those strains exotic to Australia (particularly typical *A. salmonicida*) and enzootic strains be developed.

Identification of *Aeromonas salmonicida*, based on traditional methods including classical biochemical

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testing, can be time consuming and problematic. Phenotypic variation displayed by this group of bacteria can make assigning new isolates to the existing taxonomy, based on phenotype, a difficult procedure (Austin et al. 1998). Polymerase chain reaction (PCR) technology may be able to provide a rapid means of identifying and differentiating bacterial isolates that display phenotypic properties that would otherwise hinder their timely identification. Our objective was to develop improved procedures for the detection and identification of *A. salmonicida* species and subspecies, thus enhancing the diagnostic capability for this fish pathogen.

MATERIALS AND METHODS

***Aeromonas salmonicida* isolates.** In order to undertake extensive *in vitro* validation and determination of the specificity and sensitivity of the PCR tests, the Australian Animal Health Laboratory (AAHL) Fish Diseases Laboratory (AFDL) sourced a wide variety of *A. salmonicida* isolates (n = 308) from all regions of the world where this species has been isolated (Table 1). The collection comprised type and reference cultures from recognised culture collections, and clinical and laboratory strains originating from at least 38 teleost species. Approximately 85% of the isolates in the AFDL *A. salmonicida* collection are exotic to Australia.

Cultures other than *Aeromonas salmonicida*. A variety of bacterial cultures were employed to test the specificity of the PCR assays. Isolates included representatives of the *Aeromonas* DNA hybridisation groups (Huys & Swings 1999) and a range of common fish pathogens (Table 2).

Maintenance of bacterial strains. All bacterial isolates were stored in MicroBank™ vials (Pro-Lab) at −80°C as per the manufacturer's instructions. When required, isolates were grown on Columbia Blood Agar Base (Oxoid CM331), supplemented with 5% defibrinated sheep blood, incubated aerobically at 22°C for 2 to 4 d.

Biochemical characterisation. To confirm their identity and to group the isolates according to subspecies, a biochemical profile was obtained for 243 of the 308 *Aeromonas salmonicida* isolates. The profile consisted of the following tests: Gram stain; pigment production on tryptic soy agar (TSA); cytochrome oxidase; motility; growth on sheep blood agar (SBA) at 37°C; indole; methyl red; Voges-Proskauer; arginine dihydrolase (Moeller); nitrate reduction; aesculin hydrolysis; gas production (1% carbohydrate in oxidation-fermentation [OF] basal medium) from D-glucose; and acid production from L-arabinose, D-galactose, maltose, D-mannitol, sucrose, and trehalose. Tests were conducted according to Balows et al. (1993) except that cultures were incubated at 22°C. Tests were examined at 1, 2, 3, 5, 7 and 10 d post-inoculation. Terminal end-product tests were evaluated 4 to 10 d post-inoculation.

Genomic DNA extraction. Genomic DNA was extracted from all bacterial isolates using the DNAzol® reagent (Life Technologies™) or the Puregene® DNA Isolation Kit (Gentra Systems) as per the manufacturer's instructions.

PCR protocols. PCRs were performed in 0.2 ml thin-walled PCR tubes (Quantum Scientific) in a GeneAmp® 9600 thermal cycler (Perkin Elmer-Cetus). The DNA polymerase and buffering system used was either *Taq* DNA polymerase from Promega or Platinum™ *Taq* from Life Technologies™. Unless otherwise specified, 1 to 10 ng template DNA was added.

Table 1. List of contributors to the Australian Animal Health Laboratory (AAHL) Fish Diseases Laboratory (AFDL) *Aeromonas salmonicida* collection

American Type Culture Collection, USA
AAHL Fish Diseases Laboratory
Commonwealth Scientific and Industrial Research Organisation (CSIRO) Livestock Industries, Australia
Australian Fish Health Reference Laboratory, Benalla, Australia
Dr. C. Rogers, Fish Diseases Laboratory, Centre for Environment, Fisheries and Aquaculture Science (CEFAS), UK
Dr. C. Michel, Institut National de la Recherche Agronomique, France
Dr. E. B. Shotts, Department of Microbiology, University of Georgia, USA
Dr. H. Kalnins, Commonwealth Serum Laboratories, Australia
Dr. I. Dalsgaard, Danish Institute for Fisheries and Marine Research, Denmark
Dr. J. Carson, Fish Health Unit, Mt. Pleasant Laboratory, Department of Primary Industries, Water and Environment (DPIWE), Australia
Dr. M. Hiney, Fish Disease Group, University College Galway, Ireland
Dr. O. Haenen, Institute for Animal Science and Health, The Netherlands
Dr. P. Rintamaki, University of Oulu, Finland
Dr. R. Whittington, Elizabeth MacArthur Agricultural Institute, Australia
Dr. R. B. Callinan, NSW Department of Fisheries, Australia
Dr. R. Cipriano, National Fish Health Research Laboratory, USA
Dr. S. Høie, National Veterinary Institute, Norway
Dr. S. D. Miller, Institute of Aquaculture, University of Stirling, Scotland
Dr. T. P. T. Evelyn, Pacific Biological Station, Canada
Dr. T. Meyers, Department of Fish and Game, USA
Dr. T. Wiklund, Institute of Parasitology, Abo Akademi University, Finland
Dr. B. K. Guðmundsdóttir, Institute for Experimental Pathology, University of Iceland
Mr. L D. Ashburner, Department of Conservation Forests and Lands, Australia
Mrs. J. Petrie, Fisheries Research Services, Scotland
National Collection of Industrial and Marine Bacteria (NCIMB), Scotland
Prof. B. Austin, Heriot Watt University, Scotland
Prof. T. Aoki, Tokyo University of Fisheries, Japan

Table 2. Non-target bacterial isolates employed as specificity controls during this study. NCFB: National Collection of Food Bacteria; other abbreviations in Table 1

Bacterial species	Isolate number
Yersinia ruckeri	ATCC 29473
Carnobacterium piscicola	ATCC 35586
Edwardsiella ictaluri	85:10067-1A
Lactococcus garvieae	ATCC 49156
Aeromonas hydrophila subsp. *hydrophila*	ATCC 7966
Aeromonas caviae	ATCC 15468
Aeromonas media	ATCC 33907
Aeromonas eucrenophila	ATCC 23309
Aeromonas veronii biovar *veronii*	ATCC 35624
Aeromonas veronii	ATCC 35941
Aeromonas schubertii	ATCC 43700
Aeromonas enteropelogenes	ATCC 49657
Aeromonas sobria	ATCC 43979
Aeromonas veronii biovar *sobria*	ATCC 9071
Aeromonas jandaei	ATCC 49568
Flexibacter maritimus	NCIMB 2154
Flexibacter ovolyticus	NCIMB 13127
Photobacterium phosphoreum	NCIMB 1282
Vibrio ordalii	NCIMB 2167
Vibrio tubiashii	NCIMB 1340
Vibrio anguillarum	ATCC 19264
Vagococcus salmoninarum	NCFB 2777
Vibrio alginolyticus	ATCC 17749
Vibrio harveyi	ATCC 14126
Vibrio vulnificus	ATCC 27562
Vibrio fluvialis	ATCC 33809
Vibrio natriegens	ATCC 14048

Genomic DNA from an *Aeromonas salmonicida* isolate, demonstrated to be positive by the 3 PCRs, was used as a positive control; sterile distilled water served as the negative control. A PCR was deemed positive based on product size, as determined by agarose gel electrophoresis and as compared to known standards. If a particular sample yielded a negative result, it was subsequently re-tested as follows. If a sample was negative in all 3 systems, it was tested with universal primers specific for the 16S ribosomal RNA gene. A negative result in this system indicated that amplification had been inhibited, and therefore the source bacterium was re-cultured, and the DNA isolated and re-tested. Each of the PCRs was optimised for use in our laboratory. Where reaction conditions used varied from those in previous publications they are indicated below.

Amplification with 16S rDNA primers (Dorsch & Stackebrandt 1992): Each 25 µl reaction contained 0.5 U of DNA polymerase, 2.5 µl of 10× PCR buffer, 1.5 mM $MgCl_2$, 9 pmol each of the amplification primers 27f (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′), and all 4 deoxynucleotide triphosphates at 0.2 mM each. Reaction mixes were held for 2 min at 94°C, then amplified for 28 cycles, with denaturation for 30 s at 94°C, annealing for 30 s at 49°C and elongation for 1 min 15 s at 72°C. A final extension was performed at 72°C for 3 min. The expected PCR product size was 1465 base pairs (bp).

Amplification with the PAAS primers (O'Brien et al. 1994): Each 25 µl reaction contained 0.25 U of DNA polymerase, 2.5 µl of 10× PCR buffer, 2.5 mM $MgCl_2$, 8 pmol each of the amplification primers PAAS1 (5′-CGTTGGATATGGCTCTTCCT-3′) and PAAS2 (5′-CTCAAAACGGCTGCGTACCA-3′), and all 4 deoxynucleotide triphosphates at 0.2 mM each. Reaction mixes were held for 30 s at 95°C, then amplified for 30 cycles, with denaturation for 2 min at 94°C, annealing for 30 s at 57°C and elongation for 1 min 30 s at 72°C. A final extension was performed at 72°C for 3 min. The expected PCR product size was 423 bp.

Amplification with the AP primers (Gustafson et al. 1992): Each 25 µl reaction contained 0.25 U of DNA polymerase, 2.5 µl of 10× PCR buffer, 2.5 mM $MgCl_2$, 8 pmol each of the amplification primers AP1 (5′-GGCTGATCTCTTCATCCTCACCC-3′) and AP2 (5′-CAGAGTGAAATCTACCAGCGGTGC-3′), and all 4 deoxynucleotide triphosphates at 0.2 mM each. Reaction mixes were held for 2 min at 94°C, then amplified for 30 cycles, with denaturation for 15 s at 94°C, annealing for 30 s at 57°C and elongation for 1 min 30 s at 72°C. A final extension was performed at 72°C for 3 min. The expected PCR product size was 421 bp.

Amplification with the MIY primers (Miyata et al. 1996): Each 25 µl reaction contained 0.6 U of DNA polymerase, 2.5 µl of 10× PCR buffer, 1.5 mM $MgCl_2$, 16 pmol each of the amplification primers MIY1 (5′-AGCCTCCACGCGCTCACAGC-3′) and MIY2 (5′-AAGAGGCCCCATAGTGTGGG-3′), and all 4 deoxynucleotide triphosphates at 0.2 mM each. Reaction mixes were held for 2 min at 94°C, then amplified for 35 cycles, with denaturation for 30 s at 94°C, and annealing and elongation for 1 min 30 s at 68°C. A final extension was performed at 68°C for 3 min. The expected PCR product size was 512 bp.

***In vitro* PCR specificity and sensitivity assays.** The specificity of the PCR assays was determined by screening genomic DNA prepared from non-*Aeromonas salmonicida* isolates (Table 2) with the AP, PAAS and MIY PCRs. The sensitivity (i.e. the proportion of true negative results; see discussion) of the AP, PAAS and MIY assays was determined by PCR screening of the AFDL collection of *A. salmonicida* isolates (n = 308). Each *A. salmonicida* genomic DNA sample was screened 4 times by each PCR.

Screening of *Aeromonas salmonicida* plasmids with the PAAS3 probe. Plasmids were extracted from selected *A. salmonicida* isolates using the BRESA-spin™ Plasmid Mini Kit (GeneWorks). The extracts

were denatured by boiling for 10 min, then blotted onto nylon membranes (Boehringer Mannheim) using the method of Reed & Mann (1985). The PAAS3 probe (5′-GCTAGCCAACTCTCTTTCCA-3′, O'Brien et al. 1994) was labelled with digoxygenin-11-dideoxyuridine triphosphate (ddUTP) (Boehringer Mannheim) and terminal deoxynucleotidyl transferase (Boehringer Mannheim). Pre-hybridisation was performed for 1 h at 10°C below the probe's theoretical maximum temperature (T_m) of 60°C. Hybridisation and detection of the digoxigenin-labelled oligonucleotide were performed by using alkaline phosphatase-labelled anti-digoxigenin Fab antibody fragments (Boehringer Mannheim) and the chemiluminescent alkaline phosphatase substrate CDP-Star™ (Boehringer Mannheim) according to the manufacturer's recommended procedure.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. ***Preparation of samples:*** *Aeromonas salmonicida* isolates were cultured on 5% SBA for 48 h at 25°C in air, harvested and washed 3 times in sterile phosphate buffered saline without Ca^{2+} and Mg^{2+} ions (PBSA, pH 7.4), and approximately 100 mg of bacteria were sedimented at 13 000 × *g* for 1 min. The pellet was resuspended in 1 ml SDS-glycerol buffer (10% glycerol, 2.3% SDS, 0.0625 M Tris/HCl pH 6.8, 5% mercaptoethanol) and incubated at 100°C for 10 min, and insoluble material was removed by centrifugation at 13 000 × *g* for 5 min. The supernatant was frozen at –20°C prior to electrophoresis.

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

Western blotting: Following electrophoresis, proteins were transferred to 0.22 µm nitrocellulose sheets (NitroPure, Micron Separations, #WP2HY00010) at 30 V for 16 h at 4°C using the buffer system of Towbin et al. (1979). The transferred bacterial proteins were probed with hyper-immune polyclonal antisera raised against *Aeromonas salmonicida* A-protein (supplied by Dr. Guri Eggset, Marin Bioteknologi i Tromsø, Norway) using methods described previously (Crane et al. 2000).

DNA sequencing. Products (typically 50 ng DNA) from the 16S PCRs were sequenced with the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) using the 1110f primer (5′-GCAACGAGCGCAACCC-3′; Dorsch & Stackebrandt 1992), purified using the QIAquick PCR Purification Kit (Qiagen), and run on an ABI model 377 automated sequencer (PE Applied Biosystems). The resultant sequences were compared against the GenBank database using the basic local alignment search tool (BLAST; Altschul et al. 1990) to determine their general taxonomic affiliation.

Preparation of bacterial inocula for seeded tissue studies. *Aeromonas salmonicida* isolate SFC 262 (biochemical profile shown in Table 3) was chosen for seeding experiments as it was positive by the AP, PAAS and MIY PCRs, and had been subject to minimal passage. An overnight culture grown on SBA at room temperature was used to prepare a cell suspension of 115 nephalometric turbidity units (NTUs) in 15 ml sterile PBSA as determined by the Hach nephalometer. The number of cells present in the suspension was calculated by either drop plate counts (Miles & Misra 1938) or direct counts using the WSI Counting Chamber (Weber Scientific International). Aliquots of dilutions of the suspension ranging from 10^8 colony-forming units (CFU) ml^{-1} to 10^4 CFU ml^{-1} were added to the tissue samples.

Preparation of fish tissues for seeded tissue studies. Mucus, gill, spleen, kidney and intestine samples were obtained from uninfected rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* for use in seeding experiments. Mucus was diluted 1:1 (w/v) with

Table 3. Summary of the biochemical profile of *Aeromonas salmonicida* isolate SFC 262. TSA: tryptic soy agar; SBA: sheep blood agar: +: positive; –: negative

Test	Result
Gram stain	–
Pigment production on TSA	+
Cytochrome oxidase	+
Motility	–
Growth on SBA at 37°C	–
Indole	–
Methyl red	+
Voges-Proskauer	–
Arginine dihydrolase	+
Nitrate reduction	+
Aesculin hydrolysis	+
Gas production from D-glucose	+
Acid from arabinose	+
Acid from galactose	+
Acid from maltose	+
Acid from mannitol	+
Acid from sucrose	–
Acid from trehalose	+

sterile PBSA, mixed thoroughly, and divided into 100 µl aliquots. Tissues were divided into 50 mg aliquots. All aliquots were stored at –20°C prior to use.

Extracting DNA from seeded and unseeded salmonid tissues. Unless otherwise stated, seeded tissue samples consisted of 50 µl of *Aeromonas salmonicida* cell suspension and 1 aliquot of frozen fish tissue (prepared as above) added to 200 µl of sterile distilled water in a 1.5 ml Eppendorf tube, then macerated with a Kontes Pellet Pestle® (Edwards Scientific), vortexed and processed as outlined below. Unseeded samples were processed in the same manner and contained fish tissue but no *A. salmonicida* cells. Extraction methods (boiling with or without Chelex-100 resin; BioRad® Biotechnology Grade, Sodium Form), Triton X-100 methods (Agersborg et al. 1997, Khan & Cerniglia 1997), enzymatic/chemical lysis method (Sambrook et al. 1989) and Puregene® Kit (Gentra Systems) were trialled. The method of choice, based on nucleic acid yield and purity, was the Puregene® Kit, which was used as per manufacturer's instructions, except that all reagent volumes were doubled and extracts were diluted 1:20 in sterile distilled water prior to PCR.

Calculation of the lower detection limit (LDL) of the PCRs based on purified DNA preparations. Genomic DNA was extracted from *Aeromonas salmonicida* using the Puregene® Kit and quantified using the GeneQuant II RNA/DNA calculator (Pharmacia Biotech). Serial dilutions of the DNA, ranging from 20 ng to 20 fg, were used as template directly in the AP, PAAS and MIY PCR assays. Alternatively, the *A. salmonicida* DNA was mixed with 1 to 2 µg aliquots of brown trout tissue DNA (extracted from 50 mg of either mucus, kidney, spleen, intestine or gill material using the Puregene® Kit), and then amplified in the AP, PAAS and MIY PCRs, in order to determine the effect of high levels of non-target DNA on the performance of the PCRs.

Calculation of the LDL of the PCRs based on addition of *Aeromonas salmonicida* whole cell preparations. Whole cell suspensions of *A. salmonicida* were prepared as above, and 50 µl aliquots of cells suspensions ranging from 10^7 to 10^3 CFU ml^{-1} were added to 50 mg lots of fish tissues. DNA was extracted using the Puregene® Kit and amplified using the AP, PAAS and MIY PCRs.

***Aeromonas salmonicida* overt infection in fish held at AFDL.** All fish infection experiments were conducted at 18°C. Brown trout *Salmo trutta* were anaesthetised with benzocaine (10 mg l^{-1}) and inoculated with 10^4 to 10^6 cells of *A. salmonicida* (SFC 262) in 100 µl of sterile saline by injection into the abdominal cavity. Fish were then transferred to separate 70 l aquaria and monitored daily for clinical signs of disease such as gross lesions, lethargy and abnormal swimming behaviour. Fish displaying any signs were euthanised by benzocaine overdose (100 mg l^{-1}). Uninfected control fish (n = 10), injected intraperitoneally (i.p.) with 100 µl sterile saline only, were maintained under the same conditions. Ten days after the initial exposure, surviving fish were euthanised by anaesthetic overdose and sampled.

Identification of *Aeromonas salmonicida* from experimentally infected fish tissue. Samples of mucus, gill, kidney, spleen and intestine from the brown trout were inoculated onto SBA plates and incubated for 48 h at 20°C. Colonies suspected to be *A. salmonicida* were either used directly as template in the PCR mix or subcultured and DNA extracted using the Puregene® Kit to provide a source of template for the AP, PAAS and MIY PCRs. Alternatively, the Puregene® Kit was used to directly extract DNA from 50 mg aliquots of fish tissue, which had been aseptically excised from the necropsied fish. This DNA was then used as template in the AP, PAAS and MIY PCR assays.

RESULTS

Biochemical characterisation of *Aeromonas salmonicida* isolates

A total of 243 *Aeromonas salmonicida* isolates from the AFDL reference collection were characterised using a panel of 18 phenotypic tests. Results from 14 of the biochemical tests were forwarded to Prof. Brian Austin (Department of Biological Sciences, Heriot-Watt University, Edinburgh) for computer-assisted cluster analysis. Results from this analysis grouped the isolates into 30 phenotypic clusters (data not shown), reflecting the broad phenotypic diversity of the species. Hence, on the basis of phenotypic testing alone it was not possible to accurately assign all the isolates to a recognised subspecies.

In vitro specificity and sensitivity of PAAS and AP PCR tests

The PAAS (Hiney et al. 1992) and AP (Gustafson et al. 1992) PCR tests did not produce any false positive reactions with the 27 non-target bacterial DNA extracts and, on this basis, were considered to be specific for their target, *Aeromonas salmonicida*.

The AP PCR correctly identified 288 of the 308 isolates as *Aeromonas salmonicida* and was therefore determined to have an *in vitro* sensitivity of 93.3%. The PAAS PCR correctly identified 285 of the 308 isolates as *A. salmonicida* and was therefore determined to have an *in vitro* sensitivity of 93%. When the results

of both tests were combined, 99.4 % of the isolates in the library were correctly identified as *A. salmonicida*. The 0.6 % false negative isolates were further examined in order to determine the reason(s) for these results.

As the primer binding sites for the PAAS PCR are plasmid borne, plasmid DNA was isolated from PAAS PCR-negative isolates (Fig. 1) and probed with the PAAS3 probe in order to determine whether the target primer site was present. The PAAS3 probe hybridised with a band, approximately 6 kb in size, in the plasmid profiles of the positive controls SFCs 190 and 308 (as indicated by the arrow in Fig. 1), indicating that the plasmid target site was present in these 2 isolates. Failure of the PAAS3 probe to hybridise with the remaining plasmid extracts indicated that the isolates SFC 20, 21, 22, 123, 164, 168, 170, 187, 188, 191, 192, 197, 198, 213, 222, 226, 229, 259, 480, 484, 491, 725 and 728 may lack the target plasmid.

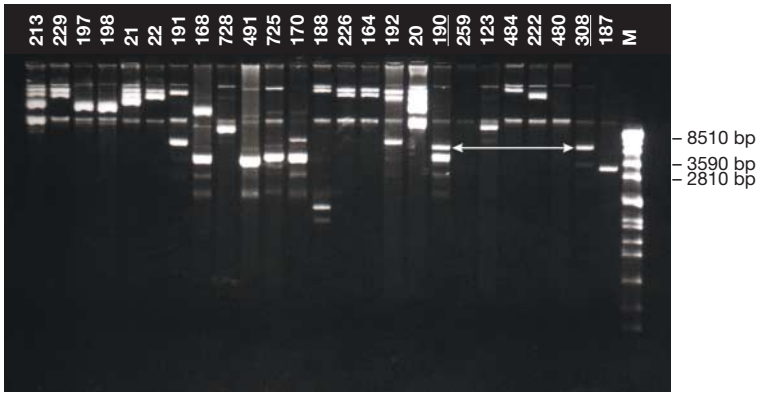

Fig. 1. *Aeromonas salmonicida*. Plasmids extracted from the previously PAAS PCR-negative *A. salmonicida* isolates using the BRESAspin™ Plasmid Mini Kit (GeneWorks). *A. salmonicida* SFC isolate numbers are marked above their corresponding plasmid profiles. The arrow indicates the plasmids of the 2 isolates (underlined) that hybridised with the PAAS3 probe. M: molecular weight marker SPP-1 DNA/*Eco*R1

Further studies using the western blotting technique with the A-protein-specific antiserum were undertaken in order to determine whether the *vap*A gene, which contains the AP PCR primer sites, was functional and being expressed. It was demonstrated that AP PCR-positive isolates did not necessarily express the A-protein, e.g. *Aeromonas salmonicida* American Type Culture Collection (ATCC) 14172 (SFC 36). It was also demonstrated that some AP PCR-negative isolates could continue to produce the A-protein, e.g. SFCs 308, 317, 481, 483 and 484 (Fig. 2).

In vitro sensitivity and specificity of the MIY PCR test

The MIY PCR correctly identified all isolates from the AFDL collection previously identified as *Aeromonas salmonicida* subsp. *salmonicida*, including all isolates submitted as 'typical' and presumed to be *A. salmonicida* subsp. *salmonicida*, and 85 of the 128 isolates that were submitted with no description but were found to be indole negative. Isolates that are negative for indole production are usually, but not always, *A. salmonicida* subsp. *salmonicida*. The test did not yield false positive results with any of the negative control extracts, any other *A. salmonicida* subspecies or any isolates from the collection that were considered to be 'atypical'.

16S rDNA sequencing

The 2 *Aeromonas salmonicida* isolates that were negative by all 3 PCR tests were subjected to partial 16S rDNA sequencing to confirm their identity. The 16S rRNA genes were amplified and partially sequenced using the 1100f primer (Dorsch & Stackebrandt 1992). The general taxonomic affiliation and likely source of the sequences, as determined using the BLAST tool available on ANGIS (Australian National Genomic Information Service), was *A. salmonicida*.

Extracting DNA from seeded and unseeded salmonid tissues

The enzymatic/chemical lysis method and the Puregene® Kit yielded better quality DNA and led to the best LDL compared to the boiling, Proteinase K, Triton X-100, Chelex or Chelex and Triton methods (Byers et

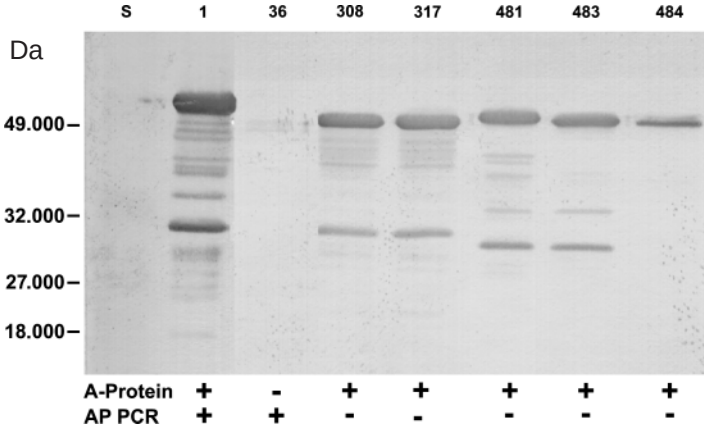

Fig. 2. *Aeromonas salmonicida*. Western blotting of protein profiles of selected isolates. Results with isolates 1, 36, 308, 317, 481, 483 and 484 demonstrate that there is no direct correlation between the presence of A-protein and the AP PCR result. Lane S contains the protein molecular weight standards

al. 2000). As the Puregene® Kit was simpler and faster to use, it became the method of choice for all future DNA extractions.

Determination of the LDL of the PCRs

The LDL of the PCRs were determined for DNA extracts and for whole cell seeded preparations. The addition of 1 µg of non-target fish tissue DNA to *Aeromonas salmonicida* DNA in the PCR mix led to a decrease in the LDL of the PCRs by about 1 order of magnitude (Table 4). When cocktails of low amounts of target DNA (<20 pg) and high levels of non-target DNA (1 to 2 µg) were tested with the PAAS PCR, some non-specific products were found to occur (example given in Fig. 3). However, these extraneous bands were of a different size from the desired product, and in these cases the target band could be excised from the gel and sequenced to confirm identity.

Culture-based identification of *Aeromonas salmonicida* in experimentally infected fish

Bacteria were readily isolated on SBA from the mucus, gills, intestine, kidney and spleen of 20 dead fish. Isolates were positive by the AP, PAAS and MIY PCRs (results not shown). Amplifying directly from the bacterial colony (i.e. aseptically transferring a small amount of isolate to the PCR mix itself) was successful 90% (n = 100) of the time. Extracting DNA from the isolate with the Puregene® Kit prior to PCR ensured a 100% success rate.

Direct PCR detection of *Aeromonas salmonicida* in experimentally infected fish

The AP and PAAS PCR assays successfully detected the presence of *A. salmonicida* in mucus, gill, muscle lesion, intestine, spleen and kidney samples obtained from each of 20 experimentally infected fish. The MIY PCR was not routinely used to screen the tissue extracts due to its poorer LDL.

In addition, it was found that using Platinum™ *Taq* (Life Technologies™), as opposed to *Taq* DNA polymerase (Promega Corporation), significantly improved the performance of all 3 PCR systems (example given in Fig. 4) and so became the DNA polymerase of choice.

Table 4. Lower detection limits (LDLs) of the *Aeromonas salmonicida* PCRs. CFU: colony-forming units

PCR	Range of LDL of PCRs		
	Target template only, per 50 µl PCR	Target template + 1 µg fish tissue DNA, per 50 µl PCR	CFU g^{-1} tissue
PAAS	2 pg to 200 fg	20 pg to 2 pg	10^4 to 10^3
AP	20 pg to 2 pg	200 pg to 20 pg	10^5 to 10^4
MIY	20 ng to 2 ng	200 ng to 20 ng	10^7 to 10^6

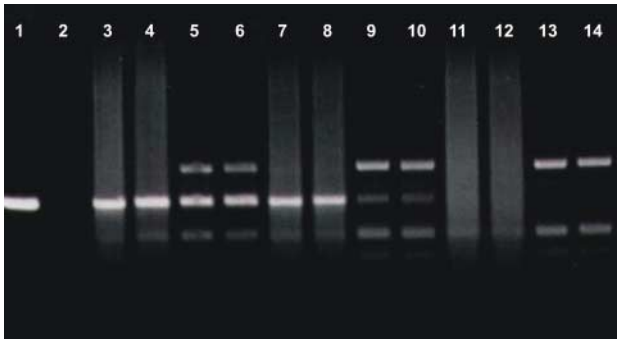

Fig. 3. PAAS PCR of kidney tissue seeded with whole cell preparations of *Aeromonas salmonicida*. Lane 1 is the positive PCR control (template was 200 pg *A. salmonicida* DNA and the PCR product is 423 bp) and lane 2 is the negative control (no *A. salmonicida* DNA added to the PCR). The templates used in lanes 3–4, 7–8 and 11–12 were extracts prepared from 25 mg brown trout kidney tissue seeded with 2×10^3, 2×10^2 and 2×10^1 *A. salmonicida* colony-forming units (CFU), respectively. The templates used in lanes 5–6, 9–10 and 13–14 were amplified from 1:20 dilutions of extracts prepared from 50 mg brown trout kidney tissue seeded with 2×10^3, 2×10^2 and 2×10^1 *A. salmonicida* CFU, respectively

DISCUSSION

An extremely broad phenotypic diversity was expressed by the *Aeromonas salmonicida* isolates, with 30 separate clusters being recognised (data not shown).

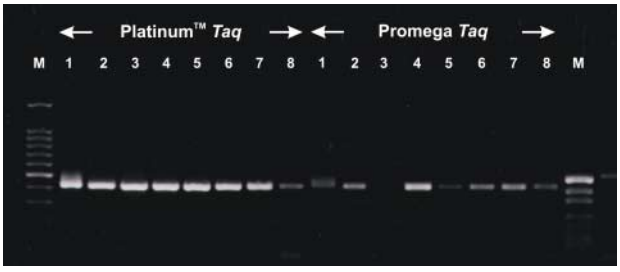

Fig. 4. PAAS PCR of extracts prepared from experimentally infected brown trout kidney tissue. Two different DNA polymerases (Platinum™ Taq and Promega Taq) were used with various DNA extraction methods: lanes 1–2, boiling method; lanes 3–4, Puregene® Kit; lanes 5–6, enzymatic/chemical lysis method; and lanes 7–8, Chelex/Triton method. Lanes M: molecular weight markers Promega 100 bp ladder (No. G210A) and pUC19/*Hpa*II, respectively

It was observed that the phenotypic tests employed in this study were often inadequate with regard to the accurate identification of *A. salmonicida* isolates to both species and subspecies levels. For example, 3 isolates were negative by the cytochrome oxidase test. Such results should have excluded these isolates from the species *A. salmonicida*, yet other tests including PCR and sequencing indicated that these isolates were indeed *A. salmonicida*. This and other, previous reports in the literature of such anomalies within this species (Chapman et al. 1991, McIntosh & Austin 1991, Teska et al. 1992, Wiklund et al. 1994, Pedersen et al. 1996) highlight the need for improved diagnostic techniques for the identification of *A. salmonicida*.

As diagnosis of *Aeromonas salmonicida* in Australia would have far-reaching consequences, it was vital that the candidate PCRs examined in this study be systematically evaluated. It is important to remember that non-culture-based methods such as PCR, enzyme-linked immunosorbent assay (ELISA) and western blotting provide us with an indication or sign that the target organism is present, as opposed to isolating the viable disease-causing agent itself. Hence, they are referred to as 'proxy' methods or measurements because they only indirectly indicate the presence of the target organism in a sample (Hiney 1997). Therefore, the application of such proxy measurements must be validated, i.e. the extent to which the technique can be legitimately used for a specified purpose must be thoroughly investigated (Hiney & Smith 1998).

The first stage of developing the diagnostic procedures involved using the candidate PCRs to screen the bacterial isolates in AFDL's reference collection. The key issues to be considered here are specificity, i.e. the proportion of true positive results, and sensitivity, i.e. the proportion of true negative results (Bernoth 1997). The putative *Aeromonas salmonicida*-specific PCR tests appeared to be 100% specific and did not cross-react with any of the non-target organisms tested. It was found that combining the results of both the AP and PAAS tests offered the best 'coverage' in terms of identifying the target organism, with only 0.6% of 308 *A. salmonicida* isolates being falsely recorded as negative. To date the MIY PCR appears to be 100% specific for *A. salmonicida* subsp. *salmonicida*, thus allowing us to distinguish between typical and atypical *A. salmonicida* isolates.

The failure of the PAAS primer set to identify 100% of the *Aeromonas salmonicida* isolates appeared to be related to the primer target site, which had previously been shown to occur on a 6.4 kb cryptic plasmid (Mooney et al. 1995) and was believed to be present in approximately 90% of *A. salmonicida* isolates (R. Powell pers. comm.). The plasmid profiles of those isolates found to be PAAS PCR-negative were probed with the PAAS3 nucleic acid probe (O'Brien et al. 1994), indicating that the primer target site may be absent in these 23 isolates. Extracts of 2 PAAS PCR-positive isolates were included and demonstrated to contain a fragment, approximately 6 kb in size (Fig. 1) that did hybridise with the PAAS probe.

The failure of the AP primer set to identify all of the *Aeromonas salmonicida* isolates is related to its primer target site, the *vap*A gene, which encodes a unique subunit protein (the 'A-protein') of the A-layer of *A. salmonicida* (Chu et al. 1991). As previously observed by Gustafson et al. (1992), it was demonstrated that AP PCR-positive isolates did not necessarily express the A-protein, e.g. SFC 36 (Fig. 2). In addition, it was demonstrated that some of the AP PCR-negative isolates were still able to express A-protein, e.g. SFC 308 (Fig. 2), which may be due to a mutation within the priming site, but still leaves the gene functional.

Once the PCR tests had been validated using a large number of purified *Aeromonas salmonicida* DNA preparations, seeded tissue studies were undertaken. Most available data regarding the trialling of PCRs to detect infectious material involves the use of seeded tissue, i.e. healthy tissue that is 'spiked' with a known pathogen. It should be remembered that there are numerous fundamental differences between infected tissues that harbour a pathogen and healthy tissue seeded with a laboratory-grown bacterium. For example, the expressed phenotype of the bacterium may differ (Fernandez et al. 1995, Garduno & Kay 1995), the physical and chemical accessibility of the pathogen may be quite different, and additional PCR inhibitors could be present in the infected tissue. However, bearing these constraints in mind, experiments involving seeded tissue proved to be a useful starting point permitting optimisation of experimental diagnostic procedures and the empirical determination of the LDLs of the PCRs.

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

monicida is exotic to Australia, sequencing the PCR product would be part of the diagnostic procedure to confirm the identity of the product.

The PAAS PCR had the lowest detection limit with regard to purified target template only, being in the range 2 pg to 200 fg per PCR. Hiney et al. (1992) reported a PAAS detection limit of 10 fg DNA, but this was achieved using a $MgCl_2$ concentration of 3.5 mM (compared to the more stringent 2.5 mM $MgCl_2$ concentration used in this study in order to maximise the specificity of the PCR) and a radioactively labelled probe to detect the PCR products. O'Brien et al. (1994) also reported a LDL of 200 genome equivalents (GE) g^{-1} sample using the PAAS PCR, which is equivalent to 1 pg target DNA g^{-1} sample based on the assumption that 1 GE is approximately 5 fg (Gustafson et al. 1992). It must be realised, however, that the study of O'Brien et al. (1994) involved either filtered effluent or faecal matter, both of which may differ in the level of inhibition they have on the PCR compared to fish tissue. More important, their measurement of the LDL was based on a comparison of PCR yield with that obtained when using pure DNA as a template. Given the non-linear kinetics of PCR, such a comparison could be inaccurate and could account for the differences between the reported LDLs and the limits determined in this study. Mooney et al. (1995), using a nested PAAS PCR, reported a LDL of 100 GE $fish^{-1}$. However, as Bernoth (1997) points out, the 100 GE limit was in fact per 10 to 100 µl of blood sample, and so actually equates to 10^3 to 10^4 GE ml^{-1} blood, which is equivalent to the level determined in this study. It is therefore considered that the PAAS PCR LDLs calculated in this study are a realistic measure of the performance of the PCR, as applied to seeded tissue samples.

Gustafson et al. (1992) had reported a LDL of 10^4 CFU g^{-1} when the AP PCR was applied to fish tissue samples seeded with known amounts of *Aeromonas salmonicida*, and our results fall within this limit. In fact, the LDLs of the AP and PAAS PCRs are approaching the theoretical limit of direct PCR detection, based on the figure of 2×10^3 cells g^{-1} fish tissue as calculated by Carson (1998). The only means of improving upon this figure is to concentrate the target DNA prior to PCR, perhaps via a cultural pre-enrichment step (Gustafson et al. 1992). Unfortunately, the MIY PCR was unable to reach the detection limits of the AP and PAAS PCRs.

It is important to note that these LDL values cannot be extrapolated to determine the performance of the PCRs in the case of infected tissues. Therefore, the PCR tests were further evaluated using tissues obtained from experimentally infected fish. The use of i.p. injection of *Aeromonas salmonicida* to generate experimental infection in fish is a highly reproducible procedure, but it does not mimic the process of natural infection. However, at least this procedure provided the target pathogen the opportunity to reproduce within the host, possibly allowing alterations of phenotype to occur. Thus, infection of fish provided a 'non-sterile incurred matrix' (Hiney 1997) in which the bacterium was embedded, as opposed to simply mixing healthy fish tissue and a laboratory-grown strain of *A. salmonicida*.

All 3 PCR tests successfully identified bacteria isolated on SBA from the mucus, gills, gut, kidney and spleen of the experimentally infected brown trout. Both the AP and PAAS PCRs yielded positive results when directly applied to overtly infected mucus, gill, intestine, muscle lesion, spleen and kidney samples. The MIY PCR, however, was less sensitive when used to screen tissue samples directly, and this PCR would require some form of pre-enrichment step to improve its performance.

Overall, the AP, PAAS and MIY PCRs appear to have a high level of specificity and sensitivity with regard to identifying pure bacterial cultures and have been incorporated into the suite of diagnostic tests used by this laboratory for the rapid identification and differentiation of isolates of *Aeromonas salmonicida*. Based on seeded tissue studies and experimental infection trials, the AP and PAAS PCRs were also demonstrated to have the potential to be used in the direct screening of fish tissue samples. However, the performance of these PCRs will still have to be validated using naturally infected fish if they are to be used routinely in the diagnosis of both overt and covert *A. salmonicida* infections.

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