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Multiplex PCR assay for detection of four major bacterial pathogens causing rainbow trout disease

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ABSTRACT: A multiplex PCR (mPCR) method was designed for the simultaneous detection of 4 major fish pathogens, *Flavobacterium psychrophilum*, *Lactococcus garvieae*, *Pseudomonas aeruginosa*, and *P. putida.* Each of the 4 pairs of oligonucleotide primers exclusively amplified the 16S rDNA gene of their targeted microorganism. The average detection limits for each organism amplified by mPCR were 2 colony-forming units (CFU) of *F. psychrophilum,* 3 CFU of *L. garvieae,* 3 CFU of *P. aeruginosa*, and 5 CFU of *P. putida* in mixed cultures. Multiplex PCR did not produce any nonspecific amplification products when tested against 28 related species of bacteria. High amounts of DNA from 1 bacterial species had a significant effect on the amplification sensitivity of the other bacterial species when these were present in lower concentrations in the multiplex reaction. The mPCR assay proved useful for the detection of the bacteria in naturally infected fish. The assay is a sensitive, specific, and reproducible diagnostic tool for the simultaneous detection of 4 pathogenic bacteria that cause disease in fish and offers a potentially useful alternative to the conventional culture-based method.

KEY WORDS: Target and non-target DNA interactions · Bacterial coldwater disease · Streptococcosis · *Pseudomonas* spp. infection · Multiplex PCR · Sensitivity test · Specificity test

INTRODUCTION

Major fish diseases of rainbow trout *Oncorhynchus mykiss* include bacterial coldwater diseases, streptococcosis, *Pseudomonas* infection, and *P. putida* infection caused by *Flavobacterium psychrophilum*, *Lactococcus garvieae*, *P. aeruginosa*, and *P. putida*, respectively. These diseases are common worldwide and result in considerable economic losses in the fish farming industry (Plumb 1999, Woo & Bruno 1999, Altinok et al. 2006, Hossain et al. 2006, Austin & Austin 2007). Disease caused by *L. garvieae, P. aeruginosa*, and *P. putida* in particular are very important because these pathogens can infect animals other than fish, including humans (Spanoghe 1984, Elliott et al. 1991, Hudson et al. 1993, Bouallegue et al. 2004, Evans et al. 2006). *F. psychrophilum* infects only fish.

Traditionally the diagnosis of a disease is obtained by culturing bacteria on agar plates followed by phenotypic and serological characterization of the pathogen, or by histological examination (Bernardet et al. 1990, Pazos et al. 1996). Biochemical tests, DNA homology, and protease variability techniques have also been used (Pyle & Shotts 1980, Bertolini & Rohovec 1992, Chen et al. 1995), but these techniques have some disadvantages, such as the need for initial isolation of the pathogen and insufficient sensitivity to detect low levels of pathogen.

Molecular techniques such as polymerase chain reaction (PCR) can be used to solve the above issues and increase sensitivity and specificity of pathogen detection. Singleplex PCR assays have been developed for detection and identification of fish pathogens. However, a large number of individual PCR reactions would be necessary if single primer sets were used to screen a large number of clinical samples, resulting in a relatively costly and time-consuming process. The simultaneous detection of several pathogens with a

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Table 1. Cultures of *Flavobacterium psychrophilum*, *Lactococcus garvieae, Pseudomonas aeruginosa*, and *P. putida* strains used in this study, and additional taxonomically and/or ecologically related bacterial species used in cross-reactivity testing of the multiplex PCR assay. ATCC: American Type Culture Collection, Rockville, MD; CECT: Coleccion Espanola de Cultivos Tipo, Universidad de Valencia, Spain; AL, AU, CA, GA, and SC strains provided by K. Hayden, Auburn University, Department of Fisheries and Allied Aquaculture, Auburn, AL; DSMZ, G27P3, M300GFFa1, PP60-1, 137/4/98, and DSMZ 20576 strains provided by A. Manfrin, Istituto Zooprofilattico Sperimentale delle Venezie, Adria, Italy; FPF strains provided by T. Wiklund, Laboratory of Aquatic Pathobiology, Abo Akademi University, Finland. The remaining strains were isolated in the Faculty of Marine Sciences, Trabzon, Turkey

Species	Strain(s)
Flavobacterium psychrophilum	ATCC 49418, FPF
Lactococcus garvieae	ATCC 49156, G27P3, M300GFFa1, PP60-1
Pseudomonas aeruginosa	ATCC 27853
P. putida	NCIB 8251, TSUR0501
Aeromonas hydrophila	ATCC 43874, AU9833, AU9738, AU0606
A. salmonicida salmonicida	ATCC 49385, GA97030, TN9716, RY0701
A. sobria	AL94232, AL9425
A. veroni	AL0548
Bacillus cereus	ATCC 11778
Edwarsiella tarda	AU0338, A19938
E. ictaluri	ATCC 33202, AL9549, AU9828, AU9738
Enterobacter faecalis	ATCC 2942
Escherichia coli	NCTC 12900
F. columnare	NCIMB 2248, CA0402, SC0406, AL0435
F. branchiophilum	ATCC 35035
Hafnia alvei	TSUR0702
Listeria monocytogenes	ATCC 7644
Plesiomonas shigelloides	AL9354, AL98051
Pseudomonas anguilliseptica	137/4/98
P. dilafialdi	RB0704
P. flourescens	AU9233, AU9738, AU9833
P. luteola	TSUC0704
Renibacterium salmoninarum	TS0601
Salmonella enterica	ATCC 14028
Streptococcus aureus	ATCC 25923
S. iniae	DSMZ 20576
Vibrio fluvialis	CECT 4217
V. alginolyticus	CECT 52
V. parahaemolyticus	CECT 511
V. mimicus	CECT 4218
Yersinia ruckeri	ATCC 29473, GA97016, ESU0701, EL0701
Y. pestis	TA0707

multiplex PCR (mPCR) approach would be relatively rapid and cost effective. Although mPCR has been widely applied to the detection of multiple viruses and bacteria in clinical specimens, it is not a common tool for the detection of fish pathogens (Brasher et al. 1998, Osorio et al. 2000, del Cerro et al. 2002, Mata et al. 2004, Altinok et al. 2008). In this investigation, an mPCR assay was developed for the simultaneous detection of *Flavobacterium psychrophilum, Lactococcus garvieae, Pseudomonas aeruginosa,* and *P. putida* from pure cultures, spiked samples, and diseased/naturally infected fish.

MATERIALS AND METHODS

Sources of fish. Rainbow trout *Oncorhynchus mykiss* from 32 farms in northeast Turkey were surveyed for *Flavobacterium psychrophilum, Lactococcus garvieae*, *Pseudomonas aeruginosa*, and *P. putida* between 2006 and 2009. Fish were sampled and examined in each season from all farms. The samples were usually comprised of 6 fish farm^{-1} season^{-1} (Dalsgaard & Madsen 2000, Altinok et al. 2008). Diseased fish were preferentially sampled if present; otherwise, asymptomatic fish were sampled.

Bacterial strains and culture conditions. The mPCR assay was evaluated using the following strains: *Flavobacterium psychrophilum* (ATCC 49418, FPF), *Lactococcus garvieae* (ATCC 49156, G27P3, M300GFFa1, PP60-1, TS0901), *Pseudomonas aeruginosa* (ATCC 27853), and *P. putida* (NCIB 8251, TSUR0501, TM0905). Strains of other species taxonomically and/or ecologically related were tested as negative controls (Table 1).

Lactococcus garvieae, *Pseudomonas aeruginosa*, and *P. putida* strains were grown in tryptic soy broth (TSB) and were incubated at 25°C for 2 d. *Flavobacterium psychrophilum* was grown in modified Anacker and Ordal broth (MAOB) and incubated at 18°C for 4 d. All of the cultures were incubated with shaking at 250 rpm in an orbital incubator.

Bacterial examination of rainbow trout. A total of 780 fish ranging between 4 and 300 g were transported to the laboratory on ice and dissected for subsequent bacteriological examination. All sampled fish were examined externally and internally. Samples from external lesions, if present, were streaked on modified Anacker and Ordal (MAO) agar (Toranzo & Barja 1993) and tryptic soy agar (TSA). The body surface of the fish was then swabbed using 70% ethyl alcohol to prevent contamination of samples taken from internal organs by normal external bacterial flora. Liver, trunk kidney, and spleen were aseptically streaked on TSA and MAO agar and incubated at 25°C for 2 d or at 18°C for 4 d, respectively. Bacteria isolated from fish were

subcultured on the same medium to check purity of the isolate. Pure cultured colonies were biochemically characterized with API Strips (Biomerieux) and the following biochemical tests: Gram staining, cytochrome oxidase, oxidation/fermentation, catalase, hemolysis on sheep blood agar, hippurate hydrolysis, pyrronidonyl arylamidase, leucine aminopeptidase, β-galactosidase, β-glucosidase, β-glucuronidase, β-mannosidase, alkaline phosphatase, N-acetyl-β-glucosaminidase, alanine-phenylanaline-proline arylamidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophane deaminase, indole production, Voges-Proskauer, and gelatinase, fermentation of glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, amygdalin, melibiose, lactose, methyl red, and arabinose tests. Isolates were identified by standard bacterial taxonomy procedures (Cowan 1974, Frerichs 1984, Krieg & Holt 1984, Lennette et al. 1985, Holt et al. 1994, Austin & Austin 2007). All isolates were stored in a broth culture supplemented with 15 to 20% glycerol at –70°C.

DNA extraction from fish tissue. For DNA extraction, from the same fish, a total weight of 0.5 g of a liver, spleen, and trunk kidney mixture was mixed with an equal volume of lysis buffer (200 mM NaCl, 10 mM Tris-HCl at pH 8.0, 1% sodium dodecyl sulfate, and 50 mM EDTA) and 5 µl of Proteinase K solution (20 mg ml^{-1}) in TE buffer (10 mM Tris-HCl and 0.1 mM EDTA at pH 8.0). If external lesions were present, 0.5 g of gill, skin, and fin tissue were also taken for DNA extraction. The lysis buffer and TE buffer were prepared with DNase- and RNase-free ultra pure autoclaved water. Samples were incubated at 60°C for 16 h. After extraction of the DNA with a mixture of phenol-chloroform-isoamyl alcohol (50:48:2; Altinok et al. 2001), the DNA was precipitated at –20°C for 2 h by adding 0.6 ml of ice-cold 100% ethanol, then centrifuged at 21 000 × *g* for 20 min at 4°C and the ethanol was decanted. The DNA was centrifuged following addition of 70% ethanol at 21 000 × *g* for 20 min at 4°C and dried overnight. DNA was suspended in 100 µl TE buffer, quantified spectrophotometrically, diluted to 100 ng $µl^{-1}$, and stored at 4°C until the PCR (Altinok et al. 2008).

DNA extraction from bacterial cultures. Bacterial cultures were centrifuged at 9000 × *g* for 5 min. Supernatant was discarded and the pellet was dissolved in 765 µl TE buffer, 50 µl of 10% SDS, and 5 µl Proteinase K (60 µg). After 1 h of incubation at 37°C, 100 µl of 5 M NaCl were added and mixed thoroughly. Then 80 µl cetyltrimethylammonium bromide were mixed with the sample, which was incubated at 65°C for 10 min (Altinok et al. 2001). The DNA was then purified as described above for tissue samples, resuspended in 100 µl TE buffer, quantified spectrophotometrically, and stored at 4°C until required.

PCR conditions. A number of primer pairs were designed from the 16S rDNA of each target pathogen using Jellyfish software (Field Scientific). The primer sequences were used to search public databases using BLAST (http://blast.ncbi.nlm.nih.gov) for homologous matches. None were found. Primer pairs were tested (data not shown) against single pure bacterial cultures of all bacterial species listed in Table 1. For each bacterium, 1 primer set was selected that did not cross-react with other bacterial DNA and did not form primer dimers. Primers for *Flavobacterium psychrophilum* (GenBank accession number D12670; FpR: 5′-CGA TCC TAC TTG CGT AG-3′; FpF: 5′- CGG CCA GAT AAG TCA GTG GT-3′), *Lactococcus garvieae* (GenBank accession number FJ915634; LgF: 5′-CCA ACT TCC GTG GTG TGA CG-3′; LgR 5′-AGT GGC TCA ACC ATT GTG TGC-3′), and *Pseudomonas putida* (GenBank accession number GU248219; PpF: 5′-CCA AAA CTG GCA AGC TAG AGT AC-3′; PpR: 5′-CAT CTC TGG AAA GTT CTC TGC-3′) were selected. Primers used in the detection of *P. aeruginosa* (PaF: 5′-GGG GGA TCT TCG GAC CTC A-3′; PaSR: 5′-TCC TTA GAG TGC CCA CCC G-3′) were described by Spilker et al. (2004).

Different annealing temperatures and $MgCl_2$ concentrations were tested to obtain the optimal specificity and sensitivity of the mPCR assay. Intensity of the amplicons for each target DNA, as well as the absence of unspecific bands, was considered in selecting the optimal mPCR conditions. The best results were obtained with an annealing temperature of 60°C and 3 mM $MgCl_2$. Each 35 µl mPCR reaction mixture (prepared on ice) contained 100 ng of the sample DNA, 17.5 µl of 2× Multiplex PCR mixture containing 3 mM $MgCl_2$ (QIAGEN Multiplex PCR kit, Qiagen Molecular Biochemicals), 3.5 µl of 5× Q solution (QIAGEN Multiplex PCR kit), and 100 ng of each primer (Iontek).

Thermal cycling was performed with a Thermo Hybaid thermal cycler (Thermo Electron) under the following conditions: an initial denaturation cycle at 95°C for 15 min, followed by 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 90 s, and extension at 72°C for 60 s), and a final 10 min extension period at 72°C. Controls consisted of the PCR mixture containing (1) no DNA template (reagent control), (2) DNA from negative control fish (negative control), or (3) DNA from *Flavobacterium psychrophilum*, *Lactococcus garvieae*, *Pseudomonas aeruginosa*, and *P. putida* cultures (positive control). After the PCR, products were transferred to a 2.0% agarose gel, electrophoresed, and DNA was visualized by ethidium bromide staining.

Sensitivity and specificity of the mPCR assay. The sensitivity and specificity of the mPCR assay (combined primer sets) was tested with pure bacterial cultures and host tissue/DNA spiked with bacterial cells. Bacterial suspensions of each target strain were prepared from log phase cultures in TSB or MAOB (APHA et al. 1998). To test sensitivity of the mPCR assay with pure cultures of the target species, the bacterial suspensions were serially diluted, mixed in equal volumes for each dilution, i.e. 1 ml of each bacterium dilution was mixed in 5 ml centrifuge tubes, the cells pelleted, and then the DNA extracted from each dilution mixture as described previously, and resuspended in 100 µl TE buffer. If extracted DNA concentrations were more than 100 ng μl^{-1}, DNA was diluted to reach 100 ng μl^{-1}, and 1 µl was used per reaction. If extracted DNA concentrations were less than 100 ng μl^{-1}, 1 µl of DNA extract was directly used per reaction without dilution. All mPCRs assessing limits of detection were performed in triplicate.

To test the sensitivity of the mPCR assay in the presence of fish tissue, spiked samples were used. One gram rainbow trout liver was homogenized with lysis buffer in a 1:10 ratio and seeded with serial dilutions of an equal mixture of pure cultures of the 4 bacterial species (del Cerro et al. 2002, Altinok et al. 2008). DNA was extracted as described previously for tissue samples.

To enumerate the target bacterial strains used for DNA extraction, 0.1 ml aliquots of the serially diluted bacteria used in the DNA extractions were spread onto TSA for *Lactococcus garvieae*, *Pseudomonas aeruginosa*, and *P. putida* and MAO agar for *Flavobacterium psychrophilum.* Bacteria were incubated at 25°C for 2 d, except *F. psychrophilum*, which was incubated at 18°C for 4 d.

To test specificity of the mPCR primers, DNA from target or non-target species (Table 1), extracted as described previously and diluted to 100 ng μl^{-1}, was tested individually in PCR reactions containing either single primer-pairs or all primer-pairs mixed together in equal concentrations (100 ng for each primer), i.e. in mPCR format.

To check specificity of the mPCR assay (combined primer sets) primers for target and non-target bacterial species in association with host DNA, 500 ng host DNA were spiked with approximately 105 colony-forming units (CFU) of each target species and 105 CFU of a non-target species from Table 1. The DNA was then purified as described above for tissue samples and diluted to 100 ng μl^{-1}. This was repeated with each non-target species in Table 1, and was again repeated using 100 mg of homogenized fish liver tissue in place of 500 ng extracted host DNA. DNA concentration was measured and diluted to 100 ng μl^{-1}. Prior to these experiments, host tissue was checked for the presence of targeted species using PCR.

To test the effect of a high amount of 1 pathogen to low amounts of the other 3 species in the mPCR reaction, 100 mg of rainbow trout liver were homogenized with lysis buffer in a 1:10 ratio and mixed with different amounts of *Flavobacterium psychrophilum*, *Lactococcus garvieae*, *Pseudomonas aeruginosa*, and *P. putida* (see Tables 2 & 3). DNA was extracted from the mixture as described for tissue samples above. All mPCR reactions assessing limits of detection were performed in triplicate.

Sequencing method. To verify that their respective specific primer pairs amplified *Flavobacterium psychrophilum*, *Lactococcus garvieae*, *Pseudomonas aeruginosa*, and *P. putida* DNA, the PCR products generated using the mPCR assay were purified with a NucleoSpin PCR purification kit (Macherey–Nagel) and directly sequenced with an ABI PRISM 310 genetic analyzer (Applied Biosystems). Both strands of the PCR templates were sequenced. The nucleotide sequences were analyzed and aligned with BioEdit Sequence Alignment Editor (North Carolina State University, Raleigh, NC). The results of the sequencing were used for homology searches following the methods of Altschul et al. (1990).

RESULTS

Each of the 4 pairs of oligonucleotide primers exclusively amplified the 16S rDNA of their specific targeted microorganism. An amplification product of the expected size was observed from pure cultures of 2 strains of *Flavobacterium psychrophilum* (685 bp), 4 strains of *Lactococcus garvieae* (857 bp), 2 strains of *Pseudomonas putida* (380 bp), and 1 strain of *P. aeruginosa* (956 bp; Fig. 1). The specificity of the assay was further verified by performing the mPCR with DNA from cultures of taxonomically and/or ecologically related non-target bacterial species. In all cases, no PCR products were obtained, demonstrating that the mPCR assay did not cross-react with these species.

The specificity of the primers, both singly and as a multiplex, was also tested against all target and non-target bacterial species in the presence of host DNA. No cross reactivity was observed.

The average detection limits in the mPCR for each species were 2 CFU for *Flavobacterium psychrophilum,* 3 CFU for *Lactococcus garvieae,* 3 CFU for *Pseudomonas aeruginosa*, and 5 CFU for *P. putida* when pure mixed species cultures were used. The detection limits for the spiked samples were 50 CFU g^{-1} of tissue for *F. psychrophilum*, 60 CFU for *L. garvieae*, 30 CFU for *P. aeruginosa*, and 45 CFU for *P. putida* (Fig. 2).

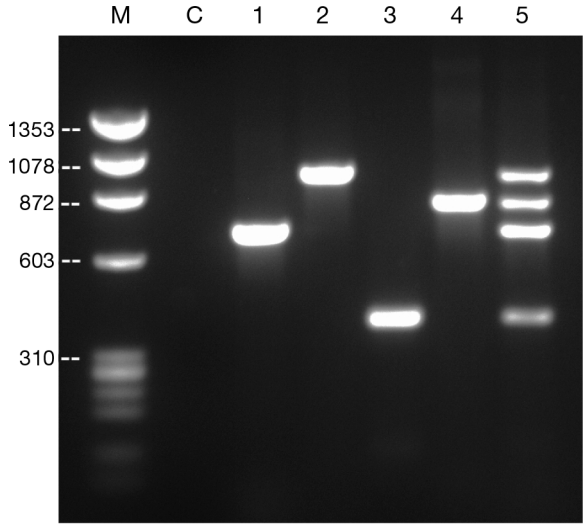

Fig. 1. Specificity of the mPCR assay developed for the detection of *Flavobacterium psychrophilum*, *Lactococcus garvieae, Pseudomonas aeruginosa*, and *P. putida*. Lane M: Phi X174 DNA/*Hae*III molecular size marker; Lane C: negative control containing PCR reagents alone; Lane 1: *F. psychrophilum* (685 bp) alone; Lane 2: *P. aeruginosa* (956 bp) alone; Lane 3: *P. putida* (380 bp) alone; Lane 4: *L. garvieae* (857 bp) alone; Lane 5: mPCR with the 4 pathogens together

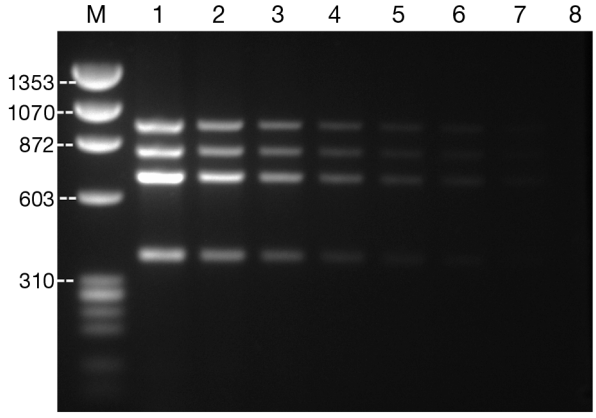

Fig. 2. Sensitivity of the detection of *Flavobacterium psychrophilum*, *Lactococcus garvieae, Pseudomonas aeruginosa*, and *P. putida* by mPCR assay. PCR product sizes obtained for *F. psychrophilum*, *L. garvieae, P. aeruginosa*, and *P. putida* were 685, 857, 956, and 380 bp, respectively. PCR amplification on tissue samples seeded with serially diluted cultures of the 4 pathogens. Lane M: Phi X174 DNA/*Hae*III molecular size marker; Lanes 1 to 8: 10^{-1} to 10^{-8} dilutions, respectively. Undiluted medium contained 50×10^7, 60×10^7, 30×10^7, and 45×10^7 CFU g^{-1} tissue of *F. psychrophilum*, *L. garvieae, P. aeruginosa*, and *P. putida*, respectively. The 10^{-7} dilution was the most dilute sample that was PCR-positive for the 4 bacteria

High amounts of DNA from 1 bacterial species had a significant effect on the amplification sensitivity of the other bacterial species when these were present in lower concentrations in the multiplex reaction. The detection limits of the mPCR for *Flavobacterium psychrophilum* and *Lactococcus garvieae* were higher when high amounts of amplification of the other target bacterial DNA occurred (Table 2). All bacterial species could be detected at a lower concentration limit of 12 CFU per 100 mg of tissue irrespective of the concentration of the other target species in the mPCR reaction (1000 CFU being the highest concentration used; Table 2). Amplified band intensity of the multiplex PCR products was the same when the reaction contained the same amounts of targeted species (Table 3). When the concentration of any of the target species was ≥25 CFU per 100 mg of tissue, a higher amount of 1 pathogen DNA (1000 CFU being the highest concentration used) did not affect the observed intensity of the products on an agarose gel (Table 3).

Table 2. Amplified band intensity in the multiplex PCR containing a high amount of 1 pathogen (1000 colony-forming units, CFU) and low amounts of the other 3 pathogens (6 CFU or 12 CFU). Band intensity was estimated by Kodak Gel logic 200 software (version 4.1). CFU of bacteria were estimated based on the McFarland standard. For band intensities, faint: intensity is <10 ng; +: 11–99 ng; +++: 200–299 ng; ++++: >300 ng

Primer set specificity	Pathogen at 1000 CFU			
	F. psychrophilum	*L. garvieae*	*P. aeruginosa*	*P. putida*
Remaining pathogens at 6 CFU				
Flavobacterium psychrophilum	++++	None	None	None
Lactococcus garvieae	None	++++	None	None
Pseudomonas aeruginosa	Faint	Faint	++++	Faint
P. putida	None	Faint	Faint	++++
Remaining pathogens at 12 CFU				
Flavobacterium psychrophilum	+++	+	+	+
Lactococcus garvieae	Faint	+++	+	+
Pseudomonas aeruginosa	+	+	+++	+
P. putida	+	+	+	++++

Of 780 rainbow trout examined from 32 hatcheries, only 25 fish showed clinical disease symptoms. *F. psychrophilum*, *L. garvieae*, and *P. putida* were respectively isolated from 1, 7, and 2 fish that had clinical signs of disease (Table 4). None of the 4 targeted species were found using culture or mPCR in the 15 remaining fish displaying clinical signs, although *Yersinia ruckeri*, *Aeromonas hydrophila*, *A. salmonicida salmonicida*, and *P. fluorescens* were subsequently isolated in cultures.

Table 3. Amplified band intensity of multiplex PCR containing various proportions of colony-forming units (CFU) each of *Flavobacterium psychrophilum*, *Lactococcus garvieae*, *Pseudomonas aeruginosa*, and *P. putida*. Band intensity was estimated by Kodak Gel logic 200 software (version 4.1). For band intensities, ++: 100–199 ng; +++: 200–299 ng

Bacteria	CFU	Band intensity	CFU	Band intensity
F. psychrophilum	50	+++	1000	+++
L. garvieae	50	+++	100	++
P. aeruginosa	50	+++	50	++
P. putida	50	+++	25	++

Flavobacterium psychrophilum, *Lactococcus garvieae*, *Pseudomonas aeruginosa*, and *P. putida* were isolated from 2, 20, 3, and 2 fish, respectively, using culture methods. Seventy-nine fish were positive for the presence of target species using the mPCR. Mixed infections of 2 and 3 bacterial species were found in 24 and 9 fish, respectively, resulting in identification of *F. psychrophilum* from 8 fish, *L. garvieae* from 45 fish, *P. aeruginosa* from 15 fish, and *P. putida* from 11 fish (Table 4). No individual fish was positive by mPCR for all 4 target bacterial species. The culture method did not pick up mixed infections for the target bacterial species. All culture-positive fish were also mPCR-positive.

Sequences of the amplified DNA products, of different expected sizes, from 3 fish matched the sequence of the 4 target bacterial species.

Some of the bacterial infections showed seasonality. While *Lactococcus garvieae* and *Pseudomonas aeruginosa* infections were common throughout the year, *P. putida* and *Flavobacterium psychrophilum* infection were common in spring and in winter, respectively.

DISCUSSION

Specific primers for *Flavobacterium psychrophilum*, *Lactococcus garvieae,* and *Pseudomonas putida* were developed and their sensitivity and specificity, in a multiplex PCR format, were tested in the present study. The assay appeared to be very sensitive, with a detection limit of as low as 60 CFU g^{-1} of tissue for all 4 bacteria. As the data were based on colony counts, it seems reasonable to expect that the number of colonies would be an underestimation of the total number of bacterial cells present (Barbosa et al. 1995); however, the mPCR detection limits are similar to PCR detection limits previously reported in the literature for other bacterial species (Brown et al. 1994, Nielsen et al. 2001, del Cerro et al. 2002). The primers described here proved to be specific under the conditions assayed both in relation to the 4 target species and the other closely related bacterial species tested, with only the specific target species showing amplification in the multiplex reaction.

PCR assays may vary in amplification efficiency, due to primer length, nucleotide content, and secondary structure. It is therefore important to see whether 1 PCR assay within the multiplex against a target in high concentration might outcompete 1 or more of the other assays to such an extent that the detection limits for the targets at lower concentrations are affected. In the present study, higher concentrations of DNA from 1 pathogen had a noticeable effect on amplification of other bacteria in the multiplex reaction when these were present at very low levels. However, bacterial concentrations of 12 CFU per 100 mg of tissue allowed detection of all target species by the mPCR in the presence of the higher competing DNA concentrations tested.

Healthy-looking fish without clinical signs or lesions can carry pathogens that create serious risks for the spread of contagious diseases in fish populations. Disease becomes evident only when stressful conditions arise, as can occur under intensive aquaculture conditions, and a significant proportion of the stock may become infected and die as a result. Therefore, detection of pathogens in carrier fish is essential for effective disease control. Prevalence of disease may change depending on the time of year and water temperature (Plumb 1999), making it difficult to identify infected fish in a population at certain times. In addition, detection of bacteria using culture methods may prove difficult in asymptomatic fish due

Table 4. Comparison of culture and mPCR methods

Bacterial species detected	No. of fish with clinical signs	No. of positive fish		No. of fish with mixed infections	
		Culture	mPCR	2 bacterial species	3 bacterial species
Flavobacterium psychrophilum	1	2	8	2	2
Lactococcus garvieae	7	20	45	13	3
Pseudomonas aeruginosa	0	3	15	5	2
P. putida	2	2	11	4	2

to low pathogen levels. From the data presented here, we conclude that the multiplex PCR assay is more sensitive than bacteriological culturing. Twenty-seven fish were positive for the target species by culture, while 79 fish were positive by mPCR, with mPCR detecting target pathogens from higher numbers of asymptomatic fish.

In addition, compared to standard bacteriological assays, the mPCR assay is much more rapid to perform. Therefore, this assay represents an important diagnostic tool to detect fish carrying the common pathogens *Flavobacterium psychrophilum*, *Lactococcus garvieae, Pseudomonas aeruginosa*, and *P. putida* especially when low prevalence is expected and large numbers of fish need to be analyzed. It may be applicable for epidemiological and transmission studies and can contribute to efforts to control or eradicate bacterial infections.

The availability of rapid, sensitive, and specific diagnostic methods for the detection of bacterial pathogens causing disease is very important in aquaculture. The results indicate that the mPCR developed within this study is a highly sensitive and specific method for detecting *Flavobacterium psychrophilum, Lactococcus garvieae, Pseudomonas aeruginosa*, and *P. putida* in fish farm outbreaks. The mPCR assay allows a rapid and reliable diagnosis of disease to be carried out in a single PCR assay.

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