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Rapid detection of columnaris disease in channel catfish (*Ictalurus punctatus*) with a new species-specific 16-S rRNA gene-based PCR primer for *Flavobacterium columnare*

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Abstract

A 16-S rRNA gene from the chromosomal DNA of the fish-pathogenic bacterium *Flavobacterium columnare* (formerly *Flexibacter columnaris*), strain ARS-I, was cloned, sequenced and used to design a polymerase chain reaction (PCR) primer set. The primer set amplified a specific 1193-bp DNA fragment from *F. columnare* strains but not from related bacteria, *F. psychrophilum*, *F. aquatile*, *F. branchiophilum*, or other bacterial pathogens of fish, *Flexibacter maritimus*, *Cytophaga johnsonae*, *Edwardsiella ictaluri*, *E. tarda*, *Aeromonas hydrophila*, and *Streptococcus iniae* or from the non-fish pathogen *Escherichia coli*. The PCR reaction conditions were optimized to permit detection of the organism from agar plates, broth culture, frozen samples, dead fish tissue, and live fish in less than 5 h (8 h, if the more sensitive nested PCR is used). DNA was extracted by a boiled-extraction method or by commercial column purification. The PCR product was detected at DNA concentrations below 0.1 ng and from as few as 100 bacterial cells. Nested PCR using universal eubacterial primers increased the sensitivity five-fold, allowing detection of *F. columnare* strains at DNA concentrations below 0.05 ng and from as few as 10 bacterial cells in apparently healthy, asymptomatic fish. The efficiency of this primer set was compared to the 16-S rRNA gene primer sets of Toyama et al. [Fish Pathol. 29 (1994) 271.] and that of Bader and Shotts [J. Aquat. Anim. Health 10 (1998) 311.]. The new primer set is as good or better than the previously published primer sets for detecting *F. columnare* in all samples and under all conditions tested.

Keywords: Gram-negative bacteria; PCR; Diagnostics; Fish diseases

1. Introduction

Flavobacterium columnare, the causative agent of columnaris disease (Plumb, 1994), infects at least 36 species of fish throughout the world, including the commercially important species: channel catfish (*Ictalurus punctatus*); common carp (*Cyprinus carpio*); goldfish (*Cyprinus auratus*); eels (*Anguilla rostrata*), (*A. japonica*), (*A. anguilla*); tilapia (*Oreochromis* spp.); rainbow trout (*Oncorhynchus mykiss*); brown trout (*Salmo trutta*); and brook trout (*Salvelinus fontinalis*) (Anderson and Conroy, 1969). Annual

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monetary losses attributed to *F. columnare* in the commercial catfish industry are second only to those losses attributed to *Edwardsiella ictaluri*, which causes enteric septicemia (Thune, 1993). Although most of these losses are associated with the commercial catfish farming industry in the United States, the organism causes economic losses throughout the world. The ubiquitous distribution of the organism in fresh water environments and the tendency for fish to acquire the disease after mechanical and/or environmental insults makes *F. columnare* among the most described pathogens in cultured, ornamental, and wild fish populations (Shamsudin, 1994; Shotts and Starliper, 1999).

Currently, effective commercial vaccines are under development, and there are no reliable means to control columnaris infections in warm water fish (Birnbaum, 1998; Bader et al., 1997). Management practices such as early intervention with topical antibiotics and chemotherapeutics, such as salt, potassium permanganate, and copper sulfate (Lau and Plumb, 1981; Avault, 1985; Chowdhury and Wakabayashi, 1988; Plumb, 1999) are often used to lessen the impact of the disease, but are not always effective. Also, most antibiotic and chemotherapeutic treatments are of limited use to the fish farmer because they are not cost-effective or are not approved for food fish use. This only leaves the fish farmer with the option of preventing an infection before it happens through the use of good management practices to minimize fish stress. Good preventative management of disease usually depends on early and accurate diagnosis of infection. Typically, *F. columnare* infection is determined by isolation of the pathogen from body surface lesions or from infected tissues. After a minimum of 24 h, colonies are evident, however, identity of the bacteria based on colony morphology requires an additional 24 h (Shotts and Teska, 1989). Additional characteristics used to identify the pathogen include wet mount observation for "haystack" formations, adherence to agar, presence of yellow-pigmented colonies, and chromoshift from yellow to red in the presence of 3% sodium hydroxide (Plumb, 1994; Shotts and Starliper, 1999).

Genetic, DNA-based diagnostic methods usually involve the detection of a pathogen through the use of a species-specific nucleic acid probe. Such methods are dependent on the amount of target gene segments present. The polymerase chain reaction (PCR) is often used to amplify segments of the target gene segment because that segment exists at or below readily detectable levels. Thus, PCR increases sensitivity. PCR can be used to both detect and identify bacteria and has been used successfully to detect and identify fish-pathogenic bacteria, including: *A. hydrophilia*, *A. salmonicida*, *E. tarda*, *Renibacterium salmoninarum*, *Vibro anguillarum*, *V. vulnificus* and *Yersinia ruckeri* (Gustafson et al., 1992; Cascon et al., 1996; Hiney et al., 1992; Aoki and Hirono, 1995; Brown et al., 1994; Martinez-Picado et al., 1996; Arias et al., 1995; Argenton et al., 1996). *F. columnare* was definitively identified in culture using species-specific PCR primer sets based on sequences from the 16-S rRNA gene (Toyama et al., 1996; Bader and Shotts, 1998). These primer sets were not easy to use because of formations of hairpins and primer–dimers and were not always specific or sensitive enough for general diagnostic use because of intra-specific genomic variation.

The objectives of this study were to design an alternative primer set to those developed by Toyama et al. (1996) and Bader and Shotts (1998) that contained less secondary structure, took in account available information of intragenetic variation, and that could be used to diagnose columnaris disease in a rapid and noninvasive manner in fish. To achieve these objectives we: (1) sequenced the small subunit 16-S rRNA gene from a reference strain of *F. columnare*, ARS-1 and compared its sequence to other *F. columnare* 16-S rRNA genes in the literature, (2) conducted a computer-aided analysis to design a new and more reliable species-specific PCR primer set for the detection of *F. columnare*, (3) determined the optimal conditions for the use of this primer set, (4) determined the ability of the primer set to detect the bacterium from cultures, frozen tissue samples, dead fish tissues, and from live fish and (5) developed a rapid and nonlethal method for the specific detection of *F. columnare*.

2. Materials and methods

2.1. Bacteria and growth conditions

Bacterial strains used in this study are listed in Table 1, together with their sources and the media used for their culture. The pathogenic *F. columnare* (ARS-1) was isolated in early 1996 from a channel

Table 1
Bacterial strains used to evaluate the 16-S rRNA primer set

Bacterial species	Strain number	Media[a]	Source[b]
Flavobacterium columnare	1	CYT	AAHRU
	43622	CYT	ATCC
	49512	CYT	ATCC
	49513	CYT	ATCC
Flavobacterium psychrophilium	49418	CYT	ATCC
	49512	CYT	ATCC
Flavobacterium branchiophilum	35035	CYT	ATCC
Flavobacterium aquatile	11947	CYT	ATCC
Flexibacter maritimus	43397	MCA	ATCC
	43398		ATCC
Cytophaga johnsonae	17061	CYT	ATCC
Escherichia coli	J-5	TSA/LB	Tyler and Klesius (1994)
Edwardsiella ictaluri	AL-93-75	TSA	AAHRU
Edwardsiella Tarda	FL6-60	TSA	AAHRU
Aeromonas hydrophila	7966	TSA	ATCC
Streptococcus iniae	98-60	TSA	AAHRU

Bacteria were grown at 28 °C for 24 h in broth with shaking or on plates.

[a] CYT—Cytophaga Agar (Anacker and Ordal, 1959) containing 1% tryptone with shaking at 100 revolutions/min; MCA—Marine Cytophaga Agar (ATCC Flexibacter medium 1559) shaking at 100 revolutions/min; TSA—Tryptic Soy broth (Difco); LB—Luria Broth.

[b] Source for these organisms: ARS—isolates from the ARS-USDA, Agriculture Research Service, Aquatic Animal Health Research Unit. ATCC—from American Type Culture Collection, Rockville, MD.

catfish (*I. punctatus*) at the United States Department of Agriculture, Agriculture Research Service, Aquatic Animal Health Research Unit (AAHRU) in Auburn, Alabama, which exhibited the characteristic clinical signs of columnaris disease, namely, saddle-back lesions and yellow pigmentation on gills and in the mouth (Plumb, 1994). The ARS-1 culture was identified according to Reichenbach (1989). It was grown at 28 °C for 24 h in Cytophaga broth (Anacker and Ordal, 1959), which contained per liter: 0.5 g of yeast extract, 0.2 g of beef extract, and 0.2 g of sodium acetate and had been modified by the addition per liter of 1.0 g of typtone. The broth was adjusted to the pH of 8.2 and were shaken at a rate of 100 revolutions/min. Plated cultures were grown on Cytophaga broth plates containing 15 g/l agar.

2.2. DNA manipulation and sequencing

Highly pure bacterial genomic DNA (260:280 ratio of 1.9) was obtained from the culture of *F. columnare*, ARS-1, using a large-scale CsCl preparation (Ausubel et al., 1987). This genomic (template) DNA was used for the production of the PCR amplified 16-S rRNA gene which was later used for cloning and sequencing.

A PCR amplified 16-S rRNA gene product was produced through the use of the universal 16-S rRNA gene PCR primers, designated pH and pA, designed by Edwards et al. (1989) to amplify the highly conserved 5′ and 3′ regions of the eubacterial 16-S rRNA gene. These primers were then used to produce a 1500-bp 16-S rRNA gene product by PCR using materials and methods similar to those described in Edwards et al. (1989). These reactions were performed in a Perkin-Elmer Cetus thermal cycler (model 2400, Norwalk, CT, USA). The 1500-bp product was gel-purified according to supplier's protocols, directly from 1% agarose gels (Promega, Madison, WI, USA).

The 1500-bp product was prepared for nucleotide sequencing by cloning the product into a sequencing plasmid vector. This product was cloned into pGEM3zf+ after blunt-ending the product with T4 polymerase, ligating a *Eco*RI phosphorylated linkers to it using T4 DNA Ligase, and then ligating the products with a 1:3 vector-to-insert ratio using T4 DNA Ligase into vector DNA (pGEM3zf+), which had been previously cut with *Eco*RI, and treated with alkaline phosphatase. The pGEM3zf+ vector is a 3.2-kbp length vector, carries both the *Lac*Z and ampicillin resistance markers, and contains binding sites for the pUC/M13 forward sequencing primer and the pUC/M13 reverse sequencing primer. The vector DNA had been previously purified from commercially obtained (Promega) host *Escherichia coli* strain HA101 (grown in LB broth or on TSA plates at 37 °C for 24 h), using plasmid miniprep kits with methods described by the manufacturer (Promega). Vector constructs were then transformed into competent *E. coli*, JM109 cells according to supplier's protocols, (Promega). Recombinants were screened for blue/white color and antibiotic activities on LB plates, supplemented with 100 μg of ampicillin/ml, 0.1 mM of ITPG, and 40 μg of X-Gal/ml, following incubation overnight at 37 °C. Several hundred white ampicillin-

resistant recombinants were selected and stored at − 80 °C. Four of the recombinants were then chosen to be sequenced, using both the M13 forward and the reverse primers by automated sequencing (model 310, Applied Biosystems, Foster City, CA, USA).

2.3. PCR primer design and production

Forward and reverse nucleic acid sequence data were used to construct a continuous sequence of inserted DNA. Continuous sequences were analyzed with the Genetics Computer Group package (GCG, version 10.1; Madison, WI). A comparison of the sequences from other *F. columnare* strains and from selected members of the genera *Flavobacterium*, *Flexibacter*, and *Cytophaga* (Table 2) was made using 16-S rRNA gene nucleotide sequence data obtained from the EMBL and GenBank databases, using FASTA algorithms.

Based on the 16-S rRNA gene sequence of *F. columnare*, ARS-1 and homologous gene sequence information from EMBL and GenBank, a new primer set was designed to amplify a species-specific 1193-bp segment of the 16-S rRNA gene. These two primers were synthesized and purified at the Genetics Core Unit of the College of Veterinary Medicine at Auburn University, on a high-throughput automated DNA synthesizer. The sequences of the primers were: FvpF1: 5′-GCCCA-GAGAAATTTGGAT-3′ and FvpR1: 5′-TGCGAT-TACTAGCGAATCC-3′.

Table 2
16-S rRNA gene sequences from the EMBL and GenBank databases used to design species-specific oligonucleotide primers

Bacterial species	Strain number	Accession number
Flavobacterium columnare	ARS-1	AY095342
	IAM 14820	AB016515
	LP-8	AB015480
	FK-401	AB010952
	IAM 14301	AB010951
	ATCC 49513	AB023660
	ATCC 43622	M58781
Flavobacterium psychrophilium	ATCC 49418	AF090991
Flavobacterium branchiophilum	IFO 15030	D14017
Flavobacterium aquatile	ATCC 11947	M62797
Flexibacter maritimus	ATCC 43398	M64629
Cytophaga johnsonae	ATCC 17061	M59051

ATCC—American Type Culture Collection; ARS-USDA, Agriculture Research Service, Aquatic Animal Health Research Unit; IAM—Institute of Fermentation, Osaka, Japan; IFO—Institute of Applied Microbiology, University of Tokyo, Japan; LK and FP—Triyanto and Wakabayashi (1999) strains.

2.4. Protocol for development of species-specific PCR

The PCR primer set was initially optimized for amplification of the 16-S rRNA gene sequence of *F. columnare*, ARS-1 using a template DNA concentration of 1 ng purified genomic DNA/50 μl reaction and 50 pmol of each primer. Amplifications were performed in a 50 μl reaction mixture that contained reaction buffer (Promega), 200 μM of each dNTP, 50 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Promega). A gradient thermocycler (Model T-gradient, Whatman Biometra, Gottingen, Germany), was used to determine an optimal annealing temperature for the specific binding of new primer set to the template DNA. A thermal gradient from 36 to 62 °C and later, a thermal gradient from 56 to 62 °C and it was determined that 59 °C was optimal for species-specific PCR. The optimal thermal parameters were as follows: denaturation for 30 s at 95 °C, annealing for 30 s at 59 °C, and extension for 1 min at 72 °C. Twenty-five cycles were followed by a final extension of 6 min at 72 °C and a 4 °C indefinite hold. The PCR products were then subjected to electrophoresis in 1% agarose and visualized by using a UV transilluminator.

After optimal PCR conditions were determined, the species-specific primers were tested to determine their ability to produce the correct product at varying concentrations of genomic template. Templates from 0.05, 0.1, 0.5, 1, 2, 5, 10, 100, to 200 ng of DNA were evaluated. Also, a single colony of *F. columnare* ARS-1 was picked from an agar plate, using a sterile 1 μl inoculating loop, and diluted with sterile phosphate-buffered saline to 10^{-9}. Each dilution was then plated to determine cells/dilution. These dilutions were used to determine a minimum level of detection at optimal PCR conditions.

2.5. Preparation of samples and species-specific PCR

To determine the usefulness of the primer set to amplify template DNA from a variety of sources,

template DNA was obtained from broth cultures, colonies on agar plates, and artificially and/or naturally infected fish tissue. Raw bacterial samples from either broth culture or agar plates were prepared for PCR by adding 40 μl volume that contained approximately 4.0×10^6 of cells either directly from 24-h culture broth, or from a single 1×1 mm 24-h colony. Each sample was picked using a sterile 1-mm plastic loop from a Cytophaga agar plate modified as described above. The 40 μl cell suspension was prepared for PCR using a boiled lysis method as follows: the cell suspension was mixed with 10 μl of protease-K (20 μg/ml) and 50 μl K-buffer (40 mM Tris–HCl, 0.2% Nonidet P-40, 0.2 mM EDTA, and 1% Tween-20, adjusted to pH 8.0). The mixture was heated at 60 °C for 20 min, then at 100 °C for 5 min, and finally, rapidly cooled on ice. Prior to use, the mixture was centrifuged at 8000 rpm for 5 min. DNA concentrations of all samples were evaluated using a spectrophotometer (GeneQuant, BioRad, Hercules, CA, USA). Template DNA for PCR was extracted from the following tissues: gill, mucus, skin, trunk, and head kidney. All tissues were obtained using sterile techniques and divided into two microcentrifuge tubes, one for fresh samples and one for frozen samples. Fresh tissue samples were processed immediately, while the frozen tissue sample tubes were stored at −80 °C until used. Mucus samples were obtained with a sterile 1 μl plastic inoculating loop or with a sterile scalpel blade by scaping the fish for 4 cm along the lateral line (Evans et al., 2001). The samples were then diluted in 100 μl sterile phosphate buffered saline. In order to obtain similarly sized samples, 1-mm cubes, or approximately 1 μg samples of the gill, skin, head, and trunk kidney were collected using aseptic technique. Then 100 μl of blood was collected via caudal vein puncture into a microcentrifuge tube, allowed to coagulate at room temperature, and spun at 3000 rpm to separate cells from sera. Mucus, blood, or tissue samples were thawed from −80 °C and processed according to the methods outlined in the DNeasy tissue kit for the isolation of DNA (Qiagen, Valencia, CA, USA). Along with the experimental samples, at least one *F. columnare* ARS-1 purified genomic DNA sample was also analyzed. Purified genomic DNA from *E. coli* was used for negative controls. Species-specific PCR amplifications were performed in a 50 μl reaction mixture that contained reaction buffer (Promega), 200 μM of each dNTP, 50 pmol of each primer, 2.5 U of Taq DNA polymerase (Promega), and 1–100 ng of sample DNA. The temperature conditions were as follows: denaturation for 30 min at 95 °C, annealing 30 s at 59 °C, and extension for 1 min at 72 °C. Twenty-five cycles were followed by a final extension of 6 min at 72 °C and a 4 °C indefinite hold. The PCR products were electrophoresed on a 1% agarose gel. Finally, the, gel was stained with ethidium bromide, visualized under UV light, and digitized onto a computer.

2.6. Animals

One hundred and fifty channel catfish (USDA 103 strain) were utilized throughout this study to produce positive tissues with *F. columnare* and to act as negative controls (tissue negative for *F. columnare*). These catfish had a mean weight of 21.3 g ($\pm$ 1.2) and mean length of 12.1 cm ($\pm$ 0.6) and were housed at the AAHRU. Prior to experimentation, fish had been stocked at 30 per 57 l aquarium in five tanks and held for 2 weeks. Aeration was supplied to each tank with compressed air through airstones. Water was circulated at 0.5 l/min, daily dissolved oxygen was 5.5 ± 0.7 mg/l, and temperature was maintained at 26.1 ± 1 °C with a light/dark photoperiod of 12:12 h (Klesius et al., 1999). Before immersion exposure, five of the fish were randomly selected from among all tanks and cultured for the presence of *F. columnare* according to the procedure of Reichenbach (1989). All five fish tested negative for *F. columnare*. Negative control fish were produced for tissue harvest by immersion exposure with Cytophaga broth only and positive fish tissues were produced using the immersion exposure methods described below.

2.7. Immersion exposure

Immersion exposure with *F. columnare* involved immersing 145 fish in 1×10^8 CFU/ml of bacteria. The proper concentration of bacteria was generated by first diluting *F. columnare* cells in 10 l of water to yield a concentration of 1×10^8 CFU/ml. Plate counts were used to verify the actual CFU in immersion water. Fish were immersed for 30 min at 29 ± 2 °C.

2.8. Evaluation of the use of the new primer set on asymptomatic animals

To provide preliminary data demonstrating the possible usefulness of the new species-specific FVpF1–FVpR1 primer set to detect *F. columnare* in apparently asymptomatic fish, PCR was performed on 27 immersed (exposed) fish. These were classified into two groups: asymptomatic and symptomatic fish. Asymptomatic fish were defined as fish that had been infected by the immersion method described earlier, but which did not show the clinical signs of columnaris disease. Three fish were sampled from each of these two treatment groups. Mucus samples from these 27 fish were collected, as previously described, and evaluated with the FVpF1–FVpR1 primer set using the previously described optimal PCR conditions. Asymptomatic fish were collected from the same tank as the symptomatic (infected) fish. All symptomatic fish were either moribund or else showed clinical signs of columnaris disease. Uninfected fish (unexposed control fish) were collected from our stock of fish and were verified as culture negative for *F. columnare* by standard methods as defined earlier.

3. Results

3.1. 16-S rRNA gene sequence and analysis

The universal 16-S rRNA gene primer set used in our study yielded the expected 1500-bp product for that gene and was able to amplify this product for all the bacteria evaluated. The specific nucleotide sequence for *F. columnare* ARS-1 was determined for 1460 bp of the 1500-bp gene. The 16-S rRNA gene sequence determined in this study has been deposited in the Genbank nucleotide sequence database under the accession number 458992. Our sequence data confirm that *F. columnare* ARS-1 has a 99% homology to *F.*

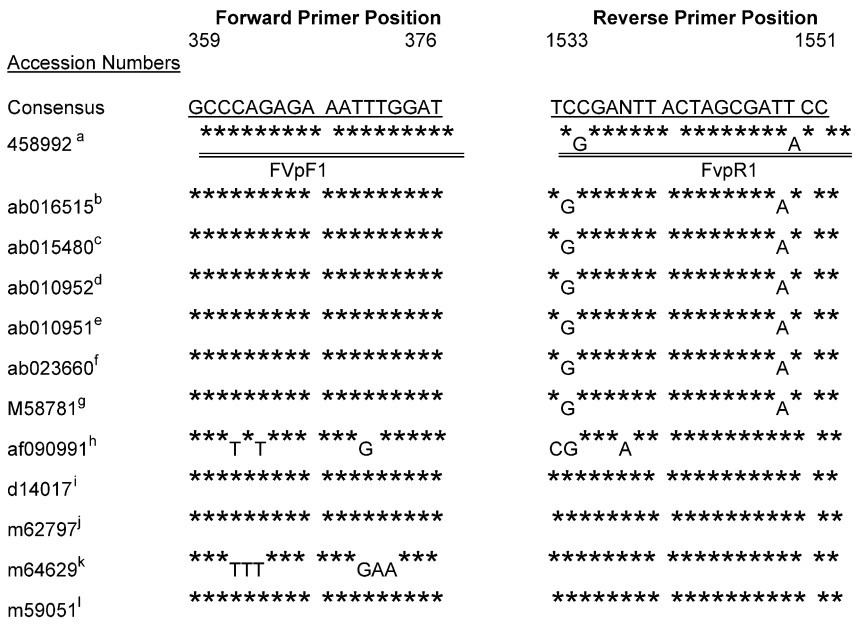

Fig. 1. Comparison of the 16-S rRNA gene sequence at the locations of the polymerase chain reaction primers, FVpF1 and FvpR1 for *F. columnare*, ARS-1 and the 11 other bacteria used to design the primer set. Sequences were obtained from the EMBL and GenBank databases of nucleic acid sequences. The double underlined text indicates primers, FvpF1 and FvpR1. Position numbers refers to the *E. coli* rRNA numbering system. *F. columnare*: (a) ARS 1, (b) IAM 14820, (c) LP-8, (d) FK-401, (e) IAM 14301, (f) ATCC 49513, (g) ATCC 43622; *F. psychrophilum*: (h) ATCC 49418; *F. branchiophilum*: (i) IFO 15030; *F. aquatile*: (j) ATCC 11947; *F. maritimus*: (k) ATCC 43398; *C. johnsonae*: (l) ATCC 17061. Abbreviation: ATCC—American Type Culture Collection; ARS-USDA, Agriculture Research Service, Aquatic Animal Health Research Unit; IAM—Institute of Fermentation; IFO—Institute of Applied Microbiology, University of Tokyo; LK and FP—Triyanto and Wakabayashi (1999) strains.

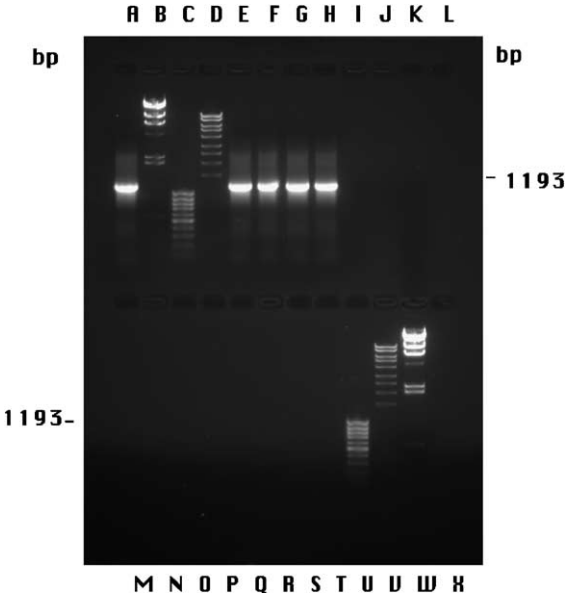

Fig. 2. Production of the 16-S rRNA species-specific gene product using purified genomic DNA templates strains of *F. columnare*, members of the genera Flavobacterium, Flexibacter, Cytophaga, Edwardsiella, Aeromonas, Escherichia, and Streptococcus, with primers FvpF1 and FvpR1. The 1193-bp product is visible on the ethidium bromide stained 1% agarose gel. Lane A, *F. columnare* ARS1 genomic DNA, as positive control; lanes B and W, Lambda *Hin*d III molecular mass ladder (Gibco, Gaithersburg, MD, USA); lanes C and U, low range mass ruler (MBI Fermentas, Hanover, MD, USA); lanes D and V, high range mass ruler (MBI Fermentas); F, ARS-1; G, IAM 14820; H, LP-8; I, FK-401; J, IAM 14301; K, ATCC 49513; L, ATCC 43622; M, ATCC 49418; N, IFO 15030; O, ATCC 11947; P, ATCC 43398; Q, ATCC 17061; R, AL-93-75; S, ARS-FL6-60; T, ATCC 7966; U, ARS-98-60; lane X, J-5, as negative control.

columnare, ATCC 49513. *F. columnare*, ATCC 49512 is considered to belong to the genomic group I of *F. columnare*, according to Triyanto and Wakabayashi (1999), and we therefore believe that ARS-1 belongs in that intra-species group. A comparison of the 16-S-rRNA gene sequence at the locations of the polymerase chain reaction primers, FVpF1 and FvpR1 for *F. columnare*, ARS-1 and the 11 other bacteria used to design the primer set is shown in Fig. 1.

3.2. Species-specific PCR test

The PCR amplification of reference organisms and *F. columnare* bacterial DNA following nested PCR (Fig. 2, lanes E–H), shows the species-specific 1193-bp product was only obtained using the FvpF1–FvpR1 primer set and *F. columnare* DNA templates. DNA from the other organisms listed in Table 1 did not yield the 1193-bp product, indicating that it is specific to *F. columnare* (Fig. 2, lanes I–T). This product was formed between 58 and 60 °C, with the best specificity at 59 °C. Nonspecific products form at annealing temperatures below this range.

The FvpF1–FvpR1 primer set is highly sensitive, and detected as few as 100 *F. columnare* cells. However, if a nested reaction was performed with the universal eubacterial primers, prior to the use of the FvpF1–FvpR1 set to detect *F. columnare*, the sensi-

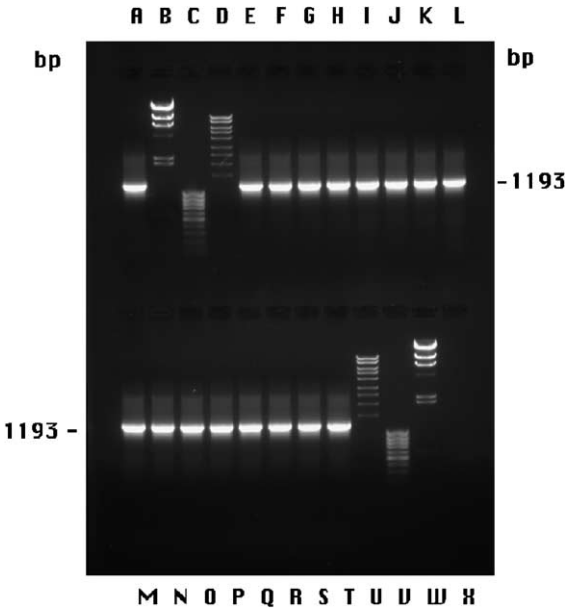

Fig. 3. Representative PCR products from various positive control fish tissues, fresh and frozen using the FvpF1–FvpR1 primer set under optimized conditions. The 1193-bp product is visible on the ethidium bromide stained 1% agarose gel. Lanes A and X, *F. columnare* ARS-1 genomic DNA, as positive control; lanes B and W, Lambda *Hin*d III molecular mass ladder (Gibco); lanes C and V, low range mass ruler (MBI Fermentas); lanes D and U, high range mass ruler (MBI Fermentas). Fresh positive control samples: E, broth; F, agar plate; G, mucus; H, gill; I, skin; J, trunk kidney; K, head kidney; L, blood. Frozen positive control samples: M, broth; N, agar plate; O, mucus; P, gill; Q, skin; R, truck kidney; S, head kidney; T, blood; lane X, J-5, as negative control. Positive control fish tissues were produced by a for 30-min immersion challenge of catfish with 1×10^8 CFU/ml *F. columnare*, ARS1 and harvesting of tissues using aseptic techniques. Fresh tissues were processed for PCR immediately. Frozen tissues were processed for PCR after being held at −80 °C.

tivity increased to 10 cells. Using the new primer set, 0.1 ng of *F. columnare* DNA was detected without nesting, while 0.05 ng of DNA could be detected using the nested PCR method.

F. columnare was detected from cultures on agar plates, broth cultures, frozen tissue samples, and live fish. Representative examples of product formation from each source are shown in Fig. 3. All tissues sampled could be used to detect the bacterium, but blood samples were highly variable in their ability to produce the properly sized amplicon. The tissue, that was most easily sampled and could be used to detect *F. columnare* infection, was the mucus. DNA was successfully extracted by a boiled-extraction method or by commercial column purification kits. The total procedure was accomplished in less than 5 h, from the point of DNA extraction to observation in an agarose gel, using the non-nested PCR reaction, but the time required increased to 8 h with nested PCR reaction.

3.3. Use of the new primer set on asymptomatic animals

Although a small number of fish were evaluated and no statistical validation was possible, our data suggest that the new PCR primer set was able to discriminate between apparently healthy and asymptomatic fish infected with *F. columnare* and uninfected. No *F. columnare* was detected in any of the nine control, uninfected fish, while the bacteria was detected in six of the apparently healthy and asymptomatic fish infected with *F. columnare* (Fig. 4). All nine fish with clinical signs of columnaris disease were PCR positive and culture positive for *F. columnare*.

4. Discussion

Intra-species heterogeneity in 16-S rRNA gene sequence has been exploited for the development of species-specific PCR-based diagnostic tools for a wide variety of both pathogenic and nonpathogenic bacteria. These tools have revolutionized the identification of bacteria, but for some bacteria, these tools are still in development in regard to species specificity, reproducibility, and ease of use. These problems have lead to the development of several different 16-S rRNA based PCR primer sets for the identification of *F. columnare*. Historically, each was constructed based on sequencing of the 16-S rRNA gene of a

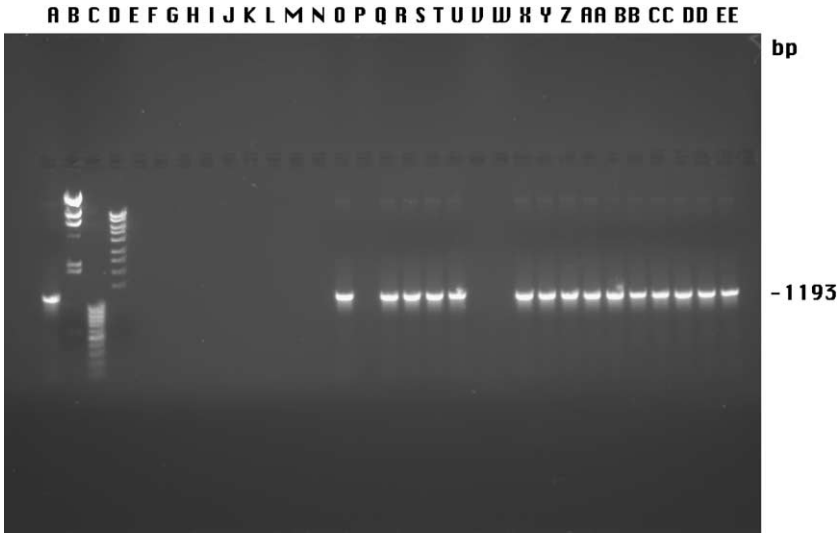

Fig. 4. Amplification in symptomatic, asymptomatic, and control fish mucus using the FvpF1–FvpR1 primer set under optimized conditions. The 1193-bp product is visible on the ethidium bromide stained 1% agarose gel. Lane A, *F. columnare* ARS-1 genomic DNA, as positive control; lanes B, Lambda *Hin*d III molecular mass ladder (Gibco); lanes C, low range mass ruler (MBI Fermentas); lanes D, high range mass ruler (MBI Fermentas); lanes, E–M, control fish; lanes, N–V, asymptomatic fish; lanes, W–EE, symptomatic fish.

given strain of *F. columnare*. The Toyama et al. (1996) primer set was based on the sequence from American Type Culture Collection (ATCC) strain 43622, and the Bader and Shotts (1998) primer set was based on the sequence from ATCC strain 14902. These primer sets work well on the isolates for which they were designed, but did not achieve the required ease of use and specificity necessary for a routine diagnostic situation. Toyama et al. (1996) designed two sets of primers (Col1-1500R, 1320-bp product; Cola-Colb, 1088-bp product) to identify the eight strains of *F. columnare*, but did not design a single primer set for the identification of all the *F. columnare* isolates. *F. columnare* is known for intra-species variation in the 16-S rRNA gene (Toyama et al., 1996; Triyanto and Wakabayashi, 1999) which makes the development of a single primer set more difficult. To overcome this problem of genetic variability, Toyama et al. (1996) chose a species-specific forward primer and the universal eubacterial 16-S rRNA reverse primer in order to achieve the desired specificity. The Bader and Shotts (1998) primer set was designed to separate *F. psychrophilium* and *Flexibacter maratimus* from *F. columnare*, but required a prolonged preheating period to melt primer–dimer formations to allow specific product formation. In double blind studies, all of the 17 *F. columnare* evaluated were identified However, this set did not work as well in our laboratory, under generally accepted PCR conditions and did not always produce reproducible PCR products . This primer set amplified a 1100-bp product. Both the Toyama et al. (1996) sets and Bader and Shotts (1998) set were evaluated in our lab and were found to be specific for the detection of some *F. columnare* strains, but they were difficult to use. The Toyama et al. (1996) and Bader and Shotts (1998) primer sets were reported to identify the organism in broth or from culture plates using the boiled-extraction method described in Toyama et al. (1994). Our inability to get the Toyama et al. (1996) sets or the Bader and Shotts (1998) set to work well led to a desire to design primer sets that would work reproducibly under generally accepted PCR conditions. During the course of our studies, another primer set designed to overcome the intra-species genomic variation in *F. columnare* was described Triyanto et al. (1999). This set appears to have been designed to deal with many of the demonstrated weaknesses of the other sets and has been designed to take in account for intra-species genomic variation. We acknowledge that this set may be better then our, because ours set was designed from a more limited number of intragenetically diverse sequence data sets. However, we did not evaluate the Triyanto et al. (1999) set, because it was not available to us when our set was developed and when the research was conducted. Future studies are needed to compare their set to ours.

We found that the PCR primer set, FvpF1–FvpR1, was specific for the identification of *F. columnare*, and differentiated *F. columnare* from other *Flavobacterium* species pathogenic to fish . Further, we found no cross-reactivity with the non-fish pathogen, *E. coli*; with the fish pathogens, *F. maritimus* and *Cytophaga johnsonae*, bacteria once thought to be closely related to *F. columnare* and with other important species of warm water fish-pathogenic bacteria, *E. ictaluri, E. tarda, A. hydrophila*, and *S. iniae*. We also found that this PCR primer set allowed detection of as few as 100 bacterial cells, and bacterial DNA at levels approaching 0.1 ng. Increased tissue sensitivity can be achieved with a preliminary amplification using the universal 16-S rRNA primers (Weisburg et al., 1991) as described in Toyama et al. (1996). After a preliminary amplification using the universal 16-S rRNA primers, detection of as few as 10 bacterial cells, and bacterial DNA levels approaching 0.05 ng were achieved. The FvpF1–FvpR1 primer set, unlike the Toyama et al. (1996) set, allowed identification of all four intra-specific genomic groups of *F. columnare* represented by the eight stains tested. This intra-specific specificity was possible because genomic differences in the 16-S rRNA gene were accounted for in the design of the primers. The results presented here demonstrate that this primer set is useful for identifying all other *F. columnare* isolates. We also considered the ability of the set to form primer–dimers and primer–primer dimers in the initial primer design. Consideration was made in the computer modeling of the FvpF1–FvpR1 primer set to minimize such dimers during pre-reaction and PCR. This was important because the formation of dimers was a major problem for the Bader and Shotts (1998) set, which required an extensive pre-PCR melting period and long cycles to achieve repeatable species-specific reactions. Our new primer set functions best in the more generally

accepted temperature ranges of PCR, does not require any pre-PCR melting period, and does not require long cycles. This set functions best and with the highest specificity at 57–60 °C temperature and with 25–30 cycles. Further, the new set does not require specialized buffers, reaction additives, and gives highly reproducible results.

The FvpF1–FvpR1 primer set is also useful for the identification of *F. columnare* in pure culture from colonies on an agar plate or from broth, in a similar manner to the Toyama et al. (1996) and Bader and Shotts (1998) sets. Unique to our study is the application of the PCR set to the identification of *F. columnare* in various tissues from live and dead fish. The use of PCR to identify pathogenic bacteria from human and animal tissues with little or no pre-preparation is well documented in the literature (Jones and Bej, 1994). PCR may also be useful for the detection of fish pathogens in both fresh tissues and preserved tissues as in In Situ PCR (Gustafson et al., 1992; Cascon et al., 1996; Hiney et al., 1992; Aoki and Hirono, 1995; Brown et al., 1994; Martinez-Picado et al., 1996; Arias et al., 1995; Argenton et al., 1996). We have clearly demonstrated that *F. columnare* can be detected in tissues from fresh, refrigerated, and frozen fish. Gill, mucus, skin, trunk, and head kidney are routinely collected by the diagnostic laboratory in the process of assessing fish health. We detected *F. columnare* in all tissues sampled and from frozen fish using the FvpF1–FvpR1 primer set. Further, the pathogen was found in gill, mucus, and skin of fresh fish. Mucus samples were the most reliable tissue type for the detection of *F. columnare* and blood the least reliable. Mucus samples can easily be obtained from living or freshly dead fish. We found that the method of obtaining mucus samples described in Evans et al. (2001) was extremely reliable in sampling for *F. columnare*. The combination of this sampling method and the FvpF1–FvpR1 primer set allow for a rapid, nonlethal method for detection of *F. columnare* in symptomatic and asymptomatic animals using mucus samples. The fact that the FvpF1–FvpR1 set can detect symptomatic fish is not surprising, since fish with clinical signs of columnaris typically have enough bacteria to make a determination of etiological agent via culture (unpublished data). What is useful is the fact that using the FvpF1–FvpR1 set, *F. columnare* can be detected in asymptomatic fish. Therefore, the FvpF1–FvpR1 set may allow for earlier detection of columnaris than presently possible and may help in earlier intervention of columnaris disease.

Unfortunately, while our newly designed primer set is very useful, it still carries with it several of the inherent problems associated with the other 16-S rRNA gene-based diagnostic sets for the detection of columnaris disease. It produce a large product, 1193 bp the order of thousand of bases as opposed to a product in the hundreds of base-pairs. Such longer products are often problematic because they tend to be less sensitive in highly fragmented DNA template environments found in diagnostic samples. In such environments, high sensitivity can truly only be achieved with amplicons of smaller size (< 300 bp). However, at present, the longer product forming 16-S rRNA gene-based primer sets are the best we have available. As we identify more genes or intragenetically diverse regions in *F. columnare*, better sets will surely be developed to rapidly diagnose *F. columnare* in fish.

In conclusion, while our results are encouraging, the use of PCR for the primary detection of fish pathogens, including *F. columnare* is presently widely debatable among microbiologists and diagnosticians in diagnostic laboratories evaluating clinical fish samples (Hiney and Smith, 1998). At best most primer sets, including ours, await a prolonged period of systematic validation before having any chance of becoming common place in diagnostic laboratories. Polymerase chain reaction-based assays for bacterial gene detection have many pitfalls and extensive validation studies need to be conducted before they can be widely used (Johnson, 2000). Therefore, future studies are certainly warranted to: (1) compare our primer set to other 16-S rRNA gene-based diagnostic primer sets for the detection of *F. columnare*, (2) evaluate the specificity of the set in real world diagnostic situations, such as direct isolation from fish populations and (3) determine the exact value of this set to detect columnaris disease in asymptomatic animals. Our study has demonstrated that the newly designed 16-S rRNA gene-based FvpF1–FvpR1 primer set is a valuable tool for the detection of *F. columnare*. It provides a rapid, highly reliable, and reproducible means of detection that can be used directly by diagnosticians and fish farmers alike. The successful use of the set to detect columnaris disease in

asymptomatic animals, without sacrificing the animal, has the potential to revolutionize how the fish farmer manages columnaris disease and may have a positive economic impact on warm water fish culture.

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