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Transmission and detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus*

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ABSTRACT: A specific and rapid PCR detection method for *Flavobacterium columnare* based on the 16S-23S rDNA intergenic spacer region (ISR) of the ribosomal RNA operon has been developed. The ISR of 30 *F. columnare* strains and other *Flavobacterium* species was amplified using universal primers and sequenced. Once *F. columnare* specific sequences within the ISR were recognized, specific PCR primers were designed against them (FCISRFL and FCISRR1). The primers were sensitive and able to detect as low as 7 colony forming units from pure culture by PCR. The new PCR detection method was applied to experimentally infected channel catfish. Two different experiments in which channel catfish fingerlings were infected by intramuscular injection or by immersion bath showed the advantage of the PCR method over standard culture techniques. *F. columnare* was detected by PCR in both tank water and catfish tissue samples with a higher frequency and in less time than standard microbiological methods. Furthermore, PCR detection confirmed that *F. columnare* can be transmitted horizontally indirectly through the water column without fish-to-fish contact. The newly developed PCR detection method for *F. columnare* was more sensitive and rapid than standard culture on bacteriological media for detection of *F. columnare* in channel catfish tissues and in tank water.

KEY WORDS: *Flavobacterium columnare* · Columnaris disease · PCR · Channel catfish · Detection

INTRODUCTION

Flavobacterium columnare is a yellow-pigmented, filamentous gram-negative bacterium of the family *Flavobacteriaceae*. Originally described by Davis (1922), the nomenclature has changed several times from *Bacillus columnaris*, *Chondrococcus columnaris*, *Cytophaga columnaris*, and *Flexibacter columnaris* to the present day *F. columnare* (Bernardet et al. 1996). *F. columnare* is ubiquitous in freshwater environments and is an opportunistic pathogen that may survive for prolonged periods in water (Groff & LaPatra 2000). At least 36 species of fish are susceptible to columnaris disease (Shoemaker et al. 2003), and *F. columnare* is second only to *Edwardsiella ictaluri* in economic impact on the commercial channel catfish *Ictalurus punctatus* Rafinesque industry in the United States (Wagner et al. 2002), causing losses in the millions of dollars each year.

Columnaris disease is characterized as an acute to chronic infection of the gills and integument, including the fins (Wolke 1975), that produces external lesions of the gills, oropharynx, and skin (Bullock et al. 1971, Wolke 1975, Austin & Austin 1993, Wakabayashi 1993). Lesions often occur along the dorsal midline and extend posterior to the dorsal fin and along the lateral flanks, commonly referred to as saddleback lesions (Wolke 1975, Wakabayashi 1993, Groff & LaPatra 2000). Transmission of *Flavobacterium columnare* can be indirect via the environment or by cohabitation with carrier fish which shed the bacterium, or direct through contact with infected fish (Austin & Austin 1993, Wakabayashi 1993, Groff & LaPatra 2000).

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Isolation and identification of *Flavobacterium columnare* is difficult using standard methods (Groff & LaPatra 2000). Methods for identification of *F. columnare* often involve the interpretation of several phenotypic tests, which include: colony morphology (yellow, rhizoid colonies that are flat and strongly adherent on *Cytophaga* agar), reduction of nitrate, hydrolysis of lecithin, adsorption of Congo red dye, production of flexirubin-type pigments, and an inability to hydrolyze carbohydrate (Shamsudin & Plumb 1996). Biotyping bacteria with commercially developed biochemical kits (API 50CH and ZYM Kits, Sociétés Analytab Products) has been used successfully to identify *F. columnare* (e.g. Bernardet & Grimont 1989, Sakai et al. 1992). These and other traditional tests can be laborious, and results can be equivocal, taking days to complete. Molecular identification methods have the potential to offer a quick and definitive choice for identification of pathogenic bacteria, either in pure culture or from environmental samples.

Because columnaris disease is initially manifested as an external infection, peripheral tissues are probably the best choice for detection in fish. *Flavobacterium columnare* exists in natural aquatic environments, and bacterial shedding from carrier fish also occurs, making detection in culture water possible using molecular techniques. *Flavobacterium psychrophilum*, a close relative of *F. columnare* and the causative agent of bacterial coldwater disease, has been successfully detected in water samples using using the polymerase chain reaction (PCR) (Wiklund et al. 2000). Several researchers have developed molecular identification methods for *F. columnare* using PCR (Toyama et al. 1996, Bader & Shotts 1998, Triyanto et al. 1999, Bader et al. 2003); however, all these methods are based on the 16S ribosomal RNA gene (rDNA) and require a nested-PCR or an amplified product restriction analysis to achieve optimal sensitivity and/or specificity.

The intergenic spacer region (ISR) between 16-23S rRNA genes, although a stable and conserved area, contains non-coding regions that exhibit a considerable degree of sequence variation. Such variable regions make the ISR a good choice for the development of molecular methods able to discriminate between closely related species (Zavaleta et al. 1996). The objectives of this study were to design a sensitive and specific molecular method for the detection of *Flavobacterium columnare* and to examine the transmission characteristics of columnaris disease.

MATERIALS AND METHODS

Experimental bacteria and culture conditions. A total of 44 bacterial isolates were used in this study. Thirty isolates were previously identified as *Flavobacterium columnare* (Table 1) based on biochemical tests according to Bernardet & Grimont (1989). Most *F. columnare* isolates were cultured from diseased channel catfish. *F. columnare* reference strains American Type Culture Collection (ATCC) 23463 (type strain) and ATCC 49512 were also included in this study. The remaining bacteria were comprised of 6 *Flavobacterium* spp., *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*), and 5 other non-*Flavobacterium* bacteria pathogenic to fish. Pure cultures of bacteria used for PCR primer testing and infection experiments were grown overnight under optimum conditions. *Flavobacterium* spp. were cultured in Shieh broth (Shieh 1980). Cultures were checked for purity by microscopic examination and phenotypic evaluation of colonies on Shieh agar. Isolation of putative *F. columnare* colonies from water and fish samples was carried out by direct plating on Shieh agar followed by incubation at 28°C. Presence of *F. columnare* on agar plates was confirmed by PCR using the procedure described below.

Primer design and PCR conditions. Universal primers 16S-14F (5′-CTTGTACACACCGCCCGTC-3′, position 1389 to 1407, *E. coli* numbering) and 23S-1R (5′-GGGTTTCCCCATTCGGAAATC-3′, position 124 to 110, *E. coli* numbering) against highly conserved regions were used to amplify the 16S-23S rDNA ISR (Zavaleta et al. 1996) from 30 *Flavobacterium columnare* isolates and 6 non-columnaris *Flavobacterium* spp. A single colony was resuspended in sterile water, boiled for 5 min, cooled on ice, and centrifuged briefly. Five µl of supernatant were used as template DNA for PCR amplification. PCR conditions were as described previously by Zavaleta et al. (1996). Amplified products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostic Corporation) and sequenced by the Auburn University Genomics and Sequencing laboratory. After multiple sequence alignments were conducted using the software package Vector NTI v. 9.0, *F. columnare* species-specific regions were identified within the ISR, and specific primers were designed (Forward, FCISRFL 5′-TGCGGCTGGATCACCTCCTTTCTAGAGACA-3′; Reverse, FCISRR1 5′-TAATYRCTAAAGATGTTCTTTCTACTTGTTTG-3′). PCR conditions to specifically amplify *F. columnare* ISR sequence were as follows: 30 µl PCR amplifications were used containing approximately 50 ng template DNA, 20 pmol of each primer, 1X PCR premix (buffer H; Epicentre), 3 U of Taq DNA polymerase (Promega), and nuclease-free water. Thermocycler conditions were: 5 min at 94°C; 40 cycles of 30 s at 94°C (denaturation), 45 s at 55°C (annealing), and 60 s at 72°C (primer extension); 7 min at 72°C (final extension); 4°C indefinitely.

Primer specificity. Specificity of PCR primers was screened against the bacteria listed in Table 1. All cultures were grown overnight under optimum conditions. Conditions for PCR amplifications were as described in the above paragraph.

Primer sensitivity. Serial dilutions of a pure overnight culture of *Flavobacterium columnare* ARS-1 (1×10^8 colony forming units [CFU] ml^{-1}) grown at 28°C were made in 1% phosphate buffered saline (PBS). Five µl of 10-fold dilutions ranging from 1×10^0 to 1×10^{-11} of the original culture were used in 50 µl PCR amplifications using the conditions outlined above. The number of CFUs assayed ranged from 0 to 7×10^4 as determined from plate counts.

In order to test the sensitivity of the method in fish samples, gill, liver, skin, and blood (approximately

Table 1. Bacteria used in testing the specificity of the FCISRFL and FCISRR1 primer pair. USDA-ARS: United States Department of Agriculture Research Service, Aquatic Animal Health Laboratory; Auburn University: Fish Disease Diagnostic Laboratory, Dept. of Fisheries and Allied Aquacultures; AFFC: Alabama Fish Farming Center; Stoneville: Thad Cochran National Warmwater Aquaculture Center, College of Veterinary Medicine, Mississippi State University; n.k.: not known; NS: no sequence

Reference ID	Microorganism	GenBank access no.	PCR (+/–)	Source of isolate	Location isolated
1. ARS-1	*Flavobacterium columnare*	AY754371	+	*Ictalurus punctatus*	USDA-ARS, Auburn, AL, USA
2. GZ	*F. columnare*	AY754379	+	*I. punctatus*	Auburn University, Auburn, AL, USA
3. ALG-00-513	*F. columnare*	AY754365	+	*I. punctatus*	AFFC, Greensboro, AL, USA
4. ALG-00-515	*F. columnare*	AY754366	+	*I. punctatus*	AFFC, Greensboro, AL, USA
5. ALG-00-521	*F. columnare*	AY754367	+	*I. punctatus*	AFFC, Greensboro, AL, USA
6. ALG-00-522	*F. columnare*	AY754368	+	*I. punctatus*	AFFC, Greensboro, AL, USA
7. ALG-00-530	*F. columnare*	AY754370	+	*I. punctatus*	AFFC, Greensboro, AL, USA
8. ALG-057	*F. columnare*	AY754362	+	*I. punctatus*	AFFC, Greensboro, AL, USA
9. ALG-063	*F. columnare*	AY754363	+	*I. punctatus*	AFFC, Greensboro, AL, USA
10. ALG-069	*F. columnare*	AY754364	+	*I. punctatus*	AFFC, Greensboro, AL, USA
11. MO 02 23	*F. columnare*	AY754383	+	*Micropterus salmoides*	Lake of the Ozarks, MO, USA
12. MS 463	*F. columnare*	AY754384	+	*I. punctatus*	Stoneville, MS, USA
13. MS 465	*F. columnare*	AY754385	+	*I. punctatus*	Stoneville, MS, USA
14. MS 467	*F. columnare*	AY754386	+	*I. punctatus*	Stoneville, MS, USA
15. MS 475	*F. columnare*	AY754387	+	*I. punctatus*	Stoneville, MS, USA
16. 27	*F. columnare*	AY754360	+	*I. punctatus*	USDA-ARS, Auburn, AL, USA
17. HS	*F. columnare*	AY754380	+	*I. punctatus*	AFFC, Greensboro, AL, USA
18. IR	*F. columnare*	AY753072	+	*Cyprinus carpio*	Israel
19. TN-02-01	*F. columnare*	AY753073	+	*I. punctatus*	Normandy Fish Hatchery, TN, USA
20. GA 02 14	*F. columnare*	AY754378	+	*Oncorhynchus mykiss*	Buford Trout Hatchery, GA, USA
21. BioMed	*F. columnare*	AY754374	+	n.k.	Bellvue, WA, USA
22. BZ-01	*F. columnare*	AY753071	+	*Oreochromis niloticus*	Brazil
23. BZ-02	*F. columnare*	AY754375	+	*Or. niloticus*	Brazil
24. BZ-04	*F. columnare*	AY754376	+	*Or. niloticus*	Brazil
25. BZ-05	*F. columnare*	AY754377	+	*Or. niloticus*	Brazil
26. LSU	*F. columnare*	AY754382	+	*I. punctatus*	Baton Rouge, LA, USA
27. PT 14	*F. columnare*	AY754388	+	*I. punctatus*	MS, USA
28. ALG-036	*F. columnare*	AY754361	+	*M. salmoides*	Auburn University, Auburn, AL, USA
29. ATCC 23463	*F. columnare*	AY754372	+	*Oncorhynchus tshawytscha*	Snake River, WA, USA
30. ATCC 49512	*F. columnare*	AY754373	+	*Salmo trutta*	France
31. ATCC 43622	*F. johnsoniae*	AY753070	–	Salmonid?	n.k.
32. ATCC 35035	*F. branchiophilum*	AY753069	–	*Oncorhynchus masou*	Guma, Japan
33. ATCC 11947	*F. aquatile*	AY753066	–	Deep well	Kent, England
34. ATCC 29551	*F. hydatis*	AY753068	–	Salmon	MI, USA
35. ATCC 43397	*Tenacibaculum maritimum*	NS	–	Black porgy	Japan
36. ATCC 17061	*F. johnsoniae*	AY753067	–	Soil	England
37. ATCC 49418	*F. psychrophilum*	AY757361	–	*Oncorhynchus kisutch*	WA, USA
38. NC-03-01	*F. psychrophilum*	NS	–	*On. mykiss*	NC, USA
39. SC-03-01	*F. psychrophilum*	NS	–	*On. mykiss*	SC, USA
40. AL-93-75	*Edwardsiella ictaluri*	NS	–	*I. punctatus*	Auburn University, Auburn, AL, USA
41. AL-93-68	*Edwardsiella tarda*	NS	–	*I. punctatus*	Auburn University, Auburn, AL, USA
42. ARS-60	*Streptococcus iniae*	NS	–	*Or. niloticus*	USDA-ARS, Auburn, AL, USA
43. 35KU	*S. agalactiae klunzingeri*	NS	–	*Liza klanzingeri*	Kuwait Bay, Kuwait
44. MN-01-01	*Aeromonas hydrophila*	NS	–	*Or. niloticus*	MN, USA

25 mg) samples from healthy individuals were spiked with 1×10^2, 1×10^3, and 1×10^4 CFU ml^{-1} dilutions of an overnight culture of *Flavobacterium columnare* ARS-1 resuspended in 1% PBS. Concentrations of *F. columnare* mg^{-1} tissue were approximately 40 to 60 CFU for the 1×10^4 treatment, 4 to 6 CFU for the 1×10^3 treatment, and 0.4 to 0.6 CFU for the 1×10^2 treatment. PBS was used as a PCR negative control. DNA was extracted from the tissue by spin column method (DNeasy Tissue Kit, Qiagen). Tissues (25 to 35 mg) were homogenized with a sterile, DNase-free pestle in a 1.7 ml microcentrifuge tube containing 180 µl homogenization buffer. The homogenate was treated with Proteinase K and incubated overnight in a 55°C water bath. Manufacturer's instructions were followed for the remainder of the DNA isolation procedure. Genomic DNA was eluted from the spin-column with 50 µl of nuclease-free water and stored at –20°C. Fifty ng of DNA was used for PCR detection.

Detection of PCR products. PCR products were detected by electrophoresing 15 µl of PCR product in a 1.5% agarose gel (2.0 % agarose gel for testing specificity of primers).

Fish and culture conditions. Channel catfish (NWAC 103 strain) were used as host fish and reared at the United States Department of Agriculture, Aquatic Animal Health Research Laboratory, Auburn, Alabama. In the study conducted in April 2003, fingerling channel catfish weighed 12.1 g; fry used in the July experiment weighed 3.2 g. Channel catfish were acclimatized for 14 d prior to experiments in flow-through 57 l glass aquaria supplied with 1 l min^{-1} dechlorinated water maintained at 26 ± 2°C with a central water heater. Fish were fed daily to satiation with Aquamax Grower 400 (PMI Nutrition International). Feed was withheld 10 d prior to infection in the April experiment and 14 d in the July experiment through their completion to increase susceptibility of channel catfish to *Flavobacterium columnare* (Shoemaker et al. 2003).

Intramuscular challenge. In early April 2003, channel catfish fingerlings were challenged with 100 µl 1×10^8 CFU ml^{-1} of *Flavobacterium columnare* ARS-1 by intramuscular (i.m.) injection. The interior of 4 tanks was bisected with Tank-Divider™ perforated partitions (Penn-Plax). Water flowed from the rear to the front section. Challenged individuals (i.m.-Challenge; n = 10) were fin-clipped and placed in the rear section of tanks with unmarked, unchallenged cohabitants (i.m.-Challenge Cohab; n = 10). The front of 3 tanks was stocked with 10 unchallenged control (i.m.-Sham Cohab) and 10 sham-injected (100 µl sterile Shieh broth; i.m.-Sham). The fourth tank served as a negative control and was stocked with 10 unchallenged individuals in both the front and rear sections. Three fish were randomly sampled from each tank section 9 d post-challenge when clinical signs of columnaris disease became evident.

Immersion challenge. Channel catfish fry were challenged in early July 2003 with *Flavobacterium columnare* ARS-1 (1.5×10^7 CFU ml^{-1}) by immersion bath. Four tanks were again divided with partitions as described above. In 3 tanks, challenged individuals (n = 25) were placed in the rear section, and 25 unchallenged fish were stocked in the front. One tank served as a negative control and was stocked with 25 unchallenged individuals in the front and rear sections.

Tank clearance. After completion of the immersion infection experiment, *Flavobacterium columnare* ARS-1 was added to tank water at a concentration of 1×10^3 CFU ml^{-1}. Water and biofilm (scraped from tank walls) samples were collected at Day 0 (prior to spiking), Days 1 to 4 (after spiking) and assayed by PCR to determine clearance of *F. columnare*.

Water sampling and preparation. Fifty ml of tank water was collected from front and rear sections of aquaria and aseptically transferred to a sterile plastic centrifuge tube. One hundred µl of sampled water was cultured on Shieh agar using the spread-plate method for identification of *Flavobacterium columnare* by standard culture methods. Samples were then immediately processed by centrifuging (4000 × *g*) at 4°C for 20 min. Water was decanted leaving pelleted cells and debris. The water and debris were resuspended in 100 µl sterile water, and DNA was extracted by the Qiagen spin-column method.

Tissue sampling and preparation. Tissues were sampled from fish after external clinical signs of columnaris disease were evident. Fish were killed with a lethal dose of MS-222 (tricaine methane sulfonate; 200 mg l^{-1}). Gill, skin, and liver samples were first swabbed with a sterile loop and streaked onto Shieh agar plates and then placed into a cryovial and frozen on dry ice. Long-term storage was at –20°C. DNA was isolated from tissue samples using the spin-column method. DNA from tissues (25 to 35 mg) was extracted as described above.

Statistical analyses. Data from the challenge experiments were analyzed by general linear model analysis of variance (ANOVA), and differences between means were determined by least significant difference analysis (LSD). Tissue or water samples positive or negative for *Flavobacterium columnare* were scored a 1 or a 0, respectively, which were averaged for each tank for use in statistical analyses. Comparisons were made between PCR and culture detection of *F. columnare* for each tissue type sampled (challenge treatment and detection method were independent factors) and also between tissue types for detection by both PCR and culture (challenge treatment and tissue type were independent factors). Detection in water samples by PCR and culture were compared in both studies (tank

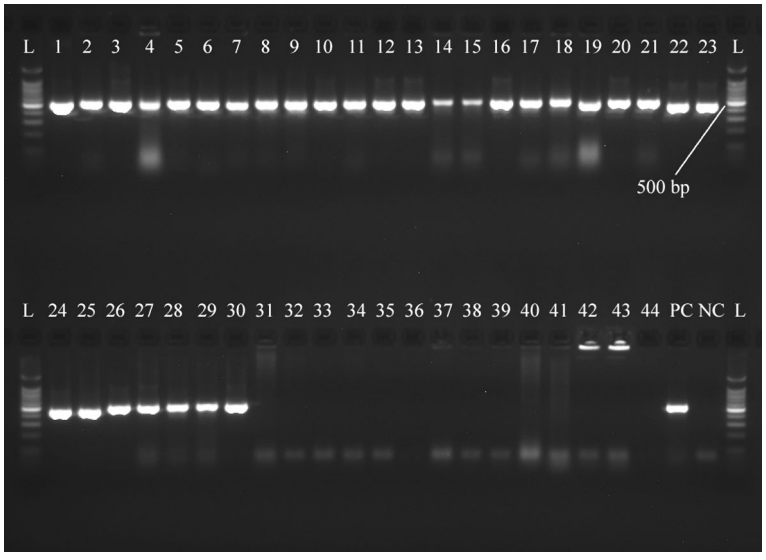

Fig. 1. Specificity of the FCISRFL and FCISRR1 primer pair for the detection of *Flavobacterium columnare*. Lane numbers correspond to the number of the bacterium in Table 1. Lanes marked L contain the 100 bp DNA ladder. PC is the positive control (*F. columnare* ARS-1 DNA), and NC is the negative control (no template DNA). Five µl of each PCR amplification was loaded into a well of an agarose gel (2.0%) containing ethidium bromide

section and detection method were independent factors). The control tank, used only to ensure fish were not exposed to columnaris disease from the system, was excluded from analyses in both challenge experiments. A significance level of $\alpha = 0.05$ was used for all statistical analyses.

RESULTS

Primer specificity

The FCISRFL and FCISRR1 primer pair only yielded the expected amplified fragment when using DNA from *Flavobacterium columnare* isolates and not those of closely related species or other bacterial fish pathogens (Fig. 1 & Table 1). ISR amplicons from *F. columnare* ranged in size from 500 to 550 bp, except for the BZ-1, BZ-2, BZ-4, and BZ-5 isolates, which produced 2 PCR products that were approximately 450 and 475 bp. The 2 products are not easily discernable by gel electrophoresis (Fig. 1).

Primer sensitivity

Primers FCISRFL and FCISRR1 were able to detect as little as 7 CFU of *Flavobacterium columnare* (Fig. 2) when pure cultures were used. It was also possible to detect *F. columnare* from artificially inoculated fish samples. After total DNA extraction, blood, gill, skin, and liver samples spiked with *F. columnare* ARS-1 were also positive by PCR (Table 2). As expected, sensitivity decreased when a more complex sample was used. Actual concentrations in PCR positive amplifications were: 40.0 CFU mg^{-1} whole blood, 58.5 CFU mg^{-1} gill, 40.3 CFU mg^{-1} skin, and 29.7 CFU mg^{-1} liver. Controls and samples treated with lower concentrations of *F. columnare* ARS-1 were PCR negative.

Intramuscular challenge

Water samples were PCR and culture negative for *Flavobacterium columnare* ARS-1 until 4 d post-challenge (Table 3). However, water samples but not fish from 2 tanks tested positive by PCR on Days 0, 1, and 2 post-challenge. DNA sequencing later confirmed that the positive samples were *F. columnare* (*F. columnare* strain LP8; GenBank Accession #AB031221; 97% sequence homology) but were not the same strain used for the experimental challenge. The ISR amplicon of the LP8 strain was approximately 650 bp and larger than the fragment normally associated with the isolates we tested. All PCR positives from water samples thereafter had ISR sequences identical to the challenge organism, *F. columnare* ARS-1. On Days 4 and 5 post-challenge, a greater proportion of water samples from the rear sections of aquaria (containing challenged individuals) were PCR and culture positive for *F. columnare* compared to the front sections, but overall,

Fig. 2. Sensitivity of the FCISRFL and FCISRR1 primer pair for the detection of *Flavobacterium columnare* ARS-1. Numbered lanes represent: Lanes 1 and 15, 100 bp DNA ladder; Lane 2, PCR containing 7×10^4 CFU; Lane 3, 7×10^3 CFU; Lane 4, 7×10^2 CFU; Lane 5, 7×10^1 CFU; Lane 6, 7×10^0 CFU; Lane 7, 7×10^{-1} CFU; Lane 8, 7×10^{-2} CFU; Lane 9, 7×10^{-3} CFU; Lane 10, 7×10^{-4} CFU; Lane 11, 7×10^{-5} CFU; Lane 12, 7×10^{-6} CFU; Lane 13, positive control; Lane 14, negative control. Fifteen µl of each PCR amplification was loaded into a well of an agarose gel (1.5%) containing ethidium bromide

Table 2. *Ictalurus punctatus.* Concentrations of *Flavobacterium columnare* ARS-1 used in the tissue spiking experiment. Bacterial dilutions were made in 1% phosphate buffered saline (PBS)

Tissue	CFU mg^{-1} tissue	PCR (+/–)
Blood	40.0	+
	4.0	–
	0.4	–
	0.0	–
Gill	58.5	+
	5.8	–
	0.6	–
	0.0	–
Skin	40.3	+
	4.0	–
	0.4	–
	0.0	–
Liver	29.7	+
	3.0	–
	0.3	–
	0.0	–

Table 3. Proportion of water samples PCR and culture positive for *Flavobacterium columnare* in the intramuscular challenge experiment. (Tanks 2–4). All water samples for the control tank (Tank 1) were PCR and culture negative. TSF: tank section front; TSR: tank section rear; Avg: average

Days post-challenge	PCR			Culture		
	TSF	TSR	Avg	TSF	TSR	Avg
0	0[a]	0	0	0	0	0
1	0[a]	0[a]	0	0	0	0
2	0[a]	0[a]	0	0	0	0
3	0	0	0	0	0	0
4	0.66	1.00	0.83	0.33	0.66	0.50
5	0.66	1.00	0.83	0.66	0.66	0.66
6	1.00	1.00	1.00	1.00	0.66	0.83
7	1.00	1.00	1.00	1.00	1.00	1.00
9	1.00	1.00	1.00	1.00	1.00	1.00

[a]Positive PCR. Sequence results indicated a *F. columnare* isolate (650 bp; BLAST search and comparison to intergenic spacer region (ISR) sequences from USDA *F. columnare* isolates) different from the challenge organism

there was no difference in detection rate between tank sections by PCR ($p = 0.06$) or culture ($p = 0.82$). PCR detection in water samples proved to be more sensitive than culture on Shieh agar plates before clinical signs of columnaris disease were evident in fish (especially Days 4 and 5) (Table 3); however, the overall detection rate of PCR and culture was not different ($p = 0.60$). After Day 5, nearly all tanks, regardless of section tested, were PCR and culture positive.

Signs of columnaris disease were evident 8 d post-challenge, and fish survival decreased significantly from 8 to 11 d post-challenge (Fig. 3). Survival was

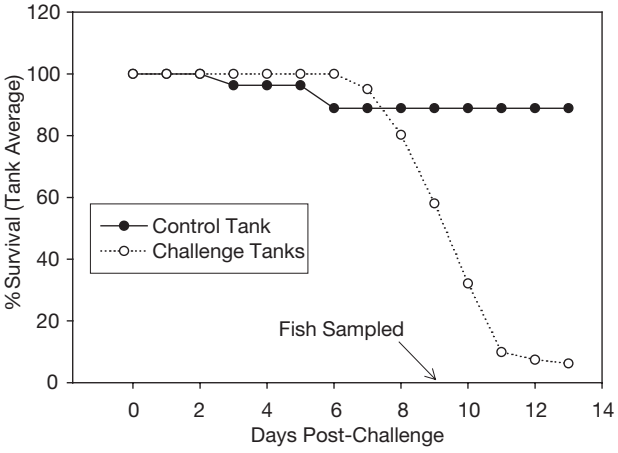

Fig. 3. *Ictalurus punctatus.* Percent survival of fish challenged with *Flavobacterium columnare* ARS-1 by intramuscular (i.m.) injection

approximately 10% by the end of the study. Infection rates did not appear to be different between experimental treatments; fish, regardless of challenge protocol, contracted columnaris disease. Tissues were sampled 9 d post-challenge. Tissues from individuals in the control tank were PCR and culture negative for the presence of *Flavobacterium columnare*. Challenge treatment did not have an effect on detection of columnaris disease by either PCR ($p = 0.06$) or culture ($p = 0.80$). Detection of *F. columnare* in gill by PCR was 100% regardless of experimental treatment or section of tank sampled and was significantly greater than by culture ($p < 0.001$) (Table 4). The detection rate for *F. columnare* by culture was higher than PCR for the skin ($p = 0.05$). The liver produced few positive identifications for *F. columnare* with either detection method. The gill was the best site for detection of *F. columnare* by PCR ($p < 0.001$) and by culture ($p < 0.001$) followed by skin and liver; all possible comparisons of mean detection values were significant for both PCR and culture (Table 4). To confirm positive PCR identification of the challenge organism in tissue and water samples, 1 positive PCR amplification for each tissue in each tank section, if available, and 1 positive water sample for each tanks section on all sampling days, if available, were sequenced directly from PCR. In addition, colonies isolated on agar plates from tissue and water samples and from moribund fish were picked and used for PCR detection of the challenge organism. A maximum of 3 colonies were tested per plate (n = 1 plate per tissue or water sample). All positive PCR samples had ISR sequence homologies greater than 99% when compared to the challenge organism.

Table 4. *Ictalurus punctatus.* Proportion of tissue samples PCR and culture positive for *Flavobacterium columnare* in the intramuscular challenge experiment. All treatments listed below were in challenge tanks (Tanks 2–4). All tissue samples for the control tank (Tank 1) were PCR and culture negative for the challenge organism (not shown). i.m.-Sham Cohab: unchallenged fish and cohabitants of IM-Sham; i.m.-Sham: intramuscular injection with 100 ul sterile Shieh broth and a cohabitant of the unchallenged control; i.m.-Challenge Cohab: unchallenged cohabitant of the challenged fish; i.m.-Challenge: intramuscular injection with 100 ul 1×10^8 CFU *F. columnare* ARS-1. Tissue samples (n = 9/tissue) were collected 9 d post challenge. Tissue average: mean values for challenge tanks. Values with different superscript letters (a, b, c) are significantly different by least significant difference (LSD) post-hoc analysis

Treatment	Tank section	PCR			Culture		
		Gill	Skin	Liver	Gill	Skin	Liver
i.m.-Sham Cohab	Front	1.00	0	0	0.44	0.33	0
i.m.-Sham	Front	1.00	0.22	0.11	0.33	0.44	0.11
i.m.-Challenge Cohab	Rear	1.00	0.11	0	0.67	0.11	0
i.m.-Challenge	Rear	1.00	0.22	0	0.67	0.22	0
Tissue average		1.00[a]	0.14[b]	0.03[c]	0.53[a]	0.28[b]	0.03[c]

Table 5. Proportion of water samples PCR and culture positive for *Flavobacterium columnare* in the immersion challenge experiment (Tanks 2–4); rear of tanks held challenged fish; water flowed from rear to front. All water samples for the control tank (Tank 1) were PCR and culture negative. Tank water was spiked with *F. columnare* (final concentration 1×10^3 CFU ml^{-1}). Water was sampled (Time 0) just prior to treatment. Tank partitions were removed for the tank clearance experiment. TSF: tank section, front; TSR: tank section, rear; Avg: average

Days post-challenge	PCR			Culture		
	TSF	TSR	Avg	TSF	TSR	Avg
0	0	0	0	0	0	0
1	0.66	0.33	0.50	0	0	0
2	1.00	0.66	0.83	1.00	0.66	0.83
3	1.00	1.00	1.00	0.66	0.33	0.50
7	0.66	0.66	0.66	0.66	0.33	0.50

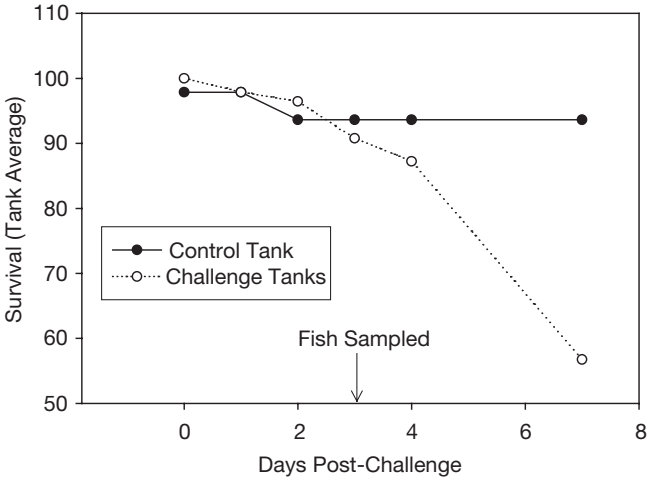

Fig. 4. *Ictalurus punctatus.* Percent survival of fish challenged with *Flavobacterium columnare* ARS-1 by immersion

Table 6. *Ictalurus punctatus.* Proportion of tissue samples PCR and culture positive for *Flavobacterium columnare* in the immersion challenge experiment (Tanks 2–4). All tissue samples for the control tank (Tank 1) were PCR and culture negative for the challenge organism (not shown). Unchallenged: unchallenged fish in front section of challenge tanks; Immersion: challenged by immersion with 1.5×10^7 CFU ml^{-1} *F. columnare* ARS-1 (rear of tank). Proportion of samples positive (Tanks 2–4). Tissue samples (n = 9/tissue) were collected 3 d post challenge. All tissue samples for the control tank (Tank 1) were PCR and culture negative. Tissue average: mean values for challenge tanks. Values with different superscript letters (a, b, c) are significantly different by LSD post-hoc analysis

Treatment	Tank section	PCR		Culture	
		Gill	Skin	Gill	Skin
Unchallenged	Front	1.00	0.22	0.67	0.11
Immersion	Rear	0.78	0.22	0.45	0.22
Tissue average		0.89[a]	0.22[b]	0.56[a]	0.16[b]

Immersion challenge

Flavobacterium columnare was detected in tank water on Day 1 and Day 2 post-infection by PCR and culture on Shieh agar, respectively (Table 5). Detection by PCR was more sensitive than detection by culture ($p = 0.04$), with a higher proportion of tanks testing positive for *F. columnare* regardless of tank section (front or rear; $p = 0.30$) sampled. All water samples from the control tanks were negative for *F. columnare*.

Signs of columnaris disease began on Day 2 post-challenge. Fish survival decreased steadily from Day 2 to the end of the experiment (Fig. 4). Percent survival was below 60% by Day 7 post-challenge. Fish were sampled 3 d post-challenge. PCR detection of *Flavobacterium columnare* was higher in gill samples compared to culture on Shieh agar ($p = 0.03$) (Table 6). The

number of *F. columnare* positives for skin samples was the same for both detection methods (p = 0.63). Detection of *F. columnare* by PCR (p = 0.001) and also by culture (p = 0.03) was significantly greater in gill compared to skin.

Water and tissue samples were tested as outlined above to confirm the presence of the challenge organism in PCR and culture positives. The ISR sequence homology between the PCR positive reactions or cultured *Flavobacterium columnare* isolates and the challenge organism was greater than 99%. In addition, infection rates, as defined by positive culture (p = 0.75) or PCR (p = 0.06) and survival rate, were not different between unchallenged (front of challenge tanks) and infected fish (rear of challenge tanks).

Tank clearance

Flavobacterium columnare was detected by PCR in tank water 1 d post-treatment but not thereafter. After 4 d but not before, *F. columnare* was detected in the biofilm scraped from tank walls.

DISCUSSION

Specificity of the FCISRFL and FCISRR1 PCR primer pair was screened against 30 isolates of *Flavobacterium columnare* cultured from fish, including the type strain, 5 other representatives from the genus *Flavobacterium*, 5 non-*Flavobacterium* bacteria pathogenic to channel catfish, and *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*). The target amplicon, a 500 to 550 bp region of the 16-23S rDNA ISR, was only amplified from *F. columnare*. However, the BZ-1, BZ-2, BZ-4, and BZ-5 *F. columnare* isolates, cultured from tilapia in Brazil, produced 2 PCR products of approximately 450 and 475 bp. *F. columnare* has at least 2, probably 3, ribosomal operons as determined by ribotyping (Arias unpubl. data). These 2 ISR fragments may result from a missing tRNA gene in the ribosomal operon. In addition, the ISR can exhibit a large degree of length variation, and multiple fragments produced by PCR targeting the ISR regions are not uncommon (Jensen et al. 1993). These Brazilian isolates, although phenotypically identified as *F. columnare* formed a unique genetic group within the species showed by different genotyping methods (Arias et al. 2004).

The ISR primers were sensitive, capable of detecting as few as 7 CFU, which is comparable to the results of Bader et al. (2003). However, a nested PCR reaction was needed using Bader's method to achieve a level of sensitivity similar to ours, and only 6 *Flavobacterium columnare* isolates were tested. Sensitivity is often lost when trying to amplify larger fragments from fragmented DNA (Bader et al. 2003). The target amplicon in our method was smaller (500 to 550 bp compared to 1193 bp) and may account for its greater sensitivity. Toyama et al. (1996) also successfully developed a PCR detection method to detect *F. columnare*. The primers amplified a portion of the 16S rRNA gene of *F. columnare*, and even though they amplified other closely related species, the size of the PCR product was unique for *F. columnare*. Two primer sets were needed to ensure detection and to discriminate *F. columnare* from other bacteria, thereby increasing the complexity of the method. Bader & Shotts (1998) designed primers that amplified an 800 bp region of the 16S rRNA gene of *F. columnare* and not of *F. psychrophilum* or *Flexibacter maritimus* (now *Tenacibaculum maritimum*). However, the columnaris specific primers also worked with *F. columnare* ATCC 43622, which has been reclassified as *F. johnsoniae* 43622. BIOLOG (Biolog) and riboprinting analyses of ATCC 43622 conducted by the ATCC (pers. comm.) and Amplified Fragment Length Polymorphism (AFLP) and ISR analyses of *Flavobacterium* spp. performed in our own lab (T. Welker unpubl. data) confirm that ATCC 43622 is probably *F. johnsoniae*. Triyanto et al. (1999) developed primers specific to *F. columnare* capable of identifying 3 genomovars. A 2-step procedure was needed, where an initial amplification using universal 16S rRNA primers was used to produce template for the specific primers. Nested PCR procedures increase the risk of cross contamination and false-positives, as well as adding extra cost and increasing detection time.

The PCR method developed in this work was more sensitive than standard culture techniques for detection of *Flavobacterium columnare* in tissues of channel catfish and in culture water. Plate cultivation may be less sensitive in part, because *F. columnare* is often overgrown by fast-growing bacteria, such as *Pseudomonas* spp. (Tiirola et al. 2002). Although other researchers have used PCR to identify *F. columnare* from fish infected with columnaris disease (e.g. Triyanto et al. 1999, Nilsson & Strom 2002, Tiirola et al. 2002, Bader et al. 2003), none have applied molecular identification methods to water samples. Successful application of PCR detection of *F. psychrophilum* in water samples has been demonstrated (Wiklund et al. 2000). PCR-based detection is a suitable tool for indicating the presence of *F. columnare* and is most likely best utilized in research studies. Because *F. columnare* is an environmental bacterium, use of PCR detection for management of the disease may be limited.

Detection of *Flavobacterium columnare* in the gill was more sensitive than in the skin and liver. Bader et al. (2003) determined mucus to be the best location for molecular detection of *F. columnare* and blood the worst. Although we did not test mucus, we did test skin samples, which did not have the same reproducibility of the gill. Adherence to the gill is an important aspect of the pathogenesis of columnaris disease (Decostere et al. 1999). Water is actively pumped across gill lamellae, and therefore, gills may be a good choice for detection of *F. columnare* in fish. Few liver samples were positive for *F. columnare*. Systemic columnaris disease is most often found with acute infections (Groff & LaPatra 2000). Because of the chronic nature of the IM injection challenge (signs became evident after 8 d compared to only 2 d for the immersion experiment), the disease was probably not systemic, and detection of *F. columnare* in liver was limited.

The pathogenesis of columnaris disease did not differ between infection modalities in either experiment. Infection rates for challenged fish, unchallenged cohabitants, and unchallenged noncohabitants were not significantly different. Fish-to-fish contact was not needed for horizontal transmission of columnaris disease (i.e. unchallenged noncohabitants separated from infected individuals by a perforated partition contracted columnaris disease). This confirms earlier results obtained by Austin & Austin (1993) and Wakabayashi (1993). Columnaris disease occurred much faster in individuals infected by immersion. The individuals used in the immersion challenge trial were significantly smaller and starved for a longer period prior to challenge, which may have increased susceptibility to *Flavobacterium columnare* (Shoemaker et al. 2003). Unexpectedly, individuals challenged by i.m. injection did not show more symptoms of columnaris disease than unchallenged cohabitants. The disease did not appear to become systemic, and the infection most likely manifested itself in the skin or muscle at the site of injection. As the infection progressed slowly, *F. columnare* shed from challenged individuals infected unchallenged individuals.

We have developed a rapid, specific, and sensitive method for detection of *Flavobacterium columnare* from fish and water samples. Our PCR method allows detection of *F. columnare* in less than 8 h from fish and water samples and is more sensitive than standard culture on bacteriological media. Further testing should be conducted with environmental samples from aquaculture ponds and water sources, and extensive validation procedures (Hiney & Smith 1998) should also be completed to certify the practical use of this new PCR method.

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LITERATURE CITED

Arias CR, Welker TL, Shoemaker CA, Abernathy JW, Klesius PH (2004) Genetic fingerprinting of *Flavobacterium columnare* isolates from cultured fish. J Appl Microbiol 97:421–428

Austin B, Austin DA (1993) Bacterial fish pathogens: disease in farmed and wild fish, 2nd edn. Ellis Horwood, New York

Bader JA, Shotts EB Jr. (1998) Identification of *Flavobacterium* and *Flexibacter* species by species-specific polymerase chain reaction primers to the 16S ribosomal RNA gene. J Aquat Anim Health 10:311–319

Bader JA, Shoemaker CA, Klesius PH (2003) 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*. J Microbiol Methods 52:209–220

Bernardet JF, Grimont PAD (1989) Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida, and Masumura 1986. Int J Syst Bacteriol 39:346–354

Bernardet JF, Segers P, Vancanneyt M, Berthe F, Kersters K, Vandamme P (1996) Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978). Int J Syst Bacteriol 46:128–148

Bullock GL, Conroy DA, Snieszko SF (1971) Bacterial diseases of fishes. In: Snieszko SF, Axelrod HR (eds) Diseases of fishes. TFH Publications, Neptune City, NJ, p 1–151

Davis HS (1922) A new bacterial disease of fresh-water fishes. Bull US Bureau Fisheries 38:261–280

Decostere A, Haesebrouk F, Van Driessche E, Charlier G, Ducatelle R (1999) Characterization of the adhesion of *Flavobacterium columnare* (*Flexibacter columnaris*) to gill tissue. J Fish Dis 22:465–474

Groff JM, LaPatra SE (2000) Infectious diseases impacting the commercial culture of salmonids. J Appl Aquacult 10: 17–90

Hiney MP, Smith P (1998) Validation of polymerase chain reaction based techniques for proxy detection of bacterial fish pathogens: framework, problems and possible solution for environmental applications. Aquaculture 162: 41–68

Jensen MA, Webster JA, Straus N (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl Environ Microbiol 59:945–952

Nilsson WB, Strom MS (2002) Detection and identification of bacterial pathogens of fish in kidney tissue using terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes. Dis Aquat Org 48:175–185

Sakai M, Soliman MK, Yoshida T, Kobayashi M (1992) Identification of pathogenic fish bacteria using the API ZYM system. Can J Fish Aquat Sci 50:1137–1141

Shamsudin MN, Plumb JA (1996) Morphological, biochemical, and physiological characterization of *Flexibacter columnaris* isolates from four species of fish. J Aquat Anim Health 8:335–339

Shieh HS (1980) Studies on the nutrition of a fish pathogen, *Flexibacter columnaris*. Microbios Lett 13:129–133

Shoemaker CA, Klesius PH, Lim C, Yildirim M (2003) Feed deprivation of channel catfish, *Ictalurus punctatus* (Rafinesque), influences organosomatic indices, chemical composition and susceptibility to *Flavobacterium columnare*. J Fish Dis 26:1–9

Tiirola M, Valtonen ET, Rintamäki-Kinnunen P, Kulomaa MS (2002) Diagnosis of flavobacteriosis by direct amplification of rRNA genes. Dis Aquat Org 51:93–100

Toyama T, Kita-Tsukamoto K, Wakabayashi H (1996) Identification of *Flexibacter maritimus*, *Flavobacterium branchiophilum* and *Cytophaga columnaris* by PCR targeted 16S ribosomal DNA. Fish Pathol 31:25–31

Triyanto, Kumamaru A, Wakabayashi H (1999) The use of PCR targeted 16S rDNA for identification of genomovars of *Flavobacterium columnare*. Fish Pathol 34:217–218

Wagner BA, Wise DJ, Khoo LH, Terhune JS (2002) The epidemiology of bacterial diseases in food-size channel catfish. J Aquat Anim Health 14:263–272

Wakabayashi H (1993) Columnaris disease. In: Inglis V, Roberts RJ, Bromage NR, (eds) Bacterial diseases of fish. Blackwell Scientific Publications, Oxford, p 23–39

Wiklund T, Madsen L, Bruun MS, Dalsgaard I (2000) Detection of *Flavobacterium psychrophilum* from fish tissue and water samples by PCR amplification. J Appl Microbiol 88: 299–307

Wolke R.E. (1975) Pathology of bacterial and fungal diseases affecting fish. In: Ribelin WE, Migaki G (eds) The pathology of fishes. University of Wisconsin Press, Madison, WI, p 33–116

Zavaleta AI, Martínez-Murcia AJ, Rodrígues-Valera F (1996) 16S-23S rDNA intergenic sequences indicate that *Leuconostoc oenos* is phylogenetically homogeneous. Microbiology 142:2105–2114

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