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Multiplex-PCR for simultaneous detection of 3 bacterial fish pathogens, *Flavobacterium columnare*, *Edwardsiella ictaluri*, and *Aeromonas hydrophila*

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ABSTRACT: A multiplex PCR (m-PCR) method was developed for simultaneous detection of 3 important fish pathogens in warm water aquaculture. The m-PCR to amplify target DNA fragments from *Flavobacterium columnare* (504 bp), *Edwardsiella ictaluri* (407 bp) and *Aeromonas hydrophila* (209 bp) was optimized by adjustment of reaction buffers and a touchdown protocol. The lower detection limit for each of the 3 bacteria was 20 pg of nucleic acid template from each bacteria per m-PCR reaction mixture. The sensitivity threshold for detection of the 3 bacteria in tissues ranged between 3.4×10^2 and 2.5×10^5 cells g^{-1} of tissue (channel catfish *Ictalurus punctatus* Rafinesque). The diagnostic sensitivity and specificity of the m-PCR was evaluated with 10 representative isolates of each of the 3 bacteria and 11 other Gram-negative and 2 Gram-positive bacteria that are taxonomically related or ubiquitous in the aquatic environment. Except for a single species (*A. salmonicida* subsp. s*almonicida*), each set of primers specifically amplified the target DNA of the cognate species of bacteria. m-PCR was compared with bacteriological culture for identification of bacteria in experimentally infected fish. The m-PCR appears promising for the rapid, sensitive and simultaneous detection of *Flavobacterium columnare*, *E. ictaluri* and *A. hydrophila* in infected fish compared to the time-consuming traditional bacteriological culture techniques.

KEY WORDS: Multiplex-PCR · Fish · *Edwardsiella* · *Flavobacterium* · *Aeromonas*

INTRODUCTION

Edwardsiella ictaluri, *Flavobacterium columnare* and *Aeromonas hydrophila* are 3 major bacterial pathogens of fish that cause diseases with significant economic impact on the aquaculture industry. *E. ictaluri*, the primary cause of enteric septicemia in channel catfish (*Ictalurus punctatus* Rafinesque), has also been frequently associated with disease in other food fish (Plumb & Sanchez 1983). *Flavobacterium columnare*, the cause of columnaris disease, is comprised of 4 distinct genogroups (Arias et al. 2004) and has a broad host range, with known infections in over 36 species of fish throughout the world (Anderson & Conroy 1969). *A. hydrophila* infects many species of fish and other terrestrial animals (Colwell et al. 1986), including humans (Janda & Duffey 1988). Particular importance is attached to *A. hydrophila* from a public health perspective due to its involvement in food-borne gastroenteritis and various opportunistic infections in immunocompromised human patients (Altwegg & Geiss 1989, Janda & Abbott 1998, Ullmann et al. 2005).

Enteric septicemia of catfish (ESC), reported by 67% of operations within 1 yr, and columnaris disease, reported by 50% of operations within 1 yr, are the 2

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most important bacterial diseases affecting the aquaculture industry in the USA (United States Department of Agriculture 2003). The prevalence and impact of septicemic disease in fish caused by *Aeromonas hydrophila* (referred to as motile aeromonad septicemia, MSA), is less known. However, *A. hydrophila* is ubiquitous in aquatic ecosystems (Holmes et al. 1996) and recent studies have shown that catfish latently infected with *A. hydrophila* (carriers) could shed the organism when co-infected with *Edwardsiella ictaluri* (Nusbaum & Morrison 2002). Since all 3 species of bacteria (*Flavobacterium columnare, E. ictaluri*, and *A. hydrophila*) are ubiquitous in the aquatic environment and fish are reared in extensive pond acreages with high stocking densities (20 000 to 30 000 fish ha^{-1}), the occurrence of coinfections or multiple infections in the same host should not be overlooked (Jack et al. 1992). Traditional methods of diagnosing bacterial infections using culture techniques require several days to arrive at a definitive diagnosis, resulting in delayed implementation of control measures and increased potential for spreading of disease. Because PCR can target unique genetic sequences of microorganisms, PCR-based nucleic acid amplification techniques have gained recognition as rapid, sensitive, and specific methods for detection of disease-causing pathogens in various aquaculture species (Vantarakis et al. 2000, Del Cero et al. 2002, Bader et al. 2003, Bilodeau et al. 2003). When multiple bacterial pathogens are likely to occur, as in the aquatic environment, amplification of multiple target genes in a single reaction mixture is possible with the multiplex PCR (m-PCR) method (Brasher et al. 1998, Del Cero et al. 2002, Panicker et al. 2004), thus reducing cost, time and effort without compromising the test utility. In this study, we developed an m-PCR method for simultaneous identification of *F. columnare*, *E. ictaluri*, and *A. hydrophila*, 3 of the most important bacterial pathogens causing extensive losses in the channel catfish aquaculture industry.

MATERIALS AND METHODS

Bacteria isolates and culture conditions. The bacterial isolates used in this study are listed in Table 1. The majority of the fish-pathogenic bacteria were originally isolated from diseased fish, characterized into their respective genera and species using standard methods (Arias et al. 2004, Panangala et al. 2005), and maintained in the archived culture repository of the Aquatic Animal Health Research Unit, Auburn, Alabama, USA. Additional cultures were kindly provided by John M. Grizzle (Department of Fisheries and Allied Aquaculture, Auburn University, Alabama, USA), Andrew E. Goodwin (Department of Aquaculture/Fisheries, University of Arkansas, Pine Bluff, Arkansas, USA) and Ronald D. Schultz (Department of Pathobiology, University of Wisconsin, Madison, Wisconsin, USA). The bacterial isolates were cultured directly from glycerol stocks on brain heart infusion agar or blood agar plates (tryptic soy agar with 5% v/v defibrinated sheep blood: Difco) for *Edwardsiella ictaluri* and *Aeromonas hydrophila,* and Shieh agar (Shieh 1980) for *Flavobacterium columnare.* Single colonies picked after incubation for 36 h were transferred to 10 ml brain heart infusion broth and cultured to log-phase growth at 28°C in a shaker water bath. For isolation of bacteria from tissues of experimentally infected fish, Shieh medium modified by replacing peptone with tryptone (Difco) was used for selective isolation of *F. columnare.* For isolation of *E. ictaluri*, Shotts selective medium (Shotts & Waltman 1990) was used, and for isolation of *A. hydrophila,* blood agar plates were used. Results were recorded after 24 to 48 h of incubation at 28°C. Isolates were distinguished as *F. columnare*, *E. ictaluri* and *A. hydrophila* on the basis of their morphological appearance on selective media and Gram-stain characteristics. Select isolates were characterized biochemically to species level using API 20E test strips (bioMerieux) and additional biochemical tests (when necessary) according to established criteria (Decostere et al. 1998, Altwegg 1999, Farmer 2003). Occasionally, contaminating organisms were also isolated on culture media, but these organisms were readily distinguished from *F. columnare*, *E. ictaluri* and *A. hydrophila* on the basis of morphological and biochemical characteristics.

Infection of fish and sample collection. Fingerling (~12 to 14 g) channel catfish *Ictalurus punctatus* Rafinesque, National Warmwater Aquaculture Center Strain 103, obtained from disease-free stock reared at the Aquatic Animal Health Research Unit, were used for experimental infection. The fish were maintained in 58 l glass aquaria with flow-through dechlorinated tap water, constant aeration, a water temperature of 26 ± 2°C and a 12:12 h light:dark photoperiod. A commercial diet (Aquamax Grower 400 Brentwood) was fed daily to satiation. Initially, fish were randomized into 3 groups, with 7 fish per group. Fish in Group 1 were injected intraperitoneally with 0.1 ml of a log-phase broth culture of *Flavobacterium columnare* ARS-1, containing ~1×10^5 CFU ml^{-1}, Group 2 fish were similarly injected with *Edwardsiella ictaluri* (American Type Culture Collection: ATCC-33202), and Group 3 fish were injected with ~10^2 CFU ml^{-1} of *Aeromonas hydrophila* K106K. Subsequently, 3 other groups of 7 fish per group (Groups 4 to 6) were injected with a combination of the organisms to simulate mixed infections as follows: Group 4, *F. columnare* + *E. ictaluri*

Table 1. Bacteria used in this study. ATCC: American Type Culture Collection; CECT: Colección Espanola de Cultivos Tipo; BRL: GIBCO, Bethesda Research Laboratories, Maryland. Isolates with prefix AL were from diseased catfish, isolated by Auburn University Fish Diagnostic Laboratory, Auburn University, Alabama; isolates with prefix ALG were provided by William Hemstreet, Alabama Fish Farming Center, Greensboro, Alabama; isolates from Mississippi were provided by David Wise, Fish Diagnostic Laboratory, Thad Cochran National Warmwater Aquaculture Center, Stoneville, Mississippi; isolates from Arkansas were provided by Andrew E. Goodwin, Department of Aquaculture, University of Arkansas at Pine Bluff, Arkansas

Species	Isolate	Source	Origin
Edwardsiella ictaluri	ATCC-33202	Channel catfish *Ictalurus punctatus* Rafinesque	ATCC
E. ictaluri	AL-93-75	Channel catfish	Alabama
E. ictaluri	ALG-03-58	Channel catfish	Alabama
E. ictaluri	ALG-03-161	Channel catfish	Alabama
E. ictaluri	013-S99-1908	Channel catfish	Mississippi
E. ictaluri	016-S99-1911	Channel catfish	Mississippi
E. ictaluri	017-S99-1914	Channel catfish	Mississippi
E. ictaluri	003-S99-1760	Channel catfish	Mississippi
E. ictaluri	IA-30-NJ#1	Tadpole madtom *Noturus gyrinus* Mitchell	New Jersey
E. ictaluri	EILO	Walking catfish *Clarius batrachus* L.	Thailand
Flavobacterium columnare	ATCC-23463	Chinook salmon *Oncorhynchus tshawytscha* Walbaum	ATCC
F. columnare	ARS-1	Channel catfish	Alabama
F. columnare	ALG-00-530	Channel catfish	Alabama
F. columnare	ALG-00-522	Channel catfish	Alabama
F. columnare	ALG-02-036	Largemouth bass *Micropterus salmoides* Lacepede	Alabama
F. columnare	BioMed	Channel catfish	Alabama
F. columnare	HS	Channel catfish	Alabama
F. columnare	LSU	Channel catfish	Louisiana
F. columnare	MS-02-463	Channel catfish	Mississippi
F. columnare	IR	Common carp *Cyprinus carpio* L.	Israel
Aeromonas hydrophila	CECT-839	Milk	CECT
A. hydrophila	CIB	Channel catfish	Alabama
A. hydrophila	K106K	Channel catfish	Alabama
A. hydrophila	K83B	Channel catfish	Alabama
A. hydrophila	AL-05-58	Channel catfish	Alabama
A. hydrophila	AL-098-69a	Channel catfish	Alabama
A. hydrophila	PB-04-211	Channel catfish	Arkansas
A. hydrophila	PB-05-269	Largemouth bass	Arkansas
A. hydrophila	GA-93-13	Channel catfish	Georgia
A. hydrophila	NC-05-02	Channel catfish	North Carolina
A. salmonicida subsp. *salmonicida*	ATCC-33658	Atlantic salmon *Salmo salar* L.	ATCC
A. sobria	ATCC-43979	Fish (species unknown)	ATCC
A. caviae	ATCC-15468	Guinea Pig *Cavia porcellus*	ATCC
E. tarda	AL-98-87	Channel catfish	Alabama
E. hoshinae	ATCC-33379	Puffin *Fratercula artica*	ATCC
F. psychrophilum	GA-05-06#2	Rainbow trout *Oncorhynchus mykiss* Walbaum	Georgia
Vibrio anguillarium	CECT-522	Atlantic cod *Gadus morhua* L.	CECT
Yersinia rukeri	ATCC-29473	Rainbow trout	ATCC
Escherichia coli	K-12 (DH5α)	Commercial	BRL
Enterobacter sakazakii	ATCC-51329	Human	ATCC
Trabulsiella guamensis	ATCC- 49490	Dust	ATCC
Streptococcus iniae	01-AU-HC-HSB-M2	Striped bass *Morone saxatilis* Walbaum	Florida
Streptococcus agalactiae	19-Mullet-Br Mullet	*Mugil cephalus* L.	Kuwait

(~1.3×10^5 CFU ml^{-1} of each organism); Group 5, *E. ictaluri* + *A. hydrophila* (~ 1.4×10^5 CFU ml^{-1} of each organism); Group 6, *F. columnare* + *E. ictaluri* + *A. hydrophila* (~1×10^5 CFU ml^{-1} of each organism). An uninfected group (Group 7) of 7 fish were maintained as controls. Fish were monitored following infection and dead or moribund fish were promptly removed for sample collection. Blood was collected into 1 ml hypodermic syringes by direct cardiac puncture, and the kidney and gill tissues were surgically removed. Duplicate sample sets were collected to provide for nucleic acid extraction and for bacteriological culture. In addition to samples from infected fish, tissues (gills, kidney and blood) obtained from euthanized (using 200 mg l^{-1} tricaine methanesulfonate; Western Chemical) naïve fish were spiked with *F. columnare*, *E.*

ictaluri, and *A. hydrophila* in different combinations. Sterile, disposable 1 µl plastic loops dipped in broth cultures containing ~1×10^9 CFU ml^{-1} of bacteria were mixed vigorously with ~30 to 50 mg of each tissue sample in 1.5 ml microtubes and placed in an ice bath until used for bacteriological culture or nucleic acid extraction.

Nucleic acid extraction. Total nucleic acid was extracted from 200 µl pure culture of each bacteria listed in Table 1, from 30 to 50 mg tissue (blood, gills or kidney) of experimentally infected fish, and from spiked tissue samples (30 to 50 mg) using a High Pure PCR template preparation kit (Roche Diagnostics) according to the instructions provided. Tissues were first macerated with sterilized Kontes disposable pellet pestles (Fisher Scientific) in 10 µl of buffer containing 10 mM Tris HCl, 100 mM EDTA, pH 7.6. Nucleic acid was quantified with a NanoDrop ND-1000 (NanoDrop Technologies) spectrophotometer.

Target genes, primers and optimization of m-PCR amplification parameters. Oligonuleotide primers for genes of *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila* are listed and described in Table 2. Primers targeting unique sequences within the 16S rRNA gene of *E. ictaluri* were designed for this study. All primers were synthesized at the Iowa State University DNA Sequencing and Synthesis Facility, Ames. Optimal conditions for the m-PCR were empirically determined by varying template nucleic acid concentrations (60, 30 and 20 ng), primer concentrations (0.2 to 0.8 µM), reaction buffers, and annealing temperatures. Reaction buffers tested included Master Mix (Promega) and FailSafe PCR PreMixes A–L containing 50 mM KCl, 200 µM of each dNTP, 3 to 7 µM $MgCl_2$ and 0 to 8 × FailSafe PCR-enhancer (Epicentre Biotechnologies). Annealing temperatures were adjusted between 65 and 55°C and a touchdown PCR protocol (Don et al. 1991) was employed with the primer annealing temperature decreased by 0.3°C per cycle, from 63 to 54°C, for 30 cycles. Based on the results, the final m-PCR mixture (25 µl) contained 12.5 µl 1 × Epicenter FailSafe PreMix Buffer-D, 1 µl (5 U) *Taq* DNA polymerase (Promega), 1 µl (20 ng) sample nucleic acid template, 3 µl sterile distilled water, and the following concentrations of primers: 0.6 µM each of FcFd/FcRs, 0.4 µM each of EiFd-1/EiRs-1, and 0.5 µM each of AeroFd/AeroRs. The cycling parameters consisted of an initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation 95°C for 30 s, annealing at 63 to 54°C (decreased by 0.3°C each cycle) for 45 s, extension at 72°C for 30 s and a final extension at 72°C for 10 min. A negative control (no template DNA) and positive controls (purified DNA from each of *F. columnare*, *E. ictaluri* and *A. hydrophila*) were included in each batch of reactions. Amplification was performed in a Tgradient thermocycler (Whatman Biometra) and PCR products were analyzed by electrophoresis on 3% agarose gels (3:1 Nu-Sieve agarose, Cambrex Bio Science) at 5 V cm^{-1}. Gels were stained in the dark with 1× SYBR-Green-1 nucleic acid stain (Molecular Probes) and scanned and documented with a Kodak Gel Logic 440 system (Eastman Kodak).

Sensitivity and specificity of m-PCR. Analytical sensitivity of the m-PCR was assessed with nucleic acid from pure cultures of *Flavobacterium columnare* ALG-00-530, *Edwardsiella ictaluri* AL-93-75 and *Aeromonas hydrophila* K106K. Each template was standardized to

Table 2. Primer sequences, locations and predicted sizes of amplified products of target DNA, showing gene and GenBank Accession Nos. of sequences from which primer sequences were derived. Location: location within sequence; Size: size of amplified product

Primer	Oligonucleotide sequence (5′–3′)	Accession No.	Location	Size (bp)	Source
Flavobacterium columnare					
FcFd (forward)	TGCGGCTGGATCACCTCCTTTCTAGAGACA	16S–23S rRNA	1–30	504	Welker et al. (2005)
FcRs (reverse)	TAATYRCTAAAGATGTTCTTTCTACTTGTTTG[a]	intergenic spacer region AY754370	474–505		
Edwardsiella ictaluri					
EiFd-1 (forward)	GTAGCAGGGAGAAAGCTTGC	16S rRNA	183–202	407	This study
EiRs-1 (reverse)	GAACGCTATTAACGCTCACACC	AF310622	569–590		
Aeromonas hydrophila					
AeroFd (forward)	CCAAGGGGTCTGTGGCGACA	Aerolysin	645–664	209	Pollard et al. (1990)
AeroRs (reverse)	TTTCACCGGTAACAGGATTG	M16495	834–853		
Universal bacterial					
fD1 (forward)	ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG[b]	16S rRNA	1525–1544	1504	Weisburg et al. (1991)
rP1 (reverse)	cccgggatccaagcttACGGTTACCTTGTTACGACTT[b]	J01695	3009–3029		

[a]Abbreviations: Y = pyrimidine (C or T); R = purine (A or G)
[b]Each universal primer includes 2 restriction sites added to the 5′ end. The added nucleotides, not complementary to bacterial DNA, are indicated by lower-case letters

contain 20 ng μl^{-1} of nucleic acid. The nucleic acid was 10-fold serially diluted down to 2 fg μl^{-1} and 1 µl of each dilution used as template in the m-PCR assay described. To determine the sensitivity in mixed culture, initial pure cultures of each bacterium (*F. columnare*, *E. ictaluri* and *A. hydrophila*) at log-phase growth were 10-fold diluted 4 times and then 2-fold serially diluted in PBS pH 7.2. Corresponding dilutions of each bacterial species were mixed. Total nucleic acid was extracted from 200 µl of each mixture and 1µl subjected to m-PCR as described above.

For additional analytical sensitivity assessment, tissues (blood, gills and kidney) from naïve fish were spiked with serial dilutions of log-phase cultures of the 3 bacteria *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila*. The concentration of log-phase cultures was first adjusted to optical densities (OD) at 540 nm of 0.7 for *F. columnare* and 1.0 for *E. ictaluri* and *A. hydrophila*. These dilutions were determined to contain 1.6×10^9 CFU ml^{-1} *F. columnare*, 3.5×10^9 CFU ml^{-1} *E. ictaluri* , and 2.5×10^9 CFU ml^{-1} *A. hydrophila*. One hundred microliters of each bacterial dilution was mixed with 100 µl of tissue (blood) or tissue homogenate (gills or kidney) and total nucleic acid prepared from the mixture using guanidinium thiocyanate acid as previously described (Casas et al. 1995); 1 µl of the 10 µl of isolated nucleic acid from each sample was used in the m-PCR reaction mixture.

Diagnostic sensitivity of each primer set for its target species was assessed by PCR amplification of nucleic acid extracted from pure cultures of 10 isolates of each species (*Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila*). Template nucleic acids from 1 isolate of each species of bacteria were also amplified together in each possible combination. Additionally, nucleic acid extracted from 11 Gram-negative bacteria listed in Table 1, which are either taxonomically related or ubiquitous in the aquatic environment and 2 Gram-positive bacteria (*Streptococcus iniae* and *S. agalactiae*) previously isolated from diseased fish (bass or mullet) were tested in the m-PCR to confirm diagnostic specificity.

RESULTS

Optimization of the m-PCR and cycling parameters

The m-PCR amplification of all 3 target gene segments yielding comparable band intensities was achieved with the Epicentre FailSafe PreMix Buffer-D and 1 µl (5 U) of *Taq* DNA polymerase (Promega). Adjusting the cycling parameters to include the stepwise reduction of the annealing temperature from 63 to 54°C considerably improved the amplification of the 3 target gene segments and eliminated spurious bands (Fig. 1). Additionally, resolving the amplified products on 3:1 Nu-Sieve agarose (compared to LE-agarose) and staining the gel with SYBR-Green-1 nucleic acid stain (compared to ethidium bromide), improved the sharpness and intensity of the resolved bands.

Diagnostic sensitivity and specificity of m-PCR

The diagnostic sensitivity and specificity of the m-PCR was evaluated by testing purified nucleic acid templates prepared from 10 isolates representing each of the species *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila* and several other

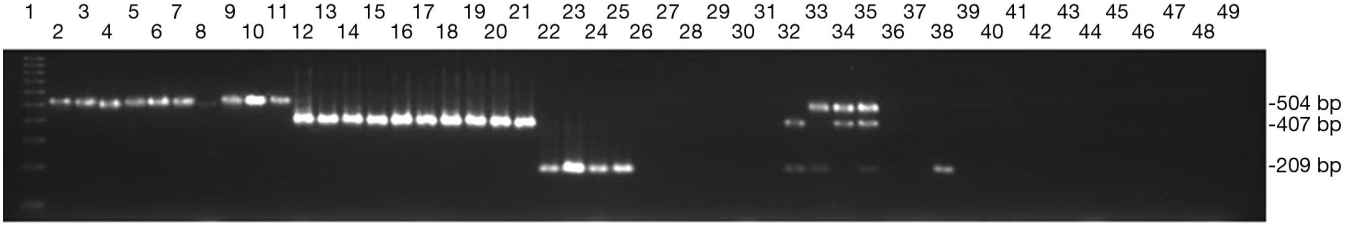

Fig. 1. *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila* multiplex-PCR (m-PCR) amplified products resolved by agarose gel electrophoresis showing diagnostic sensitivity. Lane 1: 100 bp DNA ladder (Cambrex). Lanes 2 to 11: Templates isolated from *F. columnare* isolates; 2, ATCC-23463; 3, ARS-1; 4, ALG-00-530; 5, ALG-00-522; 6, ALG-02-036; 7, BioMed; 8, LSU; 9, MS-02-463; 10, HS; 11, IR. Lanes 12 to 21: *E. ictaluri* isolates; 12, ATCC-33202; 13, AL-93-75; 14, ALG-03-58; 15, ALG-03-161; 16, 013-S99-1908; 17, 016-S99-1911; 18, 017-S99-1914; 19, 003-S99-1760; 20, IA-30-NJ#1; 21, EILO. Lanes 22 to 31: *A. hydrophila* isolates; 22, CECT-839; 23, K106K; 24, PB-05-269; 25, GA-93-13; 26, K83B; 27, AL-05-58; 28, AL-098-69a; 29, CIB; 30, NC-05-02; 31, PB-04-211. (*A. hydrophila* isolates tested in Lanes 22 to 25 are aerolysin-positive, those in Lanes 26 to 31 aerolysin-negative). Lane 32: amplicons from mixture of *E. ictaluri* and *A. hydrophila*; Lane 33: amplicons from mixture of *F. columnare* and *A. hydrophila*; Lane 34: amplicons from mixture of *F. columnare* and *E. ictaluri*; Lane 35: amplicons from mixture of *F. columnare*, *E. ictaluri* and *A. hydrophila*; Lane 36: *A. sobria* (ATCC-43979); Lane 37: *A. caviae* (ATCC-15468); Lane 38: *A. salmonicida* (ATCC-33658); Lane 39: *Edwardsiella tarda* (AL-98-87); Lane 40: *E. hoshinae* (ATCC-33379); Lane 41: *F. psychrophilum* (GA-05-06#2); Lane 42: *Vibrio anguillarium* (CECT-522); Lane 43: *Yersinia ruckeri* (ATCC-29473); Lane 44: *Escherichia coli*, K-12 (DH5α); Lane 45: *Enterobacter sakazakii* (ATCC-51329); Lane 46: *Trabulsiella guamensis* (ATCC-49490); Lane 47: *Streptococcus iniae* (01-AU-HC-HSB-M2); Lane 48: *S. agalactiae* (19-Mullet-Br); Lane 49: no template negative-control

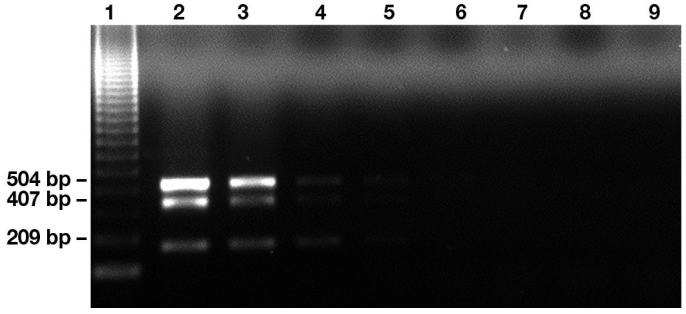

Fig. 2. *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila* multiplex-PCR amplified products resolved by agarose gel electrophoresis showing the lowest level of detection of target nucleic acid from *F. columnare, E. ictaluri* and *A. hydrophila*. Lane 1: 123 bp DNA ladder (Invitrogen) as size standard. Amplicons generated = Lane 2: 20 ng of each template; Lane 3: 2 ng of each template; Lane 4: 200 pg of each template; Lane 5: 20 pg of each template; Lane 6: 2 pg of each template; Lane 7: 200 fg of each template; Lane 8: 20 fg of each template; Lane 9: 2 fg of each template. 504 bp amplicon amplified from *F. columnare* DNA, 407 bp amplicon from *E. ictaluri* DNA, and 209 bp amplicon from *A. hydrophila* DNA

bacteria phylogenetically related or commonly occurring in the aquatic environment (Table 1). Species-specific amplicons of the predicted sizes were observed for all isolates of *F. columnare* (504 bp), *E. ictaluri* (407 bp) and aerolysin-positive strains of *A. hydrophila* (209 bp) (Fig. 1). Amplification of the target DNA sequences of all 3 bacterial species yielded comparable band intensities. With the exception of *A. salmonicida* subsp. *salmonicida*, which produced a 209 bp amplification product with the primers specific for the aerolysin gene, all other bacteria (other than the 3 targeted bacterial species) were found to be negative by m-PCR (Fig. 1). An amplification product using the set of universal bacterial primers as a positive control confirmed the presence of amplifiable bacterial DNA in the m-PCR negative samples.

Analytical sensitivity of m-PCR

The lowest limit of detection of the m-PCR amplified DNA from each of the targeted species was 20 pg nucleic acid template (Fig. 2). The detection limits for bacterial cells, determined by mixing dilutions of pure cultures, isolating nucleic acids, and using the nucleic acids as template for m-PCR, were 50 to 25 cells for *Flavobacterium columnare*, 28 to 14 cells for *Edwardsiella ictaluri* and 1300 to 650 cells for *Aeromonas hydrophila* (Fig. 3). To determine the sensitivity of the m-PCR for detection of bacteria in tissues, 10-fold serial dilutions of each bacterial species (*F. columnare*, *E. ictaluri* and *A. hydrophila*) were added to tissue homogenates from naïve fish. DNA isolated from these spiked homogenates was used as template for m-PCR. The detection limits for each bacterial species in 1 g of each tissue ranged from 3.4×10^2 to 2.5×10^5 cells g^{-1} tissue (Table 3). Differences in sensitivity varied among tissues and bacterial species. However, detection limits in blood were lower than or the same as in the 2 other tissues for all species.

Comparison of detection of bacteria in tissue samples by m-PCR and bacteriological culture

Bacteriological culture and m-PCR test results on the tissues from experimentally infected catfish are compared in Table 4. Each of the Groups 1, 2 and 3 that were infected with a single species of bacteria (*Flavobacterium columnare, Edwardsiella ictaluri*, or *Aeromonas hydrophila*) produced predicted size amplicons corresponding to the specific target bacterial DNA. With a few exceptions, the majority of tissues gave positive m-PCR results in agreement with bacteriological culture (Table 4). Bacteriological culture and m-PCR revealed the presence of indigenous *A. hy-*

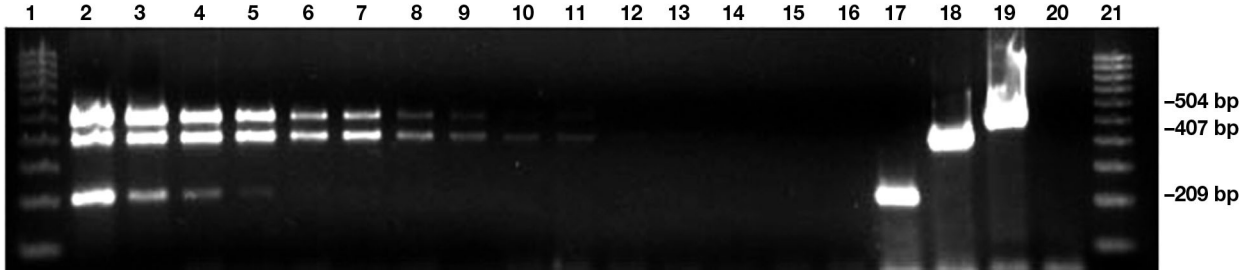

Fig. 3. *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila* multiplex-PCR products resolved by agarose gel electrophoresis showing sensitivity of detection of *F. columnare*, *E. ictaluri* and *A. hydrophila* in mixtures of pure-culture bacterial suspensions of the 3 bacteria. Lane 1: 100 bp DNA ladder; Lanes 2 to 16: Templates prepared from mixtures containing *F. columnare* (1.6×10^6, 1.6×10^5, 1.6×10^4, 1.6×10^3, 800, 400, 200, 100, 50, 25, 13, 7, 4, 2, 1 cells ml^{-1}), *E. ictaluri* (3.5×10^6, 3.5×10^5, 3.5×10^4, 3.5×10^3, 1750, 875, 437, 219, 110, 55, 28, 14, 7, 4, 2 cells ml^{-1}), *A. hydrophila* (2.6×10^6, 2.6×10^5, 2.6×10^4, 2.6×10^3, 1300, 650, 325, 163, 82, 41, 21, 11, 6, 3, 2 cells ml^{-1}). Lanes 17 to 19: template DNA from *A. hydrophila*, *E. ictaluri* and *F. columnare*, respectively (positive controls); Lane 20: no template (negative control); Lane 21: 100 bp DNA ladder

Table 3. Detection limit (cells g^{-1} tissue) of m-PCR in catfish *Ictalurus punctatus* tissue samples spiked with bacteria *Flavobacterium columnare*, *Edwardsiella ictaluri*, and *Aeromonas hydrophila*

Bacteria	Tissue		
	Blood	Gills	Kidney
F. columnare	1.6×10^3	1.6×10^3	1.6×10^3
E. ictaluri	3.4×10^2	3.4×10^3	3.4×10^4
A.hydrophila	2.5×10^4	2.5×10^4	2.5×10^5

drophila in 4 of the 7 fish that were infected with a combination of *F. columnare* and *E. ictaluri* (Group 4), in addition to the 2 organisms with which they were experimentally infected (Table 4). When tissue samples from nearly all fish that were infected with a mixture of bacteria containing *A. hydrophila* (Groups 5 and 6) were cultured, overgrowth of the cultures by *A. hydrophila* prevented the detection of *F. columnare* and sometimes of *E. ictaluri* by bacteriological culture. However, *F. columnare* and/or *E. ictaluri* were detected in these samples by m-PCR. Some variation in the fragment intensity was observed when the m-PCR amplified products were resolved by agarose gel electrophoresis. This may be due to the low copy-number of target DNA in some of the samples compared to others. Reduced fragment intensities were particularly evident in bacterial DNA extracted from blood and gill samples compared to the kidney samples where the density of bacteria was relatively high. For kidney samples the sensitivity of m-PCR was equal to or greater than bacteriological culture. The tissues collected from uninfected control fish were negative for *F. columnare*, *E. ictaluri* and *A. hydrophila* by both culture and m-PCR (Table 4).

All tissues spiked with pure cultures of *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila* (in different combinations) yielded the predicted size amplicons of the homologous bacteria with relatively equal intensities. Results are compared with bacteriological culture results in Table 5. With the exception of failure to detect *F. columnare* by bacteriological culture in tissues spiked with all 3 bacteria due to overgrowth of cultures with *A. hydrophila*, results of m-PCR were in agreement with bacteriological culture results.

DISCUSSION

The 3 species of bacteria *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila* are

Table 4. Catfish *Ictalurus punctatus* experimentally infected with *Flavobacterium columnare* (*FC*), *Edwardsiella ictaluri* (*EI*), and *Aeromonas hydrophila* (*AH*). Bacteriological culture and m-PCR results for catfish tissues. n: no. of catfish examined

Group	Bacteria injected	n	Number of fish positive by:																
			Bacteriological culture									m-PCR							
			Blood			Gills			Kidney			Blood			Gills			Kidney		
			FC	*EI*	*AH*	*FC*	*EI*	*AH*	*FC*	*EI*	*AH*	*FC*	*EI*	*AH*	*FC*	*EI*	*AH*	*FC*	*EI*	*AH*
1	*FC*	7	7	0	0	6	0	0	7	0	0	6	0	0	7	0	0	6	0	0
2	*EI*	7	0	7	0	0	6	0	0	7	0	0	5	0	0	6	0	0	7	0
3	*AH*	7	0	0	7	0	0	7	0	0	7	0	0	5	0	0	5	0	0	7
4	*FC, EI*	7	2	3	4	1	3	4	0[a]	7	4	3	4	5	2	7	3	7	7	4
5	*EI, AH*	7	0	2	7	0	0	7	0	1	7	0	4	5	0	6	6	0	7	7
6	*FC, EI, AH*	7	0[a]	2	7	0[a]	3	7	0[a]	5	7	3	3	5	1	3	5	7	7	7
7	None	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]Overgrown with *A. hydrophila* and/or other contaminating bacteria

Table 5. Bacteriological culture and m-PCR results for non-infected catfish *Ictalurus punctatus* tissue samples spiked with mixtures of bacteria *Flavobacterium columnare* (*FC*), *Edwardsiella ictaluri* (*EI*), and *Aeromonas hydrophila* (*AH*). n: no. of catfish examined

Bacteria added		Number of fish positive by:																	
		Bacteriological culture									m-PCR								
		Blood			Gills			Kidney			Blood			Gills			Kidney		
	n	*FC*	*EI*	*AH*	*FC*	*EI*	*AH*	*FC*	*EI*	*AH*	*FC*	*EI*	*AH*	*FC*	*EI*	*AH*	*FC*	*EI*	*AH*
FC, EI	7	7	7	0	5	7	0	7	7	0	7	7	0	7	7	0	7	7	0
FC, EI, AH	7	0[a]	7	7	0[a]	7	7	0[a]	7	7	7	7	7	7	7	7	7	7	7

[a]Overgrown with *A. hydrophila* and/or other contaminating bacteria

ubiquitous in the aquatic environment and the possibility of dual infections is likely (Hawke & Khoo 2004). Our detection of *A. hydrophila* in catfish *Ictalurus punctatus* injected with *F. columnaris* and *E. ictaluri* corroborate findings of others that infection with *E. ictaluri* elicits shedding of previously inapparent *A. hydrophila* (Nusbaum & Morrison 2002). In most instances, little more than anecdotal experience with mixed infections is available (Wagner et al. 2002). Thus, the need for increased awareness of mixed infections and rapid diagnosis of disease in food fish caused by these 3 important bacterial pathogens, prompted us to develop a multiplex-PCR.

In this study, 3 sets of primers, each specific for a conserved gene segment of the cognate bacterial species, were used for simultaneous detection of *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila*. We designed oligonucleotide primers corresponding to short unique segments within *the E. ictaluri* 16S rRNA gene. Recent analysis of the 16S–23S intergenic spacer regions of the rRNA operons in *E. ictaluri* has revealed that this genospecies represents a coherent homogeneous cluster within the family Enterobacteriaceae (Panangala et al. 2005). Amplification using our primers specific for the *E. ictaluri* 16S rRNA gene generated a 407 bp product from 10 archived *E. ictaluri* isolates from different geographic locations.

For identification of *Flavobacterium columnare*, we used primers that were specific for a region of the 16S–23S intergenic spacer region of *F. columnare* that had been previously designed and used by Welker et al. (2005). In contrast to *Edwardsiella ictaluri*, several studies have indicated distinctive genotypic diversity among *F. columnare* isolates (Triyanto & Wakabayashi 1999, Arias et al. 2004). Despite the genetic polymorphism, primers designed by Welker et al. (2005) were able to amplify DNA from all *F. columnare* fish isolates included in the study by Arias et al. (2004) and the 10 *F. columnare* isolates when used in the m-PCR assay in this study.

As with *Flavobacterium* and *Edwardsiella*, members of the genus *Aeromonas* are autochthonous to freshwater aquatic ecosystems world-wide and can survive over a wide range of temperatures (Altwegg & Geiss 1989). A great deal of confusion and controversy has prevailed over the taxonomic classification of *Aeromonas* species due to the lack of precise biochemical and/or other phenotypic properties and inadequacy of reliable typing schemes (Kaznowski 1998, Abbott et al. 2003). On the basis of 16S rDNA sequencing, all species of the genus *Aeromonas* are presently included under a distinct family, Aeromonadaceae. Most, if not all, fish-pathogenic *A. hydrophila* produce a cytolytic toxin aerolysin. The primer pairs previously designed for PCR amplification of a portion of the aerolysin gene (Pollard et al. 1990) were used in the m-PCR. These primers were originally designed to avoid regions of homology in the structural genes for *A. sobria* aerolysin, *Escherichia coli* hemolysin A, and/or *Staphylococcus aureus* α-toxin (Lior & Johnson 1991). The primers target a 209 bp fragment of the aerolysin gene, which was present in all aerolysin-positive fish isolates of *A. hydrophila* used in this study.

The diagnostic sensitivity and specificity of the m-PCR assay was evaluated by testing nucleic acid prepared from 10 biochemically characterized strains of each of the bacterial species *Flavobacterium columnare*, *Edwardsiella ictaluri* and *Aeromonas hydrophila* and from 13 other bacterial species that are phylogenetically related or frequently isolated from fish. Positive m-PCR amplification of the targeted DNA templates from each of the bacteria with predicted-size amplicons indicated that the test identified various isolates of the respective microbial pathogens. As expected, *A. hydrophila* isolates lacking aerolysin were negative in the m-PCR assay. With the exception of *A. salmonicida* subsp. *salmonicida*, no DNA amplification was observed with any of the other bacteria included in this study. However, amplification of *A. salmonicida* subsp. *salmonicida* DNA is relatively inconsequential to the diagnosis of bacterial infections in warm-water-cultured food fish species because typical *A. salmonicida* subsp. *salmonicida* is psychrophilic, has not been isolated from warm water cultured food fish species such as channel catfish or tilapia, and is largely a pathogen of salmonids (Dalsgaard et al. 1994).

PCR techniques have previously been developed for identification of each of the 3 bacterial species (Pollard et al. 1990, Bader et al. 2003, Bilodeau et al. 2003). All of these studies used PCR amplification of a single targeted gene segment of a given bacterium with a single set of primers. In the m-PCR, where several sets of primers are used in the reaction for amplification of multiple target genes, the diagnostic sensitivity or detection limit is generally considered to be low (Rosenfield & Jaykus 1999, Jean et al. 2004, Lee et al. 2005). In at least 1 study, a 10-fold decrease in detection sensitivity compared to single PCR was recorded (Jean et al. 2004). In an m-PCR-based method for simultaneous detection of 4 pathogens involved in warm-water streptococcosis in fish tissues, Mata et al. (2004) recorded detection limits of 5×10^3 cells g^{-1} of tissue for *Streptococcus iniae*, 1.2×10^4 cells g^{-1} for *S. difficilis*, 1×10^4 cells g^{-1} for *S. parauberis*, and 2.5×10^3 cells g^{-1} for *Lactococcus garviae*. In the present study, the m-PCR diagnostic sensitivity threshold for detection of the 3 bacteria in tissues was comparable to that obtained by Mata et al. (2004).

The m-PCR was 100% sensitive for detecting representative isolates of *Flavobacterium columnare*, *Edwardsiella ictaluri*, and the aerolysin toxin-producing *Aeromonas hydrophila*. Tissue samples spiked with approximately equal concentrations of the 3 bacteria tested positive for *E. ictaluri* and *A. hydrophila* by bacterial culture but were consistently culture-negative for *F. columnare*. In contrast, similarly treated tissue samples tested positive for all 3 species of bacteria with the m-PCR. The ability to detect specific bacteria by m-PCR, in culture-negative samples has been reported previously (Corless et al. 2001, Richtzenhain et al. 2002). A likely explanation for inability of bacterial culture to detect *F. columnare* in mixed infections is outcompetition by *A. hydrophila.* It has been documented that *A. hydrophila* suppresses the growth of *F. columnare* (Chowdhury & Wakabayashi 1989). In the present study we found that *A. hydrophila* outcompetes not only *F. columnare* but also *E. ictaluri.* These findings provide a compelling reason for employing the m-PCR as a diagnostic testing strategy when dual and/or multiple infections are suspected. Thus the m-PCR offers a rapid and reliable procedure for detection of infection and for implementing prompt measures to prevent the spread of disease from pond to pond or to neighboring farms.

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