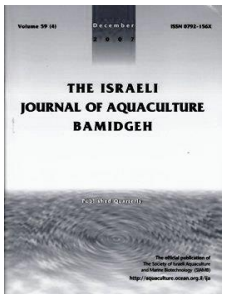

Multiplex Taqman Real-Time Pcr For Detecting *Aeromonas Hydrophila, A. Veronii* and *A. Schubertii*

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Keywords: multiplex TaqMan real-time PCR; detection; *Aeromonas hydrophila; Aeromonas veronii; Aeromonas schubertii*

Abstract

In this study, pathogenic *Aeromonas hydrophila, A. veronii,* and *A. schubertii* from fish were detected by multiplex TaqMan real-time PCR assay. The assay utilized three pairs of specific primers and three corresponding TaqMan probes designed to detect the aerolysin gene in *A. hydrophila*, the aerolysin gene in *A. veronii,* and the gyrB gene in *A. schubertii*. The specificity of the probe and primers was evaluated. The detectable concentration for the multiplex real-time PCR was 3.33×10^1 copies/μL per reaction, respectively. In addition, the coefficient of variation was less than 1.5% for both intra- and inter-assay. The assay, when screened for 120 cultured fish samples, showed 76.7% positive for *Aeromonas spp*. and could accurately identify these bacterial strains. These results indicated that this assay could be used as an effective tool for rapid detection and epidemiology investigation of *A. hydrophila, A. veronii,* and *A. schubertii*.

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Introduction

Aeromonas spp. are inhabitants of a wide range of aquatic ecosystems such as freshwater, estuarine and coastal water, sea water, and are even found in chlorinated potable water (Janda *et al.* 2010). Under environmental stress conditions, virulent representatives of *Aeromonas* spp. can cause a wide range of infections in cultured fish, whose clinical manifestations range from chronic disease with dermal and ophthalmic ulcerations to more severe symptoms including soft tissue infections, hemorrhagic septicemia, motile aeromonad septicemia (MAS), or motile aeromonad infection (MAI) (Akinbowale *et al.* 2007; Hu *et al.* 2012; Rahman *et al.* 2002). Contaminated water and foods of animal origin may be a source of human diseases such as gastroenteritis, urinary tract infections, septicemia, and peritonitis especially in immunocompromised patients and children (Parker and Shaw 2011).

Strains of the species *Aeromonas hydrophila*, *A. veronii* and *A. schubertii,* which can cause severe septicemia and ulcers with high mortality rates, have been recognized as important pathogens of cultured fish. (Pridgeon and Klesius *et al.* 2011; Cai *et al.* 2012; Liu and Li 2012). In this study, we developed a simple multiplex PCR method based on TaqMan real-time PCR to detect *A. hydrophila, A. veronii,* and *A. schubertii* in a single PCR reaction. Specific primers and probes targeting the aerolysin genes of *A. hydrophila* and *A. veronii*, and the gyrB gene of *A. schubertii* were selected for identification. Owing to its sensitivity, specificity and advantage of simultaneous detection, the method provides a rapid and effective tool for rapid detection and epidemiological investigation of these *Aeromonas* species.

Materials and Methods

Bacterial strains and DNA extraction. Type of *Aeromonas* strains used in this work included *A. hydrophila* (ATCC 7966), *A. veronii* (CCTCC 98045), and *A. schubertii* (ATCC 43700). Other bacterial pathogens such as *A. caviae* (ATCC 15468), *Escherichia Coli* (ATCC 25922), *Vibrio cholerae* (CMCC 16010), *V. vulnificus* (ATCC 27562) and some isolates of *A. salmonicida, A.jandaei, Bacillus cereus, Flavobacterium columnare, Pseudomonas fluorescens, V. parahaemolyticus* obtained from our laboratory were used to check the specificity of the assay. All bacterial species were grown on tryptic soy agar (HKM, China) or in tryptic soy broth, (HKM, China) and incubated at 28^0C for 24h-48h. Strains were maintained on TSA plates at 4^0C and were stored in 10% glycerol at – 20^0C. Total DNA from microorganisms was extracted by TIANamp Bacteria DNA Kit (TianGen, China) according to the manufacturer's recommendations. Purified DNA was quantified with Eppendorf BioPhotometer.

Primers and TaqMan probes design. Numerous sequences including the aerolysin gene, the enterotoxin gene, lipase gene, and gyrB gene of *Aeromonas* obtained from GeneBank were aligned respectively with Clustal-W (DNAStar Inc., USA), and conserved regions were identified. Then the aerolysin gene for *A. hydrophila* and *A. veronii*, and the gyrB gene for *A. schubertii* were selected and suitable primers and probes for the multiplex TaqMan real-time PCR were designed using Primer Premier 5.0 (Table 1) and synthesized by Sangon Bothech (Shanghai) Co., Ltd. All PCR products were verified by confirming their expected sizes via gel electrophoresis and observed under UV light.

Table 1. List of primers and probes in this study

Pathogens	*Primer*	*Sequence (5'-3')*	*Target gene (accession number)*	*Ampliconsize (bp)*
A. hydrophila	AH-F	GGC AGA GCC CGT CTA TCC A	Aerolysin	73
	AH-R	GCG ATA CTT GTC GCC ACA GA	(M16495)	
	AH P	FAM-ACC AGC TTC GCT TGT TTT CAT TGG GC-BHQ1		
A. veronii	AV-F	TTT GGC GTC TTC ATT GAT GCT	Aerolysin	74
	AV-R	CCG CCC ATT TGA CCT GAT C	(EF034117)	
	AV P	HEX- TGC AGG CGG CTG AAC CTG TCT ACC-BHQ2		
A. schubertii	AS-F	GGC GGT CAT TTC GGT CAA	GyrB	126
	AS-R	AGC AGG AAG TCG GCC AGC T	(JQ319030.1)	
	AS P	CY5- CCG GAT CCC AAG TTC TCC TCC CA -BHQ2		

Note: F = forward primer; P = probe; R = reverse primer.

Construction of standard recombinant plasmids. Products of PCR were purified using Agarose Gel DNA Purification Kit (Takara, China), ligated into PMD-19T vector and cloned into E. coli DH5α competent cells according to the manufacturer's instructions. Positive clones were screened via blue-white selection and detected by PCR with M13+/- primers. Finally bacteria liquid was sequenced by Sangon Bothech (Shanghai) Co., Ltd. The plasmid DNA was isolated and purified using the E.Z.N.A.TM Plasmid Mini Kit II and Zymoclean™ Gel DNA Recovery Kit (Zymo, USA), respectively. Concentration of constructed plasmids, named PMD-AH-Aer, PMD-AV-Aer and PMD-AS-gyrB, was determined by ultraviolet spectrophotometer.

Sensitivity and specificity of the multiplex TaqMan real-time PCR assay. The assays were first optimized in singleplex real-time PCR reactions; subsequently, multiplex real-time PCR was performed. The sensitivity of the multiplex TaqMan real-time PCR assay was conducted as follows: the reaction consisted of 10 μL 2×SG probe MasterMix, 0.5 μL Probe (10Mm) , 0.5 μL Forward Primer (10μM) , 0.5 μL Reverse Primer (10μM) . In the analysis, a tenfold dilution series of the purified plasmid DNA with known copy numbers ranging from 3.33×10^0 to 3.33×10^9 copies/μL and a no-template control were tested in triplicate under the optimized FQ-PCR conditions, deionized water was added to a final volume of 20 uL. PCR was performed with Mx3000P (Stratagene) followed by 95°C for 10 min (inactivation reverse transcriptase/activation Taq polymerase), 45 cycles of 95°C for 10s (denaturation), and 60°C for 30s (annealing). The sensitivity of the detection of the FQ-PCR was determined to be the highest dilution (lowest concentration of template) that resulted in the presence of a detected amplification signal. The DNAs of reference bacterial strains were also tested with this assay in order to examine assay specificity.

Reproducibility of the multiplex TaqMan real-time PCR assay. The reproducibility of multiplex TaqMan real-time PCR within each run (intra-assay) and between runs (inter-assay) was assessed separately. The real-time PCR assay was performed in triplicate under optimal conditions with 3 different concentrations of mixed plasmids DNA. The reproducibility was evaluated based on the calculation of the coefficient of variation (C.V) values, which are equal to the percentage of the standard deviation (SD) to the mean of the Ct, for the diluted DNA samples.

Application of multiplex real-time PCR as a screening method. 120 fish samples (including *Ctenopharyngodon idellus, Ophiocephalus argus* and *Pelteobagrus fulvidraco)* were collected from different farms and brought to the laboratory. The muscle, intestine, spleen and kidney tissues from fish were disrupted with sonication on ice for extracting DNA. DNA was extracted by the above-mentioned method and tested using the multiplex TaqMan real-time PCR assay to determine whether these samples were infected or contaminated with *A. hydrophila, A. veronii,* and *A. schubertii*. Meanwhile, the conventional culture/biochemical-based methods were used as a reference control (Carnahan *et al.* 1992).

Results

Specificity and sensitivity of detection. The primers and probes were specifically designed to identify pathogenic *A. hydrophila, A. veronii,* and *A. schubertii*, respectively. PCR amplification with these primers yielded amplicons of the expected molecular weights (Fig. 1).

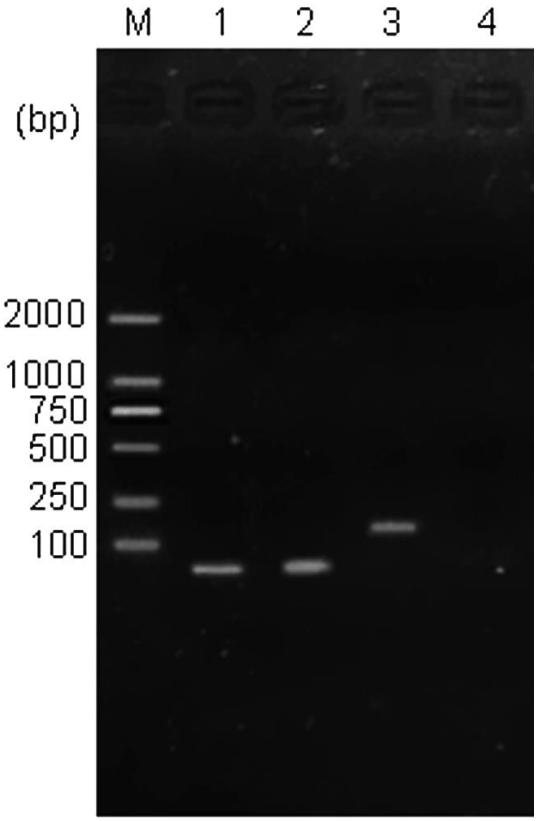

Fig. 1. The PCR results of primers for multiplex assays
M: DL2000 Marker; 1: The results of primer AH; 2: The results of primer AV; 3: The results of primer AS; 4: Blank controller

The specificity of the primers and probes against bacteria strains mentioned above was tested by multiplex real-time PCR. It was confirmed by PCR amplification of the aerolysin genes of *A. hydrophila*, aerolysin genes of *A. veronii,* and gyrB gene of *A. schubertii* respectively, but no amplification was detected for any other *Aeromonas* spp. or non-*Aeromonas* spp. (Fig. 2). During development of the assay, no cross-reactivity between the tested bacteria was observed.

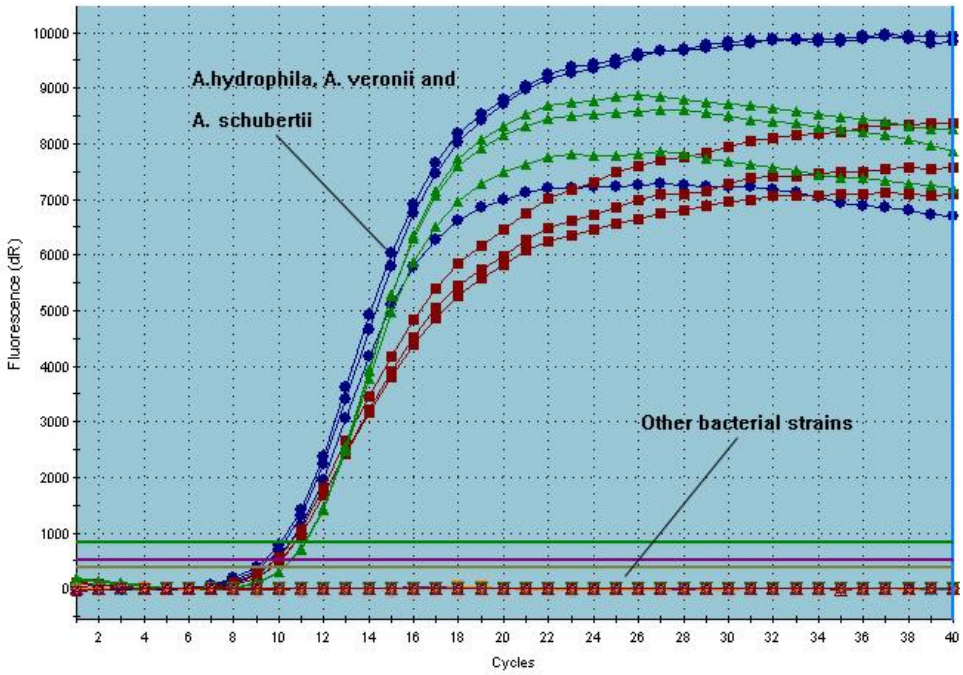

Fig. 2. Amplification curves demonstrating the specificity of the multiplex TaqMan real-time PCR assay

To determine the detection limit of the multiplex real-time PCR and to establish a standard curve for quantification, standard recombinant plasmids from 10-fold serial dilutions of these three *Aeromonas* spp. at final concentration of 3.33×10^{0} to 3.33×10^{9} copies/µL reaction was analyzed by real-time PCR (Fig. 3). Standard curves were constructed using a single target DNA in the mixture of these primer sets and probes. When simultaneous detection of these three *Aeromonas* species using a mixture of plasmid DNA, the detection limits were 3.33×10^{1} copies/µL. The amplification efficiencies were more than 99% indicating this assay effectively quantified each target (Fig. 4).

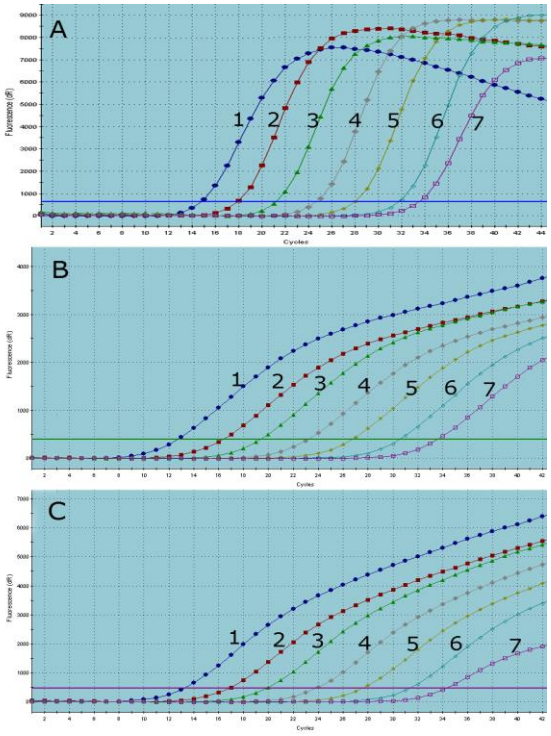

Fig. 3. Dynamic curves of the multiplex TaqMan real-time PCR for sensitivity detection
A: Detection of *A. hydrophila;* B: Detection of *A. veronii;* C: Detection of *A. schubertii*
1-7: 3.33×10^{7} copies/µL-3.33×10^{1} copies/µL

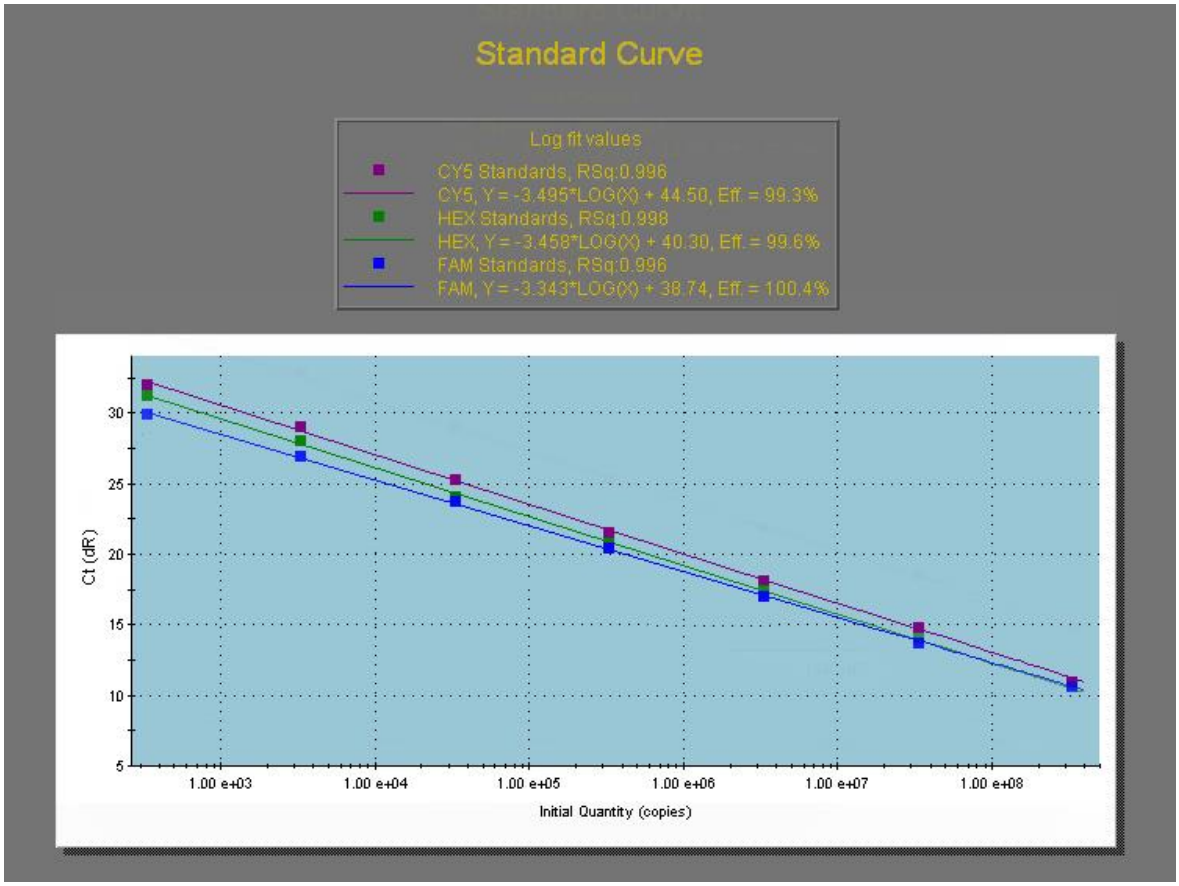

Fig. 4 Multiplex TaqMan real-time PCR standard curves. The concentrations of standard recombinant plasmids were 3.33×10^8 copies/μL-3.33×10^2 copies/μL. The equations of the lines obtained for *A. hydrophila A. veronii*, and *A. schubertii* were y = -3.343×LOG(x) + 38.74 (R^2 = 0.996), y = -3.458×LOG(x) + 40.30 (R^2 = 0.998), and y = -3.495×LOG(x) + 44.50 (R^2 = 0.996), respectively.

Reproducibility of the multiplex TaqMan real-time PCR assay. The intra-assay and inter-assay reproducibility tests of the assay using mixture of plasmid DNA template with 3 different concentrations resulted in all CV values were less than 1.5%, respectively (Table 2). This result indicates that the newly developed multiplex TaqMan real-time PCR assay was highly reproducible and stable.

Table 2. Reproducibility assay of the multiplex TaqMan real-time PCR

Standard plasmid	*Concentration (copies/ul)*	*Intra-assay Ct Mean±SD*	*CV(%)*	*Outra-assay Ct Mean±SD*	*CV (%)*
PMD-AH-Aer	$3.33x10^8$	11.43±0.09	0.79	11.23±0.12	1.07
	$3.33x10^6$	18.18±0.16	0.88	18.53±0.15	0.81
	$3.33x10^4$	24.77±0.19	0.77	25.11±0.23	0.92
	$3.33x10^2$	31.88±0.33	1.03	32.09±0.31	0.96
PMD-AV-Aer	$3.33x10^8$	10.69±0.13	1.22	10.76±0.08	0.74
	$3.33x10^6$	16.65±0.10	0.60	16.83±0.11	0.65
	$3.33x10^4$	23.06±0.18	0.78	23.64±0.22	0.93
	$3.33x10^2$	30.42±0.32	1.05	30.53±0.29	0.95
PMD-AS-Aer	$3.33x10^8$	10.22±0.07	0.68	10.38±0.09	0.87
	$3.33x10^6$	17.22±0.15	0.87	17.60±0.20	1.14
	$3.33x10^4$	24.13±0.21	0.87	24.25±0.22	0.91
	$3.33x10^2$	31.08±0.36	1.15	31.13±0.25	0.80

Detection of cultured fish samples. The multiplex real-time PCR assay was used to screen 120 commercial fishes to determine the ability to detect *A. hydrophila, A. veronii* and *A. schubertii*. As shown in Table 3, 92 of 120 (76.7%) fish tissue samples were *Aeromonas* spp. positive, including 40 positive samples of *A. hydrophila*, 43 positive samples of *A. veronii*, 33 positive samples of *A. schubertii* which 14 mixed infection of *A. hydrophila* and *A. veronii*, 10 mixed infection of *A. veronii* and *A. schubertii*. Our data indicate that the multiplex TaqMan real-time PCR assay had higher detection rate and obvious advantages for the detection of mixed infection samples compared with the biochemical reactions (Table 3).

Table 3. Detection of *Aeromonas* spp. from fish samples

Target of detection	*Positives (ratio)*	*Mixed infections*	*CT range*
Multiplex real-time PCR			
AH	40 (33.3%)	AV-14	16-29
AV	43 (35.8%)	AH-14 AS-10	14-28
AS	33 (27.5%)	AV-10	14-28
Total	92 (76.7%)	24	
Conventional method			
AH	31 (33.3%)	AV-6	-
AV	33 (35.8%)	AH-6 AS-3	-
AS	24 (27.5%)	AV-3	-
Total	79 (65.8%)	9	-

Note: AH= *A. hydrophila*, AV= *A. veronii*, AS= *A. schubertii.*

Discussion

A. hydrophila, A. veronii and *A. schubertii* are major bacterial fish pathogens belonging to *Aeromonadaceae* family. A number of epidemiological surveys have shown that they are also the common species closely related to several human diseases (Chen *et al.* 2012; Martins *et al.* 2002). Consequently, it is important to monitor the pathogenic *Aeromonas* species present in cultured fish. Conventional identification is achieved through standard biochemical testing which is time consuming, laborious, and not always conclusive. Additionally, it is difficult to discriminate among *Aeromonas* species on selective media because of the phenotypic variability of wild strains and the close relationship between them. *Aeromonas* species secrete many virulence factors such as protease, lecithinase, elastase, gelatinase, amylase, chitinase, lipase, cytotoxic enterotoxin, α-hemolysin and aerolysin (β-hemolysin) (Merino *et al.* 1995; Pemberton *et al.* 1997; Pidiyar *et al.* 2003). These virulence factors contribute to their pathogenicity and provide the ability to attach to host cells in the development of diseases; they can also be used for detection and characterization of the bacteria. To date, a number of molecular biological tools have been developed to detect *Aeromonas* species. These reports were available for PCR amplification of conserved virulence genes of *Aeromonas* spp.*,* such as the aerolysin gene, hemolysin gene, and lipase gene (Singh *et al.* 2007; Nam and Joh 2007; Xia *et al.* 2004). These methods were usually employed to detect only one species; even when multiplex PCR assays were used to detect multiple species (Persson *et al.* 2015), they were performed using conventional PCR methods, which have limited sensitivity and specificity.

In the current study, a TaqMan probe-based multiplex real-time PCR assay was developed and tested to detect *A. hydrophila, A. veronii,* and *A. schubertii*. This method utilizes three fluorescent probes simultaneously in a single PCR reaction, to increase specific amplification and sensitivity up to an order of magnitude. It also provides results within 3 hours since there is no need for post-amplification analysis, such as agarose gel electrophoresis, for confirmation of real-time detection. Moreover, the threshold cycles afford a further advantage of semi-quantification. A retrospective detection of fish samples showed that multiplex real-time PCR increased pathogen detection.

In conclusion, considering the increasing worldwide incidence of *A. hydrophila, A. veronii,* and *A. schubertii* infection in cultured fish, and its wide spread distribution in marine and freshwater environments, establishment of an accurate, early, rapid detection method for these pathogens is imperative. The multiplex real-time PCR provides rapid, sensitive, and highly specific means for simultaneous detection of *A. hydrophila, A. veronii,* and *A. schubertii* in aquatic animals. This has greatly improved microbial detection and diagnosis of fish diseases in the laboratory.

Acknowledgments

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