

Molecular Detection, Quantification, and Toxigenicity Profiling of *Aeromonas* spp. in Source- and Drinking-Water

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Abstract: *Aeromonas* is ubiquitous in aquatic environments and has been associated with a number of extra-gastrointestinal and gastrointestinal illnesses. This warrants monitoring of raw and processed water sources for pathogenic and toxigenic species of this human pathogen. In this study, a total of 17 different water samples [9 raw and 8 treated samples including 4 basin water (partial sand filtration) and 4 finished water samples] were screened for *Aeromonas* using selective culturing and a genus-specific real-time quantitative PCR assay. The selective culturing yielded *Aeromonas* counts ranging 0 – 2 x 10³ CFU/ml and 15 *Aeromonas* isolates from both raw and treated water samples. The qPCR analysis indicated presence of a considerable nonculturable population (3.4 x 10¹ – 2.4 x 10⁴ cells/ml) of *Aeromonas* in drinking water samples. Virulence potential of the *Aeromonas* isolates was assessed by multiplex/singleplex PCR-based profiling of the hemolysin and enterotoxin genes *viz* cytotoxic heat-labile enterotoxin (*act*), heat-labile cytotoxic enterotoxin (*alt*), heat-stable cytotoxic enterotoxin (*ast*), and aerolysin (*aerA*) genes. The water isolates yielded five distinct toxigenicity profiles, *viz. act, alt, act+alt, aerA+alt, and aerA+alt+act*. The *alt* gene showed the highest frequency of occurrence (40%), followed by the *aerA* (20%), *act* (13%), and *ast* (0%) genes. Taken together, the study demonstrated the occurrence of a considerable population of nonculturable *Aeromonas* spp. potentially pathogenic to humans. This emphasizes the importance of routine monitoring of both source and drinking water for this human pathogen and role of the developed molecular approaches in improving the *Aeromonas* monitoring scheme for water.

Keywords: *Aeromonas*, drinking water, real-time qPCR, singleplex and multiplex PCR, virulence genes.

INTRODUCTION

Aeromonas is a gram-negative bacterium of the family *Vibrionaceae* and is widely distributed across clinical, food, and environmental niches. Environmental *Aeromonas* constitute a high percentage of heterotrophic microbes in a variety of aquatic and terrestrial systems [1]. *Aeromonas* spp. have been implicated as the etiological agents of extra-intestinal and intestinal infections, including, but not limited to community acquired infection, nosocomial infection, traveler's diarrhea and infections associated with natural disasters such as hurricanes, tsunamis, and earthquakes [1,2-4]. *Aeromonas* spp. are infectious as well as enterotoxigenic infecting people of all age groups. A variety of potential virulence factors and toxins have been characterized [1,5-15] and associated with different illnesses in humans. The various toxins that have been associated with *Aeromonas* pathogenesis include a cytotoxic heat-labile enterotoxin (*act*), a cytotoxic heat-stable enterotoxin (*ast*), a cytotoxic heat-labile enterotoxin (*alt*), a

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cytotoxic heat-stable enterotoxin (*ast*) and an aerolysin toxin (*aerA*). These toxins may therefore serve as potential markers for differentiation of pathogenic from non-pathogenic *Aeromonas* species.

While majority of the studies on pathogenic *Aeromonas* have been focused on clinical sources, little is known on pathogenic species from environmental sources including water supplies. Understanding the prevalence of pathogenic *Aeromonas* species in water sources is critical in assessing the risk of water-to-human transmission and imminent infections [16]. Rapid and specific methods for isolation, identification, and enumeration may assist in the timely control and prevention of potentially pathogenic *Aeromonas* associated with water sources from reaching the human population. The conventional US EPA-recommended culture-based method (number 1605) currently used to monitor *Aeromonas* load in drinking water is time-consuming, laborious, and yields only the culturable viable cells of *Aeromonas*. Culture-independent methods, such as PCR could therefore offer a better measure for the detection, quantification and monitoring in environmental samples [17] and could be a reliable and effective alternative for early detection of *Aeromonas* population in water samples [18]. Identification of *Aeromonas* isolates using the DNA-DNA

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hybridization analyses [19, 20] and/or PCR assays based on common phylogenetic markers such as 16S rRNA [13] cannot reveal the pathogenic versus nonpathogenic character of the unknown *Aeromonas* isolates. A promising approach for such differentiation of potentially pathogenic *Aeromonas* isolates may require the use of virulence gene markers [16, 21-23].

In light of the above background, the current study was focused on the following specific aspects: (i) Reliable was detection and estimation of *Aeromonas* spp. in water sources (source water and municipal drinking water) using genus-specific PCR approach; (ii) isolation of *Aeromonas* strains from water samples and their profiling for the presence and distribution of virulence genes critical in conferring pathogenicity.

MATERIALS AND METHODS

Strains

Reference strains of eight *Aeromonas* species viz. *A. hydrophila* (type strain ATCC 7966), *A. jandaei* (ATCC 49568), *A. caviae* (ATCC 15468), *A. media* (ATCC 35950), *A. trota* (ATCC 49659H1), *A. veronii* (ATCC 35624), *A. eucrenophila* (ATCC 23309) and *A. sobria* (ATCC 43979) were obtained from American Type Culture Collection (ATCC, Manassas, VA).

Sampling of Source Water and Drinking Water

Source water and drinking water samples were collected from several previously determined sampling sites on the Alabama River and from the Montgomery municipal water treatment plant. The samples were designated as Raw or Source water samples (collected Upstream and Downstream from the Alabama river) and Treated or Processed water samples from the Municipal water treatment plant, including Finished or Drinking water samples (collected after

chlorination), Basin water samples (collected after partial sand filtration), and Raw water storage tank samples. A total of 9 samples were collected from the Alabama River and 8 from the treatment plant. River samples were taken at approximately 200 mm depth from the surface at each sampling site. All samples were processed within 24 to 48 hours of collection.

Culturing of *Aeromonads*

One hundred milliliter of source water sample (50 ml diluted to 100 ml with sterile water) and 500 ml of treated water were filtered through a sterile cellulose membrane filter of pore size 0.2 μm , diameter 47 mm (Millipore, Boston, MA) and the filters were incubated overnight either on the *Aeromonas* specific medium ADA-V with ampicillin (BD Biosciences, San Jose, CA) or Tryptic Soy Agar (TSA) (Difco Laboratories, Sparks, MD) at 35 °C in triplicates. Total colonies on either medium were counted under a dissecting microscope and distinctive yellow colonies with diameter of 1-3 mm on ADA-V medium were picked and streaked three successive times on freshly prepared ADA-V medium for isolation and purification of bacteria.

DNA Isolation from Water Samples and *Aeromonas*

A 50 ml aliquot of each water sample was centrifuged three times at 10,000 rpm for 10 min using the Sorvall RC 5B plus centrifuge (Sorvall, Asheville each, NC). The cell pellets from water samples were used for DNA extraction by following the Bactozol DNA isolation protocol modified as described in our earlier study [24]. For isolation of DNA from *Aeromonas* strains and isolates, the organism was grown to 120 Klett reading measured using Klett Photoelectric Colorimeter (Klett, New York, NY). One ml of each culture was centrifuged at 10,000 rpm for 10 min to obtain the cell pellet for DNA isolation using the same

Table 1. Primer pairs used for the detection of *Aeromonas* enterotoxin and hemolysin genes.

Primer Pair	Sequence (5'-3')	Target Gene	Location Within Gene (5' to 3' Base Pair Positions)	Size of Amplicon (bp)	Accession Number	Reference
AH-aerAF AH-aerAR	CAAGAACAAGTTCAAGTGCCCA ACGAAGGTGTGGTTCCAGT	<i>A. hydrophila aerA</i>	1323-1344 1631-1613	309	M16495	22
A16SF A16SR	GGGAGTGCCTTCGGGAATCAGA TCACCGCAACATTCTGATTTG	<i>16S rRNA</i>	1020-1041 1375-1355	356	X74677	22
AHCF AHCR	GAGAAGGTGACCACCAAGAACA AACTGACATCGGCCTTGAAGTC	<i>act</i>	1661-1682 1892-1871	232	M84709	17
AHLF AHLR	TGCTGGGCCTGCGTCTGGCGG AGGAACTCGTTGACGAAGCAGG	<i>alt</i>	686-707 1046-1025	361	L77573	21
AHSF AHSR	GACTTCAATCGCTTCTCAACG GCATCGAAGTCACTGGTGAAGC	<i>ast</i>	2579-2600 3114-3093	536	AF419157	21
gyrB-F gyrB-R	GAAGGCCAAGTCGGCCGCCAG ATCTTGGCATCGCCCGGTTTTC	<i>gyrB</i>	912-932 1109-1086	198	F074917 and AF208250- AF208260	13

protocol as for the water samples.

Genus-specific Real-time Quantitative PCR for Quantification of Total *Aeromonas*

The real-time qPCR was optimized on SmartCycler II platform (Cepheid, Sunnyvale, CA) using *Aeromonas* genus-specific primers targeting 198-bp region of *gyrB* gene (Table 1). The reaction mixture (20 μ l) contained 10 μ l of 2X Brilliant SYBR Green QPCR master mix (Stratagene, La Jolla, CA), 200 nM of each primer, and 50 ng of template DNA. Cycling conditions included initial denaturation for 10 min at 95°C, and 45 cycles of amplification, each for 20 s at 95°C, 30 s at 56°C, and 30 s at 72°C. Melting curves were generated by measuring the fluorescent signal while raising the temperature as follows: 15s at 95°C, 15s at 60°C and temperature increase from 60 to 95°C with a temperature transition rate of 0.1°C s⁻¹. The specificity of the primer set was validated against the 8 reference strains of *Aeromonas* spp. Using the above optimized real-time PCR amplification conditions, a standard curve for quantification of *Aeromonas* was prepared using increasing amount of DNA corresponding to increasing number of *A. hydrophila* cells (10⁰ through 10⁶ cells/ml); the master stock was prepared by growing the reference strain to a 120 Klett reading and quantifying the cell count by spread plating and incubation (24 h) on ADA-V agar (with ampicillin). The cycle threshold (Ct) values were plotted against the cell count (determined based on culturing). Known aliquots of the isolated DNA from water samples were used for the detection of total *Aeromonas* per ml based on the developed standard curve. The quality of amplification reactions was confirmed by analyzing the melting peaks of amplicons and comparing them to standard melting peak obtained for *Aeromonas*. Additionally the amplification quality was confirmed by electrophoresing (70 V, 1 h) the PCR product (10 μ l) using 1% Trevigel gel matrix (Trevigen, Gaithersburg, MD) in 1 x TAE buffer containing ethidium bromide (0.5 μ g/ml⁻¹) and 100 bp DNA size marker (Invitrogen Carlsbad, CA).

Virulence Gene Profiling Using Singleplex and Multiplex PCR Protocols

Two separate protocols of multiplex PCR were optimized based on two sets of target genes, set I (*16S rRNA* and *gyrB* set with or without *aerA*) to confirm the identity of individual isolates and set II (*act*, *alt* and *ast*) to understand their virulence potential (a singleplex PCR protocol was used to target the aerolysin gene *aerA* because of its differing annealing temperature requirement). All primer sets used for these protocols are listed in Table 1. The gyrase B gene (*gyrB*) was targeted for the genus-specific amplification wherein 16S rRNA served as an internal control.

Amplification Conditions

Multiplex PCR protocol I based on the target gene combination *16S rRNA-gyrB* or *16S rRNA-gyrB-aerA* and singleplex PCR protocol based on *aerA*, were performed using a 25 μ l reaction volume. The reaction mixture consisted of 20 ng template DNA, 1x reaction buffer [50 mM potassium chloride; 10 mM Tris chloride pH 8.3; 25mM magnesium chloride], 200 μ M each of dNTPs (dATP,

dCTP, dGTP, and dTTP), 20 pmol each of the primers, and 1.2 U Takara Ex Taq™ DNA polymerase (Takara Bio Inc, Dalian). Amplifications were performed on a GeneAmp 2400 DNA thermal cycler (Applied Biosystems, Foster City, CA). Amplification conditions were as follows: initial denaturation at 95°C for 30 s, 50 cycles of amplification [each involving denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s] and a final extension step at 72°C for 7 min.

Multiplex PCR protocol II based on the target gene set *act-alt-ast* was performed under similar reaction conditions (DNA template amount and reaction mixture) as protocol I. However, the amplification parameters included an initial heating step (95°C for 15 min) followed by 35 cycles of amplification using GeneAmp 2720 Thermal Cycler system (Applied Bio Systems, Foster city, CA). Each amplification cycle involved the following: denaturation (95°C for 15 s), annealing (69°C for 30 s), and extension (72°C for 30 s). To conclude the amplification process, a final elongation step was performed at 72°C for 10 min. The PCR product was resolved by gel electrophoresis using 1.5% agarose containing ethidium bromide and examined under UV. Identity of the amplicons was confirmed by comparison of the amplicon sizes with the predicted sizes, as shown in Table 3.

RESULTS

Culturable *Aeromonas* in Drinking Water and Source Water

Of the 17 water samples [9 raw samples and 8 processed samples which comprised of 4 basin water (partial sand filtration), and 4 finished water samples], 14 yielded bacterial colonies ranging 2 x 10¹ – 5.4 x 10⁶ CFU/ml when cultured on the general bacterial growth medium Trypticase Soy Agar (Table 2). Likewise, when cultured on the *Aeromonas* selective medium ADA-V, the samples yielded putative *Aeromonas* colonies (0 – 1.98 x 10³ CFU/ml) (Table 2). A total of 15 isolates were derived by successive passaging of the putative colonies on the selective medium ADA-V. Isolates originated not only from the raw water samples but also from treated water samples, and were picked based on the characteristic yellow pigmented colonies on the ADA-V agar plates. The isolates were arbitrarily designated as ADA-V I-1, ADA-V I-2, ADA-V I-3, ADA-V I-4, ADA-V I-5, ADA-V I-6, ADA-V I-7, ADA-V I-9, ADA-V I-10, ADA-V I-11, ADA-V I-12, R'1B, R'4B, R'3A, and DS'1A.

Genus-specific Real-time PCR-based Detection and Quantification of Total (Culturable and Nonculturable) *Aeromonas* in Water Samples

For quantitation of total *Aeromonas* by the genus-specific real-time PCR-based protocol optimized using reference strain of *A. hydrophila*, a standard curve was generated by varying the *Aeromonas* cell count (10⁰ to 10⁶). The quantification limit was as low as 10 cells/ml. (Fig. S1), although a minimum of 1 cell/ml was detectable based on the amplification growth curve. The standard curve showed a correlation coefficient (R²) value of 1.0 (Fig. S1 panel A). A

Table 2. Genus-specific real-time qPCR-based quantification of total *Aeromonads* (culturable and nonculturable) and selective culturing-based quantification of culturable *Aeromonads* in water samples.

Water Sample ID	Sampling Source ^c	Culturable Count (CFU/ml)		Total <i>Aeromonads</i> (qPCR-based)		Nonculturable <i>Aeromonads</i> ^d
		^a All Bacteria	^b <i>Aeromonas</i>	Ct Value	Counts/ml	Counts/ml
S-01	DS-1	5.4 x 10 ⁶	1980	19.21	2.6 x 10 ⁴	2.4 x 10 ⁴
S-02	DS-1	5.0x 10 ⁶	1965	19.88	2.4 x 10 ⁴	2.2 x 10 ⁴
S-03	DS-2	9.5 x 10 ⁵	688	22.50	8.8 x 10 ³	8.1 x 10 ³
S-04	DS-2	0.2 x 10 ⁶	735	22.14	8.5 x 10 ³	7.8 x 10 ³
S-05	US-1	5.1 x 10 ⁵	630	23.28	4.6 x 10 ³	4.0 x 10 ³
S-06	US-2	4.8 x 10 ⁵	614	23.49	4.5 x 10 ³	3.9 x 10 ³
S-07	US-2	4.4 x 10 ⁵	610	23.58	4.4 x 10 ³	3.8x 10 ³
S-08	RWST-1	2.5 x 10 ⁵	602	25.16	4.2 x 10 ³	3.6x 10 ³
S-09	RWST-2	1.5 x 10 ⁵	500	26.39	2.8 x 10 ³	2.3x 10 ³
S-10	Bas-1	2.3 x 10 ⁴	240	27.71	1.2 x 10 ³	9.6 x 10 ²
S-11	Bas-1	2.2 x 10 ⁴	234	27.99	3.3 x 10 ³	3.1x 10 ³
S-12	Bas-2	1.8 x 10 ⁴	150	28.96	9.8 x 10 ²	8.3 x 10 ²
S-13	Fin-1	0	0	30.68	3.0 x 10 ²	3.0 x 10 ²
S-14	Fin-2	0	0	33.83	3.4 x 10 ¹	3.4 x 10 ¹
S-15	Fin-1	20	0	30.78	2.9 x 10 ²	2.9 x 10 ²
S-16	Fin-2	0	0	33.22	3.5 x 10 ¹	3.5 x 10 ¹
S-17	Bas-2	2.0 x 10 ⁴	201	28.04	3.3 x 10 ³	3.1x 10 ³

^aTrypticase Soy Agar (TSA) was used for total culturable count.

^bADA-V agar supplemented with ampicillin and vancomycin enrichment was used for culturable *Aeromonas* count.

^cSampling details: DS – Down Stream, US – Up Stream, RWST – Raw Water Storage Tank, Bas- Basin water; Fin – Finished Water. Designation “1” refers to water samples collected in late spring whereas “2” refers to samples collected in mid-summer.

^dNonculturable *Aeromonad* counts were calculated by subtracting the culturable *Aeromonad* count from the total (qPCR-based) *Aeromonad* count.

typical growth curve consistent with the expected amplicon was detected (Fig. S1 panel B). Dissociation curve analysis (Fig. S1 panels C and D) showed that the melting temperature of the *Aeromonas* amplicon was 84 °C while the negative control (without DNA template) did not show such melting peak and instead showed a nonspecific background product with a minor melting peak at 81 °C. Agarose gel electrophoresis of the amplicon (198 bp) showed absence of any primer dimers confirming the desired quality of amplification. The genus-specific nature of the assay was validated using the established reference species of this genus namely *A. media*, *A. jandaei*, *A. caviae*, *A. eucrenophila*, *A. hydrophila*, *A. trota*, *A. sobria*, and *A. veronii*.

The developed genus-specific protocol enabled detection and quantification of total (culturable and nonculturable) *Aeromonas* in the DNA preparations isolated from the water samples. Of the 17 samples, only 8 samples yielded *Aeromonas* colonies on selected media whereas 10 samples yielded the PCR amplification signals (Table 2). The deduced count in the culture-negative samples ranged 3.4 x 10¹ - 3 x 10² cells/ml. All the PCR positive samples

showed amplicon melting peaks comparable to those for the standard (84 °C). Agarose gel electrophoresis confirmed the quality of amplification of the expected size amplicons for the water samples (data not shown).

Multiplex- and Singleplex- PCR- based Profiling of Toxin Genes in Reference Strains and Water Isolates

The reference strains harbored at least one of the four targeted toxin genes viz. *aerA*, *alt*, *ast*, and *act* (Table 3). The distribution was as follows: *ast* gene only in *A. hydrophila* strain, *act* gene only in *A. veronii*, *aerA* gene only in *A. hydrophila* and *A. eucrenophila*, and *alt* gene in all strains except for *A. veronii*. Only one of the three enterotoxin genes (*act*, *alt*, *ast*) occurred in the individual reference strains, except for *A. hydrophila* strain that harbored both the heat-labile and heat-stable enterotoxin genes *alt* and *ast* (Fig. S2). Some of the isolates did not yield the expected amplicons for the virulence genes as well as the genus-specific *gyrB* gene amplicon (Figs. S4 and S8) implying that not all isolates belonged to the *Aeromonad* group (Fig. S5). Furthermore, the isolates showed a variable distribution of the virulence gene(s). Specifically, cytotoxic

enterotoxin gene *act* was seen in the isolates ADA-V I-1 and DS' 1A (Fig. S3), heat-labile enterotoxin gene *alt* was present in isolates ADA-V I-2, ADA-V I-4, R'1B, R'4B, R'3A and DS' 1A (Fig. S3) whereas the *ast* gene was not detected from any of the isolates. The aerolysin gene *aerA* was detected in 3 of the isolates namely R'4B, R'3A and DS' 1A (Fig. S6) thereby showing a 20% frequency of occurrence. Co-occurrence of the genes encoding enterotoxin (*act*, *alt* and/or *ast*) and aerolysin (*aerA*) is a characteristic of *A. hydrophila*. A similar observation for R'4B, R'3, and DS' 1A (Figs. S6 and S7) therefore implies that these three isolates may belong to the *A. hydrophila* species. We observed that the *aerA* gene always occurred in combination with the enterotoxin gene *alt* of the heat-labile family. Cytotoxic enterotoxin gene *act* that was seen only in the *A. veronii*

reference strain was detected in the isolates ADA-V I-1 and DS' 1A (Fig. S7). A multi-gene profile consisting of aerolysin and two enterotoxins (*aerA/act/alt*) was observed only for the isolate DS' 1A (Table 3).

DISCUSSION

Culturable and Nonculturable Aeromonads in Water Sources

Recovery of Aeromonads from water in conventional culture-based monitoring may be masked because of several possible reasons. These may include loss of culturability as a result of prevailing nutrient-limited or harsh conditions and inappropriate storage, presence of competing or fast-growing

Table 3. Toxin gene profiles in *Aeromonas* reference strains and isolates.

Aeromonas- Reference Species or Isolate Name	Target Gene (With Amplicon Size)					
	<i>16S rRNA</i> (356 bp)	<i>gyrB</i> (198 bp)	<i>aerA</i> (Aerolysin) (309 bp)	<i>Act</i> (cytotoxic Heat-labile Enterotoxin) (232 bp)	<i>Alt</i> (Heat-labile Cytotoxic Enterotoxin) (361 bp)	<i>Ast</i> (Heat-stable Cytotoxic Enterotoxin) (536 bp)
<i>A. caviae</i>	+	+	-	-	+	-
<i>A. veronii</i>	+	+	-	+	-	-
<i>A. media</i>	+	+	-	-	+	-
<i>A. jandaei</i>	+	+	-	-	+	-
<i>A. sobria</i>	+	+	-	-	+	-
<i>A. hydrophila</i>	+	+	+	-	+	+
<i>A. eucrenophila</i>	+	+	+	-	+	-
<i>A. trota</i>	+	+	-	-	+	-
ADA-V I-1	+	+	-	+	-	-
ADA-V I-2	-	-	-	-	+	-
ADA-V I-3	-	-	-	-	-	-
ADA-V I-4	-	+	-	-	+	-
ADA-V I-5	+	-	-	-	-	-
ADA-V I-6	-	-	-	-	-	-
ADA-V I-7	-	-	-	-	-	-
ADA-V I-9	-	-	-	-	-	-
ADA-V I-10	-	+	-	-	-	-
ADA-V I-11	-	+	-	-	-	-
ADA-V I-12	+	-	-	-	-	-
R' 1B	+	+	-	-	+	-
R' 4B	+	+	+	-	+	-
R' 3A	+	+	+	-	+	-
DS' 1A	+	+	+	+	+	-

background microflora, and lack of appropriate growth media that enable revival of the nonculturable cells and detection of a low number of viable cells [25]. The genus-specific real-time PCR-based protocol optimized for water samples in this study circumvented these limitations and allowed for direct detection and quantification of total *Aeromonads*, including even the nonculturable (viable/non-viable) cells as observed in case of finished water samples. For these drinking water samples, while selective culturing method did not yield any *Aeromonas* colonies, the genus-specific real-time PCR yielded *Aeromonas* counts (3.4×10^1 to 3.0×10^2 cells/ml). This difference in the outcome by the two methods (culturing and PCR) implied the presence of considerable population of nonculturable cells (viable-but-nonculturable and non-viable cells) of *Aeromonas* in these samples.

The optimized *Aeromonas*-specific PCR assay based on the universally distributed bacterial genome target DNA gyrase subunit B (*gyrB*) gene is a promising alternative to the 16S rRNA-based PCR protocols [13, 26]. This assay offers numerous advantages over the 16S rRNA-RFLP assay [27] considering that it is rapid and has the ability to analyze in real time, the two desirable features for high throughput applications. The results demonstrated that the assay as applied to water samples is fairly rapid as it took an estimated 3-4 hours time including the DNA extraction step (70-90 min) and the real-time PCR protocol (90 min-2 hours) as compared to the conventional culturing-based approach which may take an estimated 1-2 days. This feature is particularly useful for timely monitoring of the pathogenic *Aeromonas* species which may escape chlorination in municipal water systems. The protocol utilized a 384 well microtiter plate format, which allows for testing of a large number of samples in a given run thereby offering a high throughput analysis. Also, the optimized protocol utilized fluorescent dye SYBR Green as against the cost-intensive fluorogenic probes. Another significant aspect of this protocol is the integration of our optimized method for direct DNA recovery and purification from field samples of source water and drinking water without culturing or enrichment. Source water may contain diverse co-occurring contaminants including organic and inorganic debris and metal particles which may act as inhibiting factors in microbial/DNA recovery and PCR reaction [28]. The DNA extraction procedure yielded DNA with quality and quantity suitable for downstream qPCR application on water DNA preparations. The assay showed a desirable minimum quantification limit (10 cells/ml), and the minimum detection limit (1 cell/ml).

Pathogenicity Potential of the *Aeromonas* spp. Isolated from Water Sources

In the event of poisoning or diarrheal outbreaks from food and environmental sources, recovery of an *Aeromonas* strain expressing hemolysins/enterotoxins may imply a hazard [1,4]. Conventionally, assessment of *Aeromonas* pathogenicity potential requires an evaluation of individual virulence phenotypes including production of individual hemolysins/enterotoxins. The phenotypic methods however may not always detect the presence of the toxins. For

instance, animal passage of *A. caviae* isolates that did not produce detectable cytotoxic or hemolytic activity in the phenotypic assays helped regain the toxin-expressing ability; however, they lost it again upon subsequent subculturing [29]. Likewise, other studies have demonstrated the role of specific culture conditions in production of cytotoxin by *Aeromonas* strains [30]. In this context, molecular screening approach for specific toxigenicity factors may offer the potentially effective way of assessing the virulence potential of *Aeromonas* isolates. From the repertoire of virulence factors in *Aeromonas*, the major toxins aerolysins and enterotoxins have been linked with its pathogenicity [31]. Molecular assays targeting the virulence factor genes have been extensively investigated in the last decade [17, 32-35]. While majority of these assays are based on one specific virulence gene, multiplexing using multiple targets has been reported. The multiplex PCR protocols employed in the current study incorporated the key virulence genes *alt*, *act* and/or *ast* which encode the heat-labile (non-cholera toxin cross-reacting Shiga-like toxins) and heat-stable (cross-reacting and non-cross-reacting cholera toxin) enterotoxins [7, 19].

Distribution of Toxin Genes

The tested virulence genes did not appear to be distributed universally among the different *Aeromonas* species except for *A. hydrophila* that was positive both for the aerolysin (*aerA*) as well enterotoxin genes (namely the heat labile (*alt*) and heat-stable (*ast*) cytotoxic enterotoxin genes). Of the *Aeromonas* cultures investigated, only two of the eight reference strains, and three of the 15 water isolates carried the aerolysin gene which always co-occurred with one or more enterotoxin genes. Taken together, the water isolates yielded five distinct toxigenicity profiles, viz. *act*, *alt*, *act+alt*, *aerA/alt*, and *aerA+alt+act*. The most prevalent toxin gene detected by the multiplex PCR in the water isolates was *alt* (40%) when compared to the frequency of occurrence of the cytotoxin genes *act* (13.3%) and *ast* (0%) and the aerolysin gene *aerA* (20%) (Table 3).

Act Gene

Among the *Aeromonas* species, *A. veronii* is considered more virulent because of its greater invasiveness and lower LD50 dose [7]. Hence, the presence of *act* gene in this species and in the two water isolates ADA-VI-1 and DS'1A may imply their relatively higher virulence. Absence of *act* gene in *A. caviae* is consistent with the previous reports [23, 36] which found it less cytotoxic and virulent as compared to the other groups. Though *act* is an important virulence factor, the absence of this gene marker in some of the *Aeromonas* isolates in this study does not imply their "not-virulent" nature. In this context, it is noteworthy that the 232-bp *act* amplicon was not observed for the reference strains of known pathogenic species *A. jandaei*, *A. caviae*, *A. media*, *A. sorbia*, *A. eucrenophila*, and *A. trola*. However, drawing a generalized conclusion on the distribution of hemolytic or enterotoxin genes across the subject *Aeromonas* species may not be appropriate considering that only a limited number of strains (one per species) were included.

Ast Gene

Although the *ast* gene has been reported in clinical and environmental isolates (ecotypes) of *Aeromonas* spp. [37, 38], there is limited information on the presence of this gene in water isolates. Consistent with our results on the absence of *ast* gene in water isolates, previous studies have reported the low occurrence of *ast* and *act* genes in *Aeromonas* isolates and ascribed the absence to the method used and/or geographic difference in virulence gene carriage [29].

CONCLUSION

Taken together, this study revealed the presence of a considerable nonculturable population of Aeromonads in drinking water based on a genus-specific real-time qPCR assay. On the other hand, the study revealed pathogenicity potential of *Aeromonas* strains prevalent in various water sources as determined using optimized multiplex/singleplex PCR protocols based on toxin gene markers. Relative distribution of *Aeromonas* virulence genes followed the order *alt* (40%) > *aerA* (20%) > *act* (13%) in the tested water samples. Detection of toxigenic *Aeromonas* spp. potentially pathogenic to humans in both source and drinking water samples in this study emphasizes the importance of routine molecular monitoring of various stages of water treatment process (source water through finished/tap water) involved in the generation of drinking water. This may serve as a critical factor in intervention for reducing and eliminating the risk of water-borne Aeromonads in community health.

CONFLICT OF INTEREST

This is to confirm that the authors have no conflicts of interest with the content of this article.

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REFERENCES

- Janda JM, Abbott SL. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clin Microbiol Rev* 2010; 23(1): 35-73.
- Aggar WA, McCormick JD, Gurwith MJ. Clinical and microbiological features of *Aeromonas hydrophila*-associated diarrhea. *J Clin Microbiol* 1985; 21: 909-13.
- Wu CJ, Wu JJ, Yan JJ, *et al.* Clinical significance and distribution of putative virulence markers of 116 consecutive clinical *Aeromonas* isolates in southern Taiwan. *J Infect* 2007; 54: 151-8.
- Parker JL, Shaw JG. *Aeromonas* spp. clinical microbiology and disease. *J Infect* 2011; 62:109-18.
- Chakraborty T, Huhle B, Bergbauer H, Goebel W. Cloning, expression, and mapping of the *Aeromonas hydrophila* aerolysin gene determinant in *Escherichia coli*. *J Bacteriol* 1986; 167: 368-74.
- Chopra AK, Houston CW, Peterson JW, Jin GF. Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. *Can J Microbiol* 1993; 39: 513-23.
- Chopra AK, Houston CW. Enterotoxins in *Aeromonas*-associated gastroenteritis. *Microbes Infect* 1999; 1:1129-37.
- Hirono I, Aoki T. Cloning and characterization of hemolysin genes from *Aeromonas salmonicida*. *Microb Pathog* 1993; 15: 269-82.
- Hirono I, Aoki T, Asao T, Kozaki S. Nucleotide sequences and characterization of hemolysin genes from *Aeromonas hydrophila* and *Aeromonas sobria*. *Microb Pathog* 1992; 13: 433-46.
- Howard SP, Buckley JT. Molecular cloning and expression in *Escherichia coli* of the structural gene for the hemolytic toxin aerolysin from *Aeromonas hydrophila*. *Mol Gen Genet* 1986; 204: 289-95.
- Howard SP, Garland WJ, Green MJ, Buckley JT. Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. *J Bacteriol* 1987; 169: 2869-71.
- Husslein V, Huhle B, Jarchau T, Lurz R, Goebel W, Chakraborty T. Nucleotide sequence and transcriptional analysis of the *AerCaerA* region of *Aeromonas sobria* encoding aerolysin and its regulatory region. *Mol Microbiol* 1988; 2: 507-17.
- Tacao M, Moura A, Alves A, *et al.* Evaluation of 16S rDNA and *gyrB*-DGGE for typing member of the genus *Aeromonas*. *FEMS Microbiol Lett* 2005; 246: 11-8.
- Seshadri R, Joseph SW, Chopra AK, *et al.* Genome sequence of *Aeromonas hydrophila* ATCC 7966T: the jack of all trades. *J Bacteriol* 2006; 188: 8272-82.
- Khan AA, Kim E, Cerniglia CE. Molecular cloning, nucleotide sequence, and expression in *Escherichia coli* of a hemolytic toxin (aerolysin) gene from *Aeromonas trota*. *Appl Environ Microbiol* 1998; 64: 2473-8.
- Khajanchi BK, Fadl AA, Borchardt MA, *et al.* Distribution of virulence factors and molecular fingerprinting of *Aeromonas* species isolates from water and clinical samples: suggestive evidence of water-to-human transmission. *Appl Environ Microbiol* 2010; 76: 2313-25.
- Bin Kingombe CI, Huys G, Tonolla M, *et al.* PCR detection, characterization, and distribution of virulence genes in *Aeromonas* spp. *Appl Environ Microbiol* 1999; 5: 5293-302.
- Mendes-Marques CL, Hofer E, Leal NC. Development of duplex-PCR for identification of *Aeromonas* species. *Revista de Sociedade Brasileira de Medicina Tropical* 2013; 46(3): 355-7.
- Kirov SM 2001. *Aeromonas* and *Plesiomonas* species. In: M.P. Doyle, L.R. Beuchat, Eds. *Food microbiology: fundamentals and frontiers*. 2nd ed. ASM Press, Washington, DC; pp. 265-87.
- Cascon A, Anguita J, Hernanz C, Sanchez M, Fernandez M, Naharro G. Identification of *Aeromonas hydrophila* hybridization group I by PCR assays. *Appl Environ Microbiol* 1996; 62: 1167-70.
- Bin Kingombe CI, D'Aoust JY, Huys G, Hofmann L, Rao M, Kwan J. Multiplex PCR method for detection of three *Aeromonas* enterotoxin genes. *Appl Environ Microbiol* 2010; 425-33.
- Wang G, Clark CG, Liu C, *et al.* Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. *J Clin Microbiol* 2003; 41(3): 1048-54.
- Wang G, Tyler KD, Munro CK, Johnson WM. Characterization of cytotoxic, hemolytic *Aeromonas caviae* clinical isolates and their identification by determining presence of a unique hemolysin gene. *J Clin Microbiol* 1996; 34: 3203-5.
- Khan IUH, Yadav JS. Real-time PCR assays for genus-specific detection and quantification of culturable and nonculturable mycobacteria and pseudomonads in metalworking fluids. *Mol Cell Probes* 2004; 18: 67-73.
- Figueras MJ, Soler L, Chacon MR, Guarro J, Martinez-Murcia AJ. Extended method for the discrimination of *Aeromonas* spp. by 16SrRNA RFLP analysis. *Int J Syst Evol Microbiol* 2000; 50: 2069-73.
- Yadav JS, Khan IUH, Fakhari F, Soellner MB. DNA-based methodologies for rapid detection, quantification, and species- or strain-level identification of respiratory pathogens (mycobacteria and pseudomonads) in metalworking fluids. *Appl Occup Environ Hyg* 2003; 18: 966-75.
- Gobat PF, Jemmi T. Comparison of seven selective media for the isolation of mesophilic *Aeromonas* species in fish and meat. *Int J Food Microbiol* 1994; 24: 375-84.
- Yamamoto S, Harayama S. PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of

- Pseudomonas putida* strains. Appl Environ Microbiol 1995; 61: 1104-9.
- [29] Singh, DV, Sanyal SC. Production of hemolysis and its correlation with enterotoxicity in *Aeromonas* spp. J Med Microbiol 1992; 37: 262-7.
- [30] Namdari H, Bottone EJ. Microbiological and clinical evidence supporting the role of *Aeromonas caviae* as a pediatric enteric pathogen. J Clin Microbiol 1990; 28: 837-40.
- [31] Burke V, Robinson J, Beaman J, *et al.* Correlation of endotoxicity with biotype in *Aeromonas* spp. J Clin Microbiol 1983; 18: 1196-200.
- [32] Baloda SB, Krovacek K, Eriksson L, Linne T, Mansson I. Detection of aerolysin gene in *Aeromonas* strain isolates from drinking water, fish, and foods by polymerase chain reaction. Comp Immunol Microbiol Infect Dis 1995; 18: 17-26.
- [33] Kaznowski, A. Identification of *Aeromonas* strains of different origin to the genomic species level. J Appl Microbiol 1998; 84: 423-30.
- [34] Lior H, Johnson WM. Application of polymerase chain reaction (PCR) to detection of the aerolysin gene in whole cell cultures of B-hemolysin *Aeromonas hydrophila*. Experientia 1991; 47: 421-4.
- [35] Pollard DR, Johnson WM, Lior H, Tyler SD, Rozee KR. Detection of the aerolysin gene in *Aeromonas hydrophila* by the polymerase chain reaction. J Clin Microbiol 1999; 28: 2477-81.
- [36] Watson IM, Robinson JO, Burke V, Gracey M. Invasiveness of *Aeromonas* spp. in relation to biotype virulence factors and clinical features. J Clin Microbiol 1985; 22: 48-51.
- [37] Aguilera-Arreola MJ, Hernandez-Rodríguez C, Zuniga G, Figueras MJ, Castro-Escarpulli G. *Aeromonas hydrophila* clinical and environmental ecotypes as revealed by genetic diversity and virulence genes. FEMS Microbiol Lett 2005; 242: 231-40.
- [38] Albert MJ, Ansaruzzaman M, Talukder KA, *et al.* Prevalence of enterotoxin genes in *Aeromonas* spp. isolated from children with diarrhea, healthy controls, and the environment. J Clin Microbiol 2000; 38: 3785-90.

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