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32 *The Open Microbiology Journal,* **2014,** *8,* **32-39**

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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 $0 - 2 \times 10^3$ CFU/ml and 15 *Aeromonas* isolates from both raw and treated water samples. The qPCR analysis indicated presence of a considerable nonculturable population ($3.4 \times 10^1 - 2.4 \times 10^4$ 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 cytotonic enterotoxin (*alt*), heat-stable cytotonic 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 *aer*A (20%), *act* (13%), and *ast* (0%) genes. Taken together, the study demonstrated the occurrence of a considerable population of nonculturable Aeromonads in water and prevalence of toxigenic *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 Aeromonads consitute 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 cytotonic heat-labile enterotoxin (*alt*), a cytotonic 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 Aeromonads 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 Aeromonads 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 ºC 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	CAAGAACAAGTTCAAGTGGCCA ACGAAGGTGTGGTTCCAGT	*A. hydrophila aerA*	1323–1344 1631–1613	309	M16495	22
A16SF A16SR	GGGAGTGCCTTCGGGAATCAGA TCACCGCAACATTCTGATTTG	*16S rRNA*	1020–1041 1375–1355	356	X74677	22
AHCF AHCR	GAGAAGGTGACCACCAAGAACA AACTGACATCGGCCTTGAACTC	*act*	1661–1682 1892–1871	232	M84709	17
AHLF AHLR	TGCTGGGCCTGCGTCTGGCGG AGGAACTCGTTGACGAAGCAGG	*alt*	686–707 1046–1025	361	L77573	21
AHSF AHSR	GACTTCAATCGCTTCCTCAACG GCATCGAAGTCACTGGTGAAGC	*ast*	2579–2600 3114-3093	536	AF419157	21
gyrB-F gyrB-R	GAAGGCCAAGTCGGCCGCCAG ATCTTGGCATCGCCCGGGTTTTC	*gyrB*	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 Aeromonads

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 µl) contained 10 µl of 2X Brilliant SYBR Green QPCR master mix (Stratagene, La Jalla, 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 (10^0 through 10^6 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 µl) using 1% Trevigel gel matrix (Trevigen, Gaithersburg, MD) in 1 x TAE buffer containing ethidium bromide (0.5 ug/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 *gyr*B set with or without *aer*A) 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 (g*yrB*) 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-aer*A and singleplex PCR protocol based on *aerA*, were performed using a 25 µl reaction volume. The reaction mixture consisted of 20 ng template DNA, 1x reaction buffer [50 mM potassium chloride; 10 mMTris chloride pH 8.3; 25mM magnesium chloride], 200 µM 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 Aeromonads 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 10^1 – 5.4 x 10^6 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 10^3 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) Aeromonads in Water Samples

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

[a]Trypticase Soy Agar (TSA) was used for total culturable count.
[b]ADA-V agar supplemented with ampicillin and vancomycin enrichment was used for culturable *Aeromonas* count.
[c]Sampling 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.
[d]Nonculturable 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 ºC 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 ºC. 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 10^1 - 3 x 10^2 cells/ml. All the PCR positive samples showed amplicon melting peaks comparable to those for the standard (84 ºC). 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. *aer*A, *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. hydophila* 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)					
	16S rRNA **(356 bp)**	***gyrB*** **(198 bp)**	***aerA*** **(Aerolysin) (309 bp)**	***Act*** **(cytotoxic Heat-labile Enterotoxin) (232 bp)**	***Alt*** **(Heat-labile Cytotonic Enterotoxin (361 bp)**	***Ast*** **(Heat-stable Cytotonic Enterotoxin) (536 bp)**
A. caviae	+	+	-	-	+	-
A. veronii	+	+	-	+	-	-
A. media	+	+	-	-	+	-
A. jandaei	+	+	-	-	+	-
A. sobria	+	+	-	-	+	-
A. hydrophila	+	+	+	-	+	+
A. eucrenophila	+	+	+	-	+	-
A. trota	+	+	-	-	+	-
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*) cytotonic 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 cytotoxigenic 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. trota*. 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%) > *aer*A (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.

ACKNOWLEDGEMENTS

This study was supported in part by the National Institute of Health's RIMI grant P20 MD 000547 (BKR) and University of Cincinnati funds (JSY). Carol Harden acknowledges the financial support by the MARC-USTAR fellowship program of the National Institute of Health grant T34 GM 008176.

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Received: November 09, 2013 Revised: January 17, 2014 Accepted: February 9, 2014