DNA-based real-time detection and quantification of aeromonads from fresh water beaches on Lake Ontario

Izhar U. H. Khan, Alyssa Loughborough and Thomas A. Edge

ABSTRACT

The present study was designed to develop a novel, rapid, direct DNA-based protocol to enumerate aeromonads in recreational waters. An *Aeromonas* genus-specific real-time quantitative polymerase chain reaction (Q-PCR) protocol was developed and optimized using newly designed genus-specific oligonucleotide primers derived from the gyrase B subunit (*GyrB*) gene. A standard curve was developed based on the PCR protocol with a minimum quantification limit of 10 cell equivalents ml^{-1} achieved using an autoclaved water sample from recreational water spiked with known quantities of an *Aeromonas* ATCC strain. The Q-PCR protocol was validated and applied to detect and quantify the total number of aeromonads in water samples collected from two fresh water beaches on Lake Ontario. The Q-PCR protocol revealed significantly higher numbers of aeromonads in all water samples than a culture-based assay at both beaches. Foreshore sand was found to serve as a reservoir of high concentrations of *Aeromonas* similar to this phenomenon noted for enteric bacteria like *Eschershia coli*. The new real-time Q-PCR protocol facilitated the rapid quantification of total numbers of *Aeromonas* cells present in recreational water samples in <3 hours without culturing.

Key words | aeromonads, gyrase B subunit (*GyrB*) gene, real-time Q-PCR, recreational water

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INTRODUCTION

Aeromonads are Gram-negative, non-spore forming, rod shaped waterborne bacteria that are ubiquitously found in environmental habitats such as soil, fresh and brackish water, sewage and wastewater, untreated and treated drinking water (Altwegg 1996; Brandi *et al.* 1996; Janda & Abbott 1998). Among 14 proposed species of *Aeromonas*, mesophilic and motile species such as *A. hydrophila*, *A. veronii*, *A. caviae*, *A. jandaei* and *A. schubertii* have been associated with human infections such as acute gastroenteritis, septicaemia, wound infections, endocarditis, meningitis and respiratory infections (Hanninen & Siitonen 1995; Janda & Abbott 1998; Isonhood & Drake 2002). In 1996, *Aeromonas* was included on the microbiological contaminant candidate list by the US Environmental Protection Agency (EPA 1998) The prevalence and distribution of *Aeromonas* in aquatic environments, its role as a contaminant of water, and the potential for pathogenicity mediated by mesophilic and psychrophilic *Aeromonas* spp. are all of public health concern (Borrell *et al.* 1998; Dumontet *et al.* 2000; Joseph & Carnahan 2000).

Accurate methods are needed to enumerate *Aeromonas* in aquatic ecosystems. Culture-based assays are recognized as only capable of enumerating cells that can be cultured under laboratory conditions. Viable cells that are injured, stressed or in a viable but non-culturable state might not be detected by culture-based methods (Pommepuy *et al.* 1996; Santo Domingo *et al.* 2003; Khan *et al.* 2007). Another disadvantage of culture-based methods is that they are not ideal for the rapid analysis of large numbers of samples in surveillance or outbreak investigations. In addition, the conventional biochemical and physical-based methods for the isolation and

doi: 10.2166/wh.2009.041

identification of *Aeromonas* are generally time-consuming, labour intensive and often fail to identify cultured isolates to genus and species level.

In the last two decades, molecular methods based on polymerase chain reaction (PCR) or DNA-DNA hybridization have overcome many problems associated with culture-based techniques, enabling the detection of microorganisms in more rapid, accurate and robust fashion. A number of conventional PCR assays have been developed for the detection of *Aeromonas* spp. using 16S rRNA, 23S rRNA genes (Martinez-Murcia *et al.* 1992; Ash *et al.* 1993; East & Collins 1993; Dorsh et al. 1994; Borrell *et al.* 1997; Figueras *et al.* 2000; Morandi *et al.* 2005) and several virulence associated genes such as aerolysin (Husslein *et al.* 1992; Khan *et al.* 1999), lipases, amylase, chitinase, elastase, nuclease, gelatinase, lecithinase, protease and lateral flagella genes (Cascon *et al.* 1996; Pemberton *et al.* 1997; Kingombe *et al.* 1999; Nam & Joh 2007). However, recent advances in quantitative culture-independent DNA-based methods, such as the real-time PCR approach, offer a better measure for the quantification and monitoring of pathogens and microbial water quality indicators in various environmental sources including drinking water, agricultural water and wastewater as well as food and food products (Fukushima *et al.* 2003, 2007; Lee *et al.* 2006; Zucol *et al.* 2006; Khan *et al.* 2007; Wang *et al.* 2007).

To date, studies have been reported using real-time PCR assays for monitoring *Aeromonas* spp. in drinking water, stools and fish tissues (Fukushima *et al.* 2003; Balcázar *et al.* 2007; Yu *et al.* 2008). However, no studies have been reported for monitoring aeromonads in recreational waters. *Aeromonas* species occur at freshwater and marine beaches (Marino *et al.* 1995; Dickerman *et al.* 2006) and high numbers have been found in faecal pollution sources known to contaminate beaches such as gull faecal droppings (Levesque *et al.* 1993) and municipal wastewater (Ashbolt *et al.* 1995). *Aeromonas* species have been associated with a variety of human skin and wound infections in aquatic recreational environments (Hanson *et al.* 1977; Joseph *et al.* 1979; Vally *et al.* 2004). Considering the importance of *Aeromonas* spp. as a waterborne pathogen, the aim of this study was to develop a robust, rapid and reliable, culture-independent, DNA-based, real-time quantitative PCR (Q-PCR) protocol by designing *Aeromonas* genus-specific oligonucleotide primers using the *GyrB* gene. The protocol was then applied to validate this quantitative and sensitive molecular assay for the detection and quantification of total aeromonads at two fresh water beaches on Lake Ontario. The results from the culture and Q-PCR based assays were compared and used to determine the effectiveness of this protocol for recreational waters.

MATERIALS AND METHODS

Reference strains and cultural conditions

Aeromonas hydrophila ATCC 23123 was used as a reference strain for the development and optimization of the protocol. The specificity of the developed protocol including oligonucleotide primers, PCR conditions and optimization of the quantitative real-time assay was evaluated and validated using 17 *Aeromonas* and 35 reference strains of other bacterial species isolated from different sources to serve as positive and negative controls (Table 1). *Aeromonas* reference strains were grown aerobically in tryptic soy broth (Oxoid, Canada) with an overnight incubation at 30°C. The cell density (colony forming units (CFU) ml^{-1}) was measured after spread plating with an appropriate dilution on tryptic soy agar (TSA), and counting colonies per ml. The reference strains of different species used as negative controls were grown on selective growth media according to specified culture conditions.

Collection of water samples

A total of 96 water samples were collected over the 2006 bathing season from two fresh water beaches (Bayfront Park and Pier4 Park) in Hamilton Harbour at Lake Ontario. Sampling was conducted bi-weekly along a single transect at each beach at locations for sand pore water, ankle depth water and chest depth water. Sand pore water was collected by digging a hole in the wet foreshore sand about 1 metre inland from the water's edge, and collecting the water that seeped into the hole. All water samples were collected in 2-l sterile bottles and returned on ice to the laboratory for further analysis.

Table 1 | List of ATCC and other *Aeromonas* reference strains and other bacterial species used in this study

Serial #	Species	Strains	Source
1	*Aeromonas hydrophila*	23213	River water
2	*A. hydrophila*	23211	Water supply
3	*A. hydrophila*	13444	Ditch water
4	*A. allosaccharophila*	51208	Diseased elvers
5	*A. besterium*	51108	Infected fish
6	*A. caviae*	15468	Epizootic of young guinea pigs
7	*A. encheleia*	51929	Healthy juvenile freshwater eel
8	*A. eucrenophila*	11163	–
9	*A. jandaei*	49568	Human faeces
10	*A. media*	BAA-229	Marine shellfish
11	*A. popoffi*	17541	Drinking water production plant
12	*A. salmonicida* subsp. *salmonicida*	14174	Diseased brook trout
13	*A. schubertii*	43701	Skin
14	*A. sobria*	35994	Sludge
15	*A. trota*	49658	Human faeces
16	*A. veronii* biogroup *sobria*	9071	Frog red-leg
17	*A. veronii*	35625	Amputation wound
18	*Escherichia coli*	29194	Urine
19	*E. coli*	35218	Canine
20	*E. coli*	25922	Clinical isolate
21	*E. coli*	C1	Environmental isolate
22	*E. coli* O157:H7 (Sakai)	BAA-460	Human faeces
23	*E. coli* O157:H7 (EDL 933)	700927	Human faeces
24	*Campylobacter jejuni* subsp. *jejuni*	33291	Human faeces
25	*C. jejuni* subsp. *jejuni*	33292	Human faeces
26	*C. jejuni* subsp. *jejuni*	43432	Human faeces
27	*C. jejuni* subsp. *jejuni*	49349	Human faeces
28	*C. jejuni* subsp. *jejuni*	29428	Diarrhoeic stool of child
29	*C. jejuni* subsp. *jejuni*	33560	Bovine faeces
30	*C. coli*	43136	Swine
31	*C. coli*	49941	–
32	*C. coli*	BAA-371	Human faeces
33	*C. lari*	43675	Human faeces
34	*C. lari*	35221	Herring gull cloacae swab
35	*C. upsaliensis*	49816	Human faeces
36	*C. upsaliensis*	43954	Dog faeces
37	*C. concisus*	51562	Human faeces
38	*C. helveticus*	51210	Cat
39	*C. hyointestinalis*	35217	Intestine of swine
40	*C. fetus* subsp. *fetus*	15296	Blood
41	*Helicobacter pylori*	43579	Human gastric mucosa

Table 1 | (*continued*)

Serial #	Species	Strains	Source
42	*H. pylori*	51652	Human endoscopic biopsy
43	*Mycobacterium avium* subsp. *para*TB	BAA-968	Bovine faeces
44	*M. avium* subsp. *para*TB	19851	Clinical specimen
45	*M. avium* subsp. *avium*	35712	Chicken
46	*M. avium* subsp. *avium*	35716	Bovine
47	*M. intracellulare*	23434	Chicken
48	*M. intracellulare*	35771	Bovine lymph node
49	*Klebsiella pneumoniae*	C6	Environmental isolate
50	*Pseudomonas aeruginosa*	27853	Blood
51	*Salmonella enterica* subsp. *arizonae*	13314	–
52	*S. enterica* subsp. *diarizonae*	12325	–

Isolation and enumeration of culturable *Aeromonas*

One litre of each water sample was analysed by centrifugation at 12,800 × g (Beckman, Fullerton, USA) for 20 min. For total number of culturable *Aeromonas* cells per 100 ml, the pellet was resuspended in 0.85% saline solution and spread plated on Ryan *Aeromonas*-selective growth media containing 5 μg ml^{-1}Ampicillin supplement (Oxoid) using a variable volume (range 10 – 100 μl). The plates were incubated overnight at 30°C. The putative *Aeromonas* isolates were initially confirmed by their growth characteristics, colony morphology and gram staining reaction. Putative culture isolates were confirmed as *Aeromonas* by the newly developed DNA-based PCR protocol.

DNA extraction and purification

One litre of each water sample was centrifuged at 12,800 × g (Beckman) for 20 min. To test the specificity of primers and the PCR protocol as well as check for PCR inhibition, 1 ml of a previously grown pure culture of *Aeromonas* was spiked into 1 ml of an autoclaved (121°C, 15 min) sand pore water sample and processed with the above described protocol. The pellets obtained from centrifugation were washed in TE buffer and processed for an efficient nucleic acid extraction and purification to remove potential PCR inhibitors such as humic acid and debris of other compounds using the Qiagen Mini Stool DNA kit (Qiagen, USA) according to the procedure described by Khan *et al.* (2007). The purified DNA was further concentrated using the Pellet Paint kit as per the manufacturer's instructions (Novagen, Madison, USA) and resuspended in 25 μl of TE buffer. The concentration, quality and purity of the total microbial genomic DNA extract was confirmed by agarose gel electrophoresis using 1x TAE buffer.

Oligonucleotide primers

An *Aeromonas* genus-specific PCR was developed based on a newly designed oligonucleotide primer pair selected from conserved flanking regions of the gyrase B subunit (*GyrB*) gene. The following primer sequences were used: IA- Forward 5′-CTG AAC CAG AAC AAG ACC CCG-3′ and IA- Reverse 5′-ATG TTG TTG GTG AAG CAG TA-3′. The primer pair was used to amplify a *GyrB* fragment of an expected size of 130 bp. These primers were purchased from Integrated DNA Technologies (Coralville, USA). Initially the specificity of primers and optimization of the PCR protocol was carried out by a conventional Mastercycler Gradient PCR system (Eppendorf, Hamburg, Germany).

Real-time PCR for detection of *Aeromonas*

The amplification reaction was performed using SYBR Green JumpStart *Taq* ReadyMix (Sigma, USA). The reaction mixture (25 μl) consisted of a varying amount of genomic DNA template, 10 μl of 10X master mix including buffer, $MgCl_2$, dNTPs, SYBR Green and DNA polymerase,

0.5X reference dye and 40 ng each of the forward and reverse primers. The PCR amplification programme comprising an initial melting cycle (94°C for 2 min) followed by 40 cycles of amplification each involving 94°C for 10 s (denaturation), 55°C for 10 s (annealing) and 72°C for 10 s (extension) was optimized for *Aeromonas*. The reaction was performed using the Chromo*4* Real-Time PCR Detector system (Bio-Rad, USA). To check and confirm the quality of amplification, a melting profile was generated for the amplicon over a temperature range of 65°C to 95°C. Due to expected small amplicon fragment size, the PCR amplicons obtained from *Aeromonas* reference strains and field samples were confirmed by agarose electrophoresis using a 2% agarose gel matrix (Fisher Scientifics, California) with 1X TAE buffer containing ethidium bromide ($0.5\,\mu g\,ml^{-1}$) using 100 bp DNA size marker (PGC Scientifics, USA). Gels were visualized on a UV transilluminator and photographed using the Ingenius Syngene Bioimaging gel documentation system (Perkin Elmer, USA).

Real-time quantitative PCR for *Aeromonas*

Development of standard curves

Initially, a standard curve was prepared using a pure culture of the *A. hydrophila* reference strain as a template for real-time PCR amplification with varying number of cell equivalents per ml (10^6 through 10^0). In another set of reactions, the pure culture of *A. hydrophila* was spiked into an autoclaved (121°C, 15 min) sand pore water sample and a standard curve was developed with varying number of cell equivalents (10^6 through 10^1 per ml). The more turbid sand pore water sample represented the potential for more significant PCR inhibition and was used to validate the DNA purification procedure adopted. In order to obtain a standard curve, the threshold cycle (Ct) value for each set of reaction was plotted against each cell equivalent number.

Validation of real-time assay and application to field samples

Using the optimized real-time PCR amplification reaction and conditions, varying amounts of isolated total genomic DNA collected from the three different beach sampling zones were used for the detection of *Aeromonas* and quantification of their number per 100 ml based on the standard curve.

Sequencing of Q-PCR amplicons of field samples

The oligonucleotide primers and real-time Q-PCR protocol were evaluated to determine the specificity of amplified products for aeromonads. Eighteen of 96 positive real-time PCR amplicons representing water samples from each sampling location were arbitrarily selected and purified using a QIAquick gel extraction kit (Qiagen Sciences, Maryland, USA). The purified amplicons were sequenced at McMaster University (Hamilton, Ontario, Canada). The sequence data were further analysed using a BLAST search against the global database to identify that sample at the genus and species level. The sequences for all samples were further analysed and compared by multiple alignment with the *Aeromonas* reference strain using MegAlign 1993–2006 (DNASTAR Inc., Madison, Wisconsin).

Nucleotide sequence accession numbers

Sequences for the 18 *Aeromonas* Q-PCR positive amplicons were submitted to GenBank with the following accession numbers: EU369396 to EU369413, respectively.

Data analysis

Statistical analysis to compare culturable and total numbers of *Aeromonas* (viable but non-culturable cells) per 100 ml was performed by a paired t-test using JMP® 7.0.1 (SAS Institute Inc., Cary, North Carolina). In addition, the coefficient of variation (CV) was used for comparison of the variability between conventional culture and Q-PCR-based approaches.

RESULTS

Detection and enumeration of aeromonads

Occurrence patterns for *Aeromonas* at both beaches were very similar, so results were combined. All water samples

collected from sand pore water at both beaches showed the highest culturable *Aeromonas* numbers (ranging from 18 to 1.8×10^5 CFU 100 ml^{-1}), followed by ankle depth water (ranging from 15 to 5.9×10^3 CFU 100 ml^{-1}) and chest depth water (ranging from 9 to 9.5×10^3 CFU 100 ml^{-1}). Putative *Aeromonas* isolates from water samples routinely showed typical growth patterns and colony morphology on selective growth media. PCR assays have confirmed these growth and colony characteristics are reliable for identifying *Aeromonas* spp. isolates (data not shown).

PCR optimization and amplification

The newly designed gyrase B subunit (*GyrB*) gene primer pair successfully yielded a typical amplicon with a predicted size of 130 bp and detected all reference strains of *Aeromonas* species chosen for the study. The specificity of the primers was also tested on DNA templates prepared from the 35 different bacterial reference strains serving as negative controls, and did not show any cross-reaction (Table 1). The results indicated that the primer pair showed specificities only for all *Aeromonas* species (data not shown).

Quantitative real-time PCR and development of standard curve

Based on the conventional PCR results, standard curves for quantitation were generated using the optimized Q-PCR amplification assay conditions based on cell equivalent numbers ranging from 10^0 to 10^6 per ml for laboratory water and 10^1 to 10^6 per ml for spiked autoclaved sand pore water, respectively. The standard curves showed correlation coefficients (R^2) of 0.999 and 0.998, and the quantification detection limits were 1 and 10 cell equivalents per ml, respectively (Figures 1(a) and 2(a)). Typical amplification curves for the amplicons were also detected based on the fluorescent signals measured by the Chromo*4* detection system following the annealing step at 55°C (Figures 1(b) and 2(b)). The melting curve analysis of the PCR products showed typical melt profiles at 85°C (Figures 1(c) and 2(c)), while the negative control did not show any melting curve.

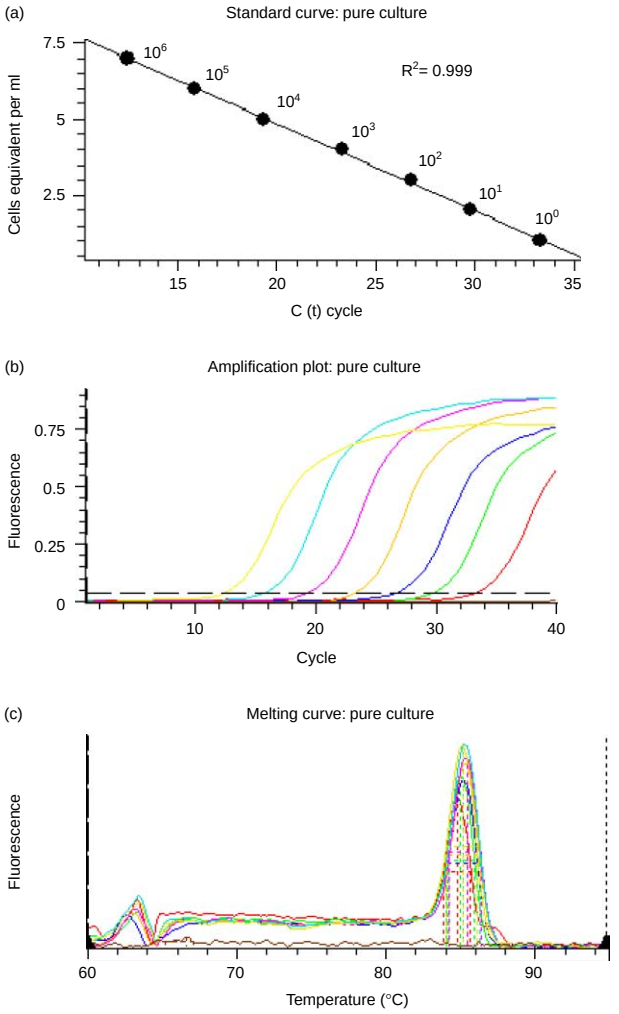

Figure 1 | (a) Development of a standard curve based on known number of cell equivalents per ml (10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0) plotted versus the cycle threshold (Ct) values using pure culture of *A. hydrophila* (ATCC 23123) reference strain; (b) real-time PCR amplification signals detecting the varying numbers of cells per ml of a pure culture of *A. hydrophila*; (c) the melting curve for the seven amplicons showing a specific relative intensity against the negative control at 85°C.

Protocol validation and quantification of total aeromonads in field water samples

The optimized real-time PCR protocol for detecting and quantifying total number of aeromonads in water samples was robustly validated. All 96 water samples yielded culturable counts on *Aeromonas* selective growth media, and showed positive real-time PCR amplification signals with specific melting profiles comparable to the standards (Figure 3(a) and (b)). Agarose gel electrophoresis confirmed the size and quality of the amplified products for all samples

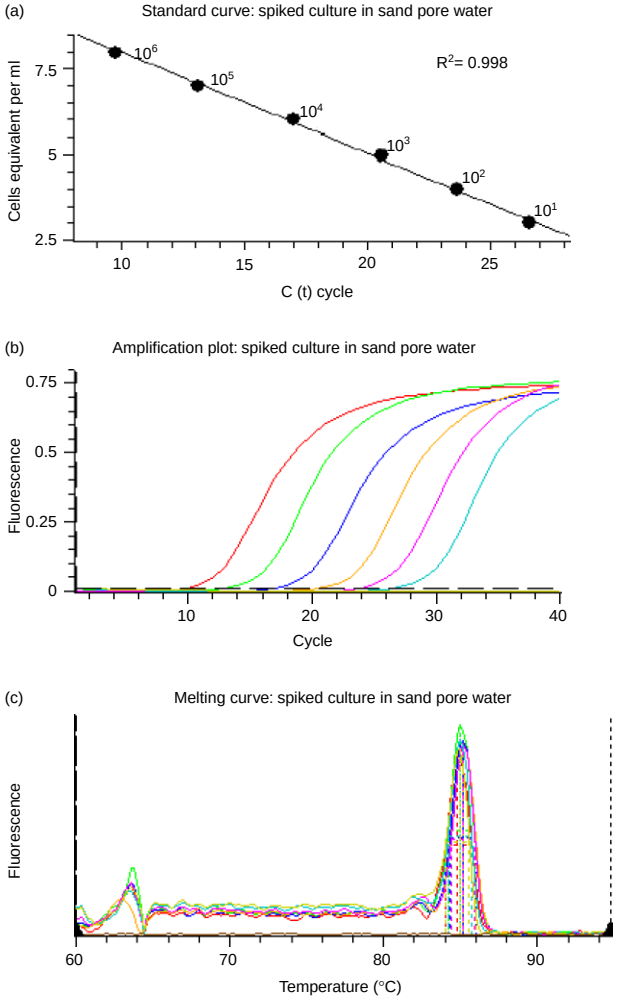

Figure 2 | (a) Standard curve based on known number of spiked cell equivalents per ml (10^6, 10^5, 10^4, 10^3, 10^2 and 10^1) plotted versus the cycle threshold (Ct) values using an autoclaved sand pore water sample; (b) real-time PCR amplification signals detecting the varying number of *A. hydrophila* cells per ml spiked into autoclaved sand pore water sample with a negative control; (c) the melting curve for the six amplicons showing a specific relative intensity similar to the pure culture of *Aeromonas* (85°C) against the negative control.

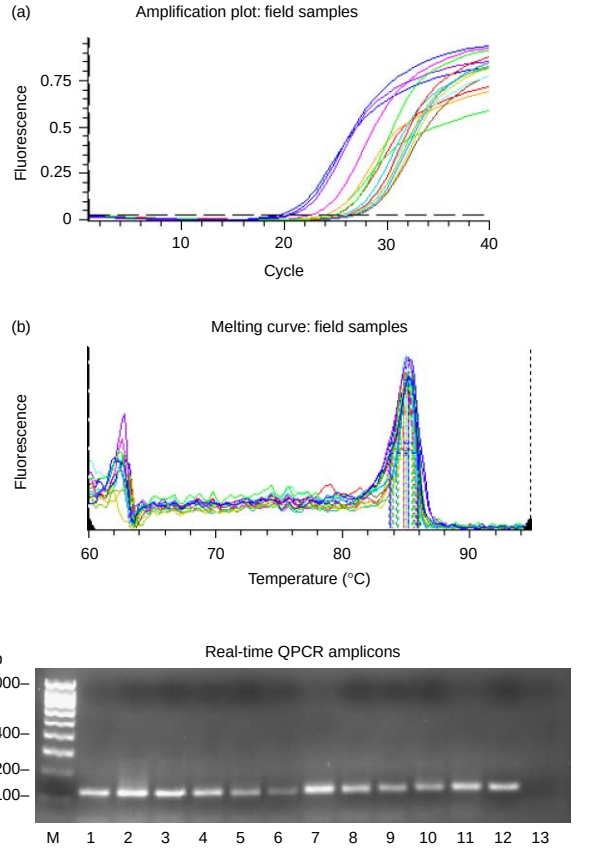

Figure 3 | (a) real-time PCR amplification signals for the detection and quantification of total numbers of *Aeromonas* cells per 100 ml in water samples collected from sand pore, ankle and chest depth waters of two Lake Ontario beaches. The varying cycle threshold (Ct) values corresponding to the total number of cells are shown in the graph (Figure 4); (b) the melting curve showing the same melting temperature (85°C) for all amplicons from water samples, corresponding to the relative intensity of *A. hydrophila* amplicons used as a standard; (c) confirmation of real-time PCR products on 2% agarose gel with an expected 130 bp fragment size (lanes 1–12 field isolates); lane 13 served as a negative control; M: 100 bp DNA size marker (PGC Scientifics, USA).

(Figure 3(c)). Total *Aeromonas* numbers in sand pore water samples ranged from 1.1×10^3 to 9.3×10^5 cell equivalents $100\,ml^{-1}$ compared with ankle depth water (ranging from 1.0×10^3 to 1.3×10^5 cell equivalents $100\,ml^{-1}$) and chest depth water (ranging from 3.2×10^2 to 1.7×10^4 cell equivalents $100\,ml^{-1}$). Even the water samples with the lowest culturable *Aeromonas* counts (as low as 9 cells $100\,ml^{-1}$) in chest depth water showed higher total counts (1,225 cell equivalents $100\,ml^{-1}$) by Q-PCR. This was also the case for sand pore (55 culturable cells $100\,ml^{-1}$ vs. 1.3×10^4 total cell equivalents $100\,ml^{-1}$) and ankle depth (15 culturable cells $100\,ml^{-1}$ vs. 4.6×10^3 total cell equivalents $100\,ml^{-1}$) water samples. The results revealed that the total cell equivalent numbers per 100 ml (including viable but non-culturable and dead cells) of aeromonads were significantly higher (paired *t*-test; $P < 0.05$) than the culturable cell numbers per 100 ml in each water sample collected from each sample location (Figure 4(a) and (b)). The coefficient of variation for the culture-independent Q-PCR protocol indicated less variability for enumerating *Aeromonas* than the culture-based method (Figure 4(c)). Across all 96 water samples, the Q-PCR protocol also

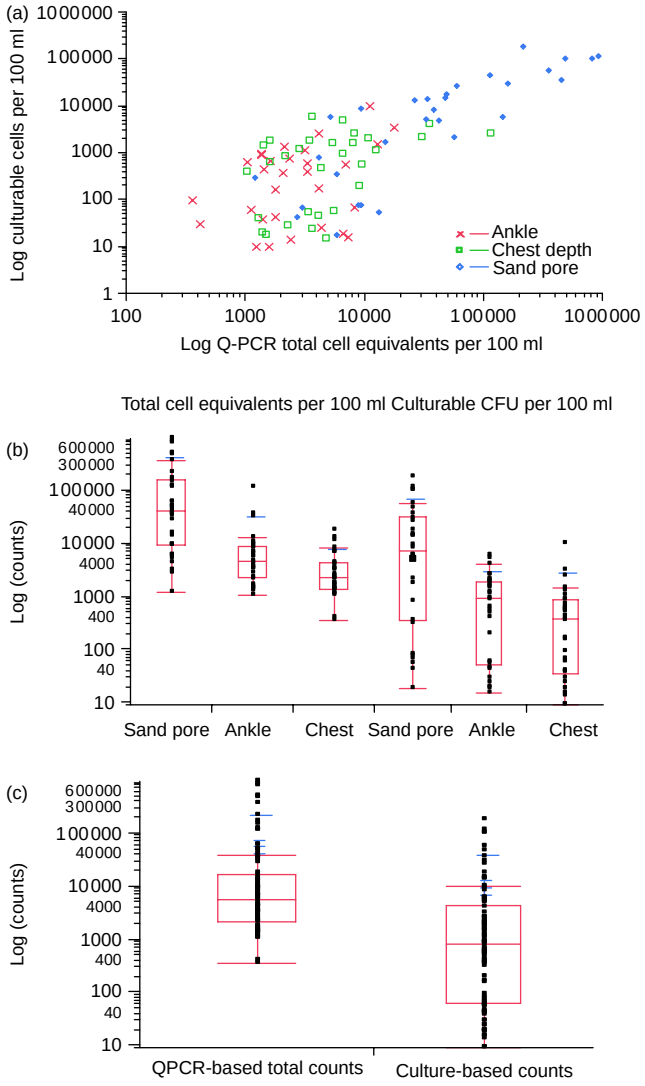

Figure 4 | Correlation (a) and comparative analysis (b) of culturable and Q-PCR total *Aeromonas* cell equivalents per 100 ml between individual samples representing each water sample location (ankle depth, chest depth and sand pore) at two Lake Ontario beaches; (c) comparative analysis of cultural and real-time Q-PCR-based assays for the detection and quantification of *Aeromonas* cells per 100 ml in water samples collected from three sample locations containing mixed bacterial flora. Several water samples with significant difference in total and culturable cells are shown as outliers.

detected significantly greater numbers (>16%) of cells than the conventional culture-based approach.

Nucleotide sequence-based identification

All 18 Q-PCR positive amplicons selected for sequencing were confirmed as *Aeromonas* to genus level by available GenBank sequence data analysis and showed >98% sequence homology with *Aeromonas* spp. by multiple sequence alignment results (data not shown).

DISCUSSION

Traditional and novel DNA-based molecular methods are improving knowledge about the occurrence of *Aeromonas* spp. in aquatic ecosystems. Unlike conventional PCR assays, real-time PCR uses short fragments that can be amplified efficiently. In addition, detection is based on hybridization to specific sequences rather than amplicon product length; hence time consuming blot and probe techniques are not necessary to confirm product identity. However, the use of PCR assays as a diagnostic tool depends on a high quality purified nucleic acid template. The present study adopted the previously optimized protocol using centrifugation concentration and rigorous DNA purification approach for PCR amplification of templates naturally contaminated with PCR inhibitors such as humic acids, and debris of other compounds (Khan *et al.* 2007). The success of this quantitative real-time Q-PCR assay was, in part, based on direct and efficient DNA recovery using a centrifugation procedure as well as the efficient purification of a high quality DNA template for sensitive PCR amplification. Our data on cell equivalents and colony forming units are presented as minimum detectable numbers given some possibility of less than 100% DNA extraction efficiency and less than 100% cell recovery. The present Q-PCR protocol was successfully developed and optimized for quantifying the total number of aeromonads in recreational water samples. The protocol was validated and applied to water samples collected from two fresh water beaches on Lake Ontario.

Several genes have been used for the molecular detection and confirmation of *Aeromonas*. However, the selection of the *GyrB* gene (encoding the B-subunit of DNA gyrase, a type-II DNA topoisomerase) in this study was based on its suitability as a phylogenetic marker for bacterial systematics (Kasai *et al.* 1998; Yamamoto *et al.* 1999; Yáñez *et al.* 2003). This protein plays a crucial role in the DNA replication process, and its gene has a mean synonymous substitution rate that is almost four times that

of the 16S rDNA gene (Yamamoto & Harayama 1996). Although the *GyrB* gene is ubiquitously distributed among bacterial species, it is rarely transmitted horizontally (Kasai *et al.* 1998). Recently this gene has been characterized in 53 *Aeromonas* strains (Yáñez *et al.* 2003). Therefore, the gene provided valuable conserved sites for designing *Aeromonas* genus-specific primers.

Following the development and optimization of the assay targeting the *GyrB* gene, it was applied to the field samples for validation purposes. The developed protocol can be used to detect a minimum of 10 cells per ml of *Aeromonas* in environmental samples. A significant difference between the results obtained from culture-based and real-time Q-PCR assays was observed. On average, the Q-PCR protocol detected >16% more cells than culture-based techniques. It is possible that viable but non-culturable *Aeromonas* spp., which may potentially be infectious, might not be detected by culture-based methods but could be detected by Q-PCR assay. A more accurate enumeration of *Aeromonas* in water samples may allow a better assessment of the likelihood that high numbers of *Aeromonas* may interfere with existing methods for enumerating coliform bacteria in water samples. Conventional cultural-based assays have shown that lactose-fermenting aeromonads can interfere with counts of total coliform bacteria in environmental and drinking water samples by yielding false positive results based on their β-galactosidase activity (Leclerc *et al.* 1977; Freier & Hartman 1987). β-galactosidase can be used in rapid and simple methods that differentiate lactose-utilizing from non-lactose-utilizing members of the family Enterobacteriaceae (Edberg *et al.* 1988; Rambach 1990); however, most *Aeromonas* spp. also showed β-galactosidase activity (Sakazaki & Balows 1981). Ciebin *et al.* (1995) reported false-positive β-glucuronidase *Aeromonas* spp. with the isolates of *E. coli*, *Klebsiella pneumoniae*, *Citrobacter freundii* and *Enterobacter* species at specific thermophilic cultural conditions.

Several other studies have also observed a significant difference between Q-PCR and culture-based methods investigating microbial pathogens and water quality indicators in various environmental and food matrices (Ibekwe & Grieve 2003; Khan & Yadav 2004; Bohaychuk *et al.* 2007; Khan *et al.* 2007). Several factors might contribute to the difference in detection between culture-based and real-time Q-PCR assays. First, injured or stressed cells which had lost their viability and/or culturability would not be detected by a culture-based method. Second, the culture-based method might underestimate the concentration of *Aeromonas* because only green centred with opaque margin colonies on *Aeromonas* selective agar plates were considered as presumptive *Aeromonas*. However, some strains of *Aeromonas* could possibly be Ampicillin-sensitive, an antimicrobial agent that was used in our media to suppress the growth of background bacteria. Previous studies have reported the presence of atypical colonies on selective growth media as *Aeromonas* as well as the occurrence of Ampicillin-sensitive *Aeromonas* strains (Havelaar *et al.* 1987; Holmes & Sartory 1993; EPA 2001). Third, after prolonged incubation of cultures, the colonies can diffuse and merge, which may lead to lower colony counts (Havelaar *et al.* 1987). On the other hand, real-time PCR may overestimate the number of viable cells due to the detection of dead cells. To estimate the number of target genes in viable cells, there is need to first quantify the live cells and dead cells in a sample before applying the developed Q-PCR assays. In a recent study, Nocker *et al.* (2006) reported the use of propidium monoazide (PMA) in combination with Q-PCR, which can quantify viable and dead cells over a wide range of bacterial pathogens. Moreover, several studies have been conducted on the use of ethidium monoazide (EMA) to quantify viable cells. However, a disadvantage of EMA is that it can penetrate into live cells of some bacterial species (Nogva *et al.* 2003; Rudi *et al.* 2005; Wang & Levin 2006). It is possible our real-time Q-PCR assay could be used in combination with either PMA or EMA in future studies to quantify the number of viable *Aeromonas* in recreational waters and other environmental matrices.

The Q-PCR protocol developed in this study can also contribute to gaining a better understanding of the prevalence and ecology of aeromonads in recreational waters. The two beaches we studied have been frequently closed in recent years due to high levels of *E. coli* (O'Connor 2003). A microbial source tracking study found that beach sand was serving as an important secondary source of *E. coli* contamination of the adjacent beach water at BayFront Beach, and that there was a significant concentration gradient of declining *E. coli* concentrations moving offshore

from the beach (Edge & Hill 2007). Our current study found a similar concentration gradient of high *Aeromonas* numbers in foreshore sand, with declining concentrations in water samples collected while wading offshore from the beach. This suggests that foreshore sand can also serve as a reservoir of high concentrations of ubiquitous bacteria such as *Aeromonas* similar to the phenomenon noted for enteric bacteria such as *E. coli*. Further research is warranted in order to better understand the relationship between Q-PCR results, *Aeromonas* health risks to humans and the ecology of *Aeromonas* in beach sand.

CONCLUSIONS

A Q-PCR protocol was developed to allow a rapid detection and quantification of the total number of aeromonad cells in water. This protocol can contribute to better understanding of various ecological factors involved in the prevalence, survival and human health risks of *Aeromonas* in recreational waters. Application of the Q-PCR protocol at two Lake Ontario beaches found that foreshore sand can serve as a reservoir of high concentrations of *Aeromonas* similar to the phenomenon noted for enteric bacteria such as *E. coli*.

ACKNOWLEDGEMENTS

The study was funded by the Great Lakes 2020 program. We thank Dr Kent Burnison (retired), Dr James Sherry and Neheli Tannis (Environment Canada, Burlington) for providing research facilities for this work. We also thank Eva Nowak, Flora Suen and Simone Vandaiyar for helping in water sample collection and laboratory assistance. Part of this study was presented at the 57th Annual Conference of the Canadian Society of Microbiologists (CSM), Québec City, Québec, 17–20 June 2007.

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First received 17 April 2008; accepted in revised form 30 July 2008. Available online February 2009