Multiplex PCR for detection of water-borne bacteria

Roohollah Kheiri, Reza Ranjbar, Mojtaba Memariani and Leili Akhtari

ABSTRACT

Microbial water-borne diseases still affect developing countries and are major water quality concerns throughout the world. Routine culture-based methods of identifying bacterial pathogens in water sources are laborious and time-consuming. Recently, the use of molecular techniques such as the polymerase chain reaction (PCR) has provided rapid and highly promising detection methods. In this study, we developed two multiplex PCR assays for simultaneous detection of six water-borne bacteria. Two triplex PCR protocols were developed to detect six target genes. The first protocol targets *uidA* (*Escherichia coli*), *int* (*Shigella* spp.), and *gyrB* (*Pseudomonas aeruginosa*) genes, while *invA* (*Salmonella* spp.), *ompW* (*Vibrio cholera*), and *lacZ* (coliforms) were amplified by the second protocol. Specificity testing was carried out for 12 reference strains. Furthermore, the applicability of the multiplex PCR assays for detection of these bacteria was investigated for 52 surface water samples. The results indicated that all primer pairs showed specificities only for their corresponding target organisms. The detection sensitivity of both multiplex PCR assays was $3 \times 10^2 - 3 \times 10^3$ colony forming units. The developed assays represent simple and efficient diagnostic procedures for co-detection of water-borne bacteria and have the potential to provide earlier warnings of possible public health threats and more accurate surveillance of these organisms.

Key words | *Escherichia coli*, multiplex PCR, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, water

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INTRODUCTION

Water-borne pathogens that cause gastroenteritis remain a major global health concern and are one of the most common infectious causes of morbidity and mortality worldwide (Khan *et al.* 2000). Water sources, drinking water supply systems and treated drinking water can become contaminated with naturally occurring microbes or toxins, but may also be the targets of sabotage and intentional contamination. Microbial agents that may be utilized in intentional contamination of drinking water comprise naturally occurring enteropathogens, eradicated or uncommon pathogens, genetically modified bacteria or viruses or microbial toxins (Kortepeter and Parker 1999; Nicolson 2002). In theory, any microbe or microbial toxin possessing the potency to cause illness or a disorder in humans can be used as a bacterial agent against a target population through the drinking water supply. Thus, regular monitoring of water-borne microbes is required to protect public health (Nicolson 2002).

Conventional diagnostic procedures for routine detection of water-borne bacteria such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., and *Vibrio cholera* involve enrichment steps, selective culture, biochemical identification, and serotyping (Rundell *et al.* 2014). These steps are laborious, tedious, time-consuming, and low throughput, often taking several days to get a final result. Furthermore, several pathogens cannot be distinguished from normal flora by morphology and biochemical properties. Other pathogens are either difficult to culture or non-culturable, but can still cause illnesses (APHA/AWWA/WEF 2012; Luyt *et al.* 2012). In comparison with conventional

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culture-based methods, molecular methods are more rapid and offer sensitive detection, and can potentially be adapted by health authorities to improve water quality monitoring (Alhamlan *et al.* 2015). The main advantage of microbial identification by genetic markers is the relative stability of the genotype rather than the phenotype (Alhamlan *et al.* 2015). Amongst molecular approaches, the polymerase chain reaction (PCR) is currently the most frequently used technique because of its versatility, high specificity and sensitivity. In this regard, it is possible to selectively amplify a fragment of DNA that has a sequence shared only by particular species or strains. However, if a single primer set is used (uniplex PCR), a large number of individual PCR assays would be necessary to detect all bacteria of interest, leading to a relatively laborious, costly, and time-consuming process. Recently, the use of multiplex PCR has provided a rapid, specific, and highly sensitive method for co-detection of water-borne pathogens in aquatic environments (Alhamlan *et al.* 2015; Ramírez-Castillo *et al.* 2015).

In this study, we have developed and applied two high-throughput multiplex PCR assays for simultaneous detection of six different water-borne bacteria including *E. coli*, coliforms, *Pseudomonas aeruginosa*, *Shigella* spp., *Salmonella* spp., and *Vibrio cholerae*.

METHODS

Bacterial strains, culture conditions, and DNA extraction

For development and optimization of the PCR assays, *Salmonella typhimurium* (American Type Culture Collection 14028), *E. coli* (ATCC 25922), *Vibrio cholerae* (ATCC 14035), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella sonnei* (ATCC 9290), as well as 12 type strains (as negative controls) were purchased from the Iranian Research Organization for Science and Technology (IROST). All of the bacterial strains were cultured in the appropriate media (BHI broth) and under optimal growth condition (37 °C, aerobic), according to the IROST recommendations. Following overnight incubation, the optical density of bacterial cultures was adjusted to a 1 McFarland standard. Then, 10 ml of the cultures were centrifuged at 2,500 rpm for 15 min. The supernatant was discarded and genomic DNA was extracted from the bacterial pellet using an AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea).

PCR primer design

Highly conserved regions within the genome of corresponding bacterial pathogens were chosen as target genes, including *uidA* (encoding for β-glucuronidase, specific for *E. coli*), *int* (encoding for putative integrase, specific for *Shigella* spp.), *gyrB* (encoding for DNA gyrase subunit B, specific for *Pseudomonas aeruginosa*), *lacZ* (encoding for β-galactosidase, specific for coliforms), *invA* (encoding for type III secretion system protein InvA, specific for *Salmonella* spp.), and *ompW* (encoding for outer membrane protein W, specific for *Vibrio cholerae*).

A total of six sets of primers were designed using Allele ID v7.6 software (PREMIER Biosoft, USA) based on available data from GenBank® (www.ncbi.nlm.nih.gov/genbank). We also used Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to confirm lack of DNA homology in bacterial species other than the mentioned pathogens. The sequences and amplicon sizes of the primers are shown in Tables 1 and 2.

Table 1 | List of gene-specific PCR primers for the first protocol

Target microorganism	Target gene	Primer sets (5′ to 3′)	Annealing temperature (°C)	Amplicon size (bp)	Primer concentration (pmol)
E. coli	*uidA*	GAACTGGCAGACTATC CGTATTCGGTGATGAT	60	1,103	8
Shigella sonnei	*int*	GCTGGATGAACGATGTCCAC ATCTGGCGGCTATGAGATGG	60	356	6
Pseudomonas aeruginosa	*gyrB*	CAAGGGCAAGATCCTCAAC CATCTGGCGGAAGAAGAAG	60	217	4

Table 2 | List of gene-specific PCR primers for the second protocol

Target microorganism	Target gene	Primer sets (5′ to 3′)	Annealing temperature (°C)	Amplicon size (bp)	Primer concentration (pmol)
Vibrio cholerae	*ompW*	TTAACGCTTGGCTATATGTT GAGGAACCAGCTATCATTG	60	345	6
Salmonella typhimurium	*invA*	GGCGATAGCGATAATATG AAATAGACCGTAAATTGTTCA	60	500	6
Coliforms	*lacZ*	TGAAGCAGAACAACTTTA CATATTTAATCAGCGACTG	60	600	6

Uniplex and multiplex PCR assays

The PCR assays were optimized using a Veriti® 96-Well Thermal Cycler (Applied Biosystems). For uniplex PCR assays, 2X PCR Master Mix (CinnaGen, Iran), different concentrations of primer sets (2–10 pmols) (all synthesized by Bioneer, South Korea), 1 μl of genomic DNA (approximately 100 ng/μl), and double-distilled water to a total volume of 20 μl was used. The PCR conditions were as follows: denaturation at 95 °C for 5 min, annealing at 58 °C to 63 °C (depending on the primer sets), 1 min at 72 °C, and a final extension at 72 °C for 7 min. The reactions resulted in a 1103-bp fragment for *uidA* (*E. coli*), a 600-bp fragment for *lacZ* (coliforms), a 217-bp fragment for *gyrB* (*Pseudomonas aeruginosa*), a 356-bp fragment for *int* (*Shigella sonnei)*, a 500-bp fragment for *invA* (*Salmonella typhimurium*), and a 345-bp fragment for *ompW* (*Vibrio cholerae*).

For multiplex PCR assays, two protocols were designed: the first protocol targets *uidA*, *int,* and *gyrB,* while *invA, ompW*, and *lacZ* were amplified by the second protocol. The multiplex PCR reactions consisted of *AccuPower*® HotStart PCR PreMix (containing a thermostable DNA polymerase, pyrophosphatase, reaction buffer, dNTPs, tracking dye, and a patented stabilizer), different concentrations of primer sets (2–10 pmols) (Bioneer, South Korea), 1 μL (approximately 100 ng/μl) of genomic DNA, and double-distilled water to a total volume of 20 μl. The PCR condition used was as follows: denaturation at 95 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 40 s at 60 °C (for both protocols), 1 min at 72 °C, and a final extension at 72 °C for 7 min. All PCR products were evaluated by electrophoresis in 1.5% agarose gel containing SYBR green and visualized by a Gel Doc™ XR$^+$ (BIORAD).

Sensitivity and specificity of primer sets

In order to evaluate the sensitivity of the multiplex PCR assays, suspension of type strains in Luria-Bertani broth (incubated for 18 h) were centrifuged at 2,500 rpm for 15 min, and washed with phosphate buffered saline (PBS). The bacterial pellet was suspended in distilled water and adjusted to a 1 McFarland standard. A 10-fold serial dilution with sterile distilled water was performed for eight consecutive concentrations (3×10^8 to 3×10^1 CFU/ml). Twenty ml of the serial dilutions ($20\times3\times10^8$ to $20\times3\times10^1$ CFU) were subjected to an AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea), and extracted DNA was dissolved in 20 μl elution water, yielding 3×10^8 to 3×10^1 copies of DNA in 1 μl of eluted water. One μl of DNA for each pathogen was subsequently mixed together and used as a template for the PCR. The multiplex and uniplex PCR assays were performed simultaneously for the same dilution series, by using the same PCR machine and in the same program as described above. All PCR products were evaluated by electrophoresis in 1.5% agarose gel containing SYBR green and visualized under ultraviolet light.

Analysis of spiked water

To spike, bacterial suspensions of *Salmonella typhimurium* (ATCC 14028), *E. coli* (ATCC 25922), *Vibrio cholerae* (ATCC 14035), *Pseudomonas aeruginosa* (ATCC 27853), and *Shigella sonnei* (ATCC 9290) were adjusted to an optical density equivalent to a 1 McFarland standard. Ninety-nine ml of sterile PBS was inoculated with 1 ml of the bacterial suspensions yielding a concentration of 3×10^6 CFU/ml for each type strain. Ten ml of the spiked water was centrifuged

at 2,500 rpm for 15 min and genomic DNA was extracted using an AccuPrep® Genomic DNA Extraction Kit.

Analysis of environmental samples

In order to evaluate the applicability of the multiplex PCR assays, we applied them to detect the mentioned bacteria in surface water samples. Following daily sampling, 52 surface water samples were collected from the Karaj River with geographic coordinates of 35°48′46.48″N; 51°0′43.03″E, between July and August 2015. Sampling bottles were immediately placed in a lightproof insulated box containing icepacks to ensure rapid cooling. The samples were shipped to the central laboratory of the Alborz Province Water and Wastewater Co. In order to validate the PCR results, all samples underwent the culture-based tests. For culture-based tests, conventional methods according to Standard Methods for the Examination of Water and Wastewater 22nd edition were used. All samples were analyzed for the presence of *E. coli* (method 9221), coliforms (method 9221), *Pseudomonas aeruginosa* (method 9213 F), *Shigella* spp. (method 9260 E), *Salmonella* spp. (method 9260 B), and *Vibrio cholerae* (method 9260 H) (APHA).

Since the number of bacteria in the water samples is not high enough for DNA extraction, samples were enriched in a highly boosting medium using GN enrichment broth (Merck KGaA). Experiments were conducted in 250 ml bottles containing 50 ml of 3X GN enrichment broth and 100 ml of water sample at 37 °C in a shaking incubator at 180 rpm for 12 hours. Confirming the turbidity, 50 ml of the sample was transferred into a 50 ml Falcon tube and centrifuged at 2,500 rpm for 15 min. Supernatant was discarded and genomic DNA was extracted using an AccuPrep® Genomic DNA Extraction Kit.

RESULTS

Specificity of multiplex PCR assays

Following the optimization of primer set concentrations in multiplex PCR assays (Tables 1 and 2), the specificity of each primer set was also evaluated. For two protocols of the multiplex PCR, each corresponding DNA and combinations of all DNA were amplified successfully without non-specific bands (Figure 1). Each PCR product could be observed as a clear band at 1,103, 600, 217, 504, 356, 500, and 345 bp generated by *uidA*, *lacZ*, *gyrB*, *int*, *invA*, and *ompW* primer sets, respectively. In addition to the above-mentioned bacteria, genomic DNA was also extracted from reference strains (listed in Table 3) and used as a template in the PCR assays. Lack of amplifications for these bacterial strains confirms the high specificity of the newly designed primer sets.

Sensitivity of multiplex PCR assays

The sensitivity of the uniplex and multiplex PCR assays was subsequently examined in 10-fold serial dilutions of bacterial DNA mixtures. The sensitivity of the uniplex PCR for each primer was (approximately) $3 \times 10 - 3 \times 10^2$ CFU of bacteria per ml (data not shown). As shown in Figure 2, a detection limit of $3 \times 10^2 - 3 \times 10^3$ CFU was noted for mixtures of all primers in both multiplex PCR assays. Although the infectious concentrations varied between pathogens, it is generally believed that most bacterial pathogens can cause infection when more than 10^3 infectious cells are ingested (Ramírez-Castillo *et al.* 2015). Hence, the detection limit of the multiplex assays described in this study was within the infectious doses of most enteric pathogens.

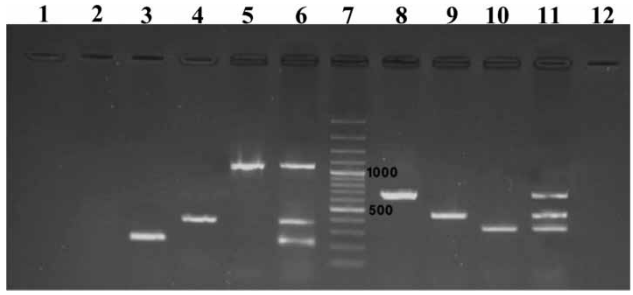

Figure 1 | Gel agarose analysis of uniplex and multiplex PCR protocols of spiked water. Lane 1, *Enterococcus faecalis* (ATCC 29212) as negative control; Lane 2, *Pseudomonas putida* (ATCC 12633) as negative control; Lane 3, *gyrB* for *Pseudomonas aeruginosa* (ATCC 27853) (217 bp); Lane 4, *int* for *Shigella sonnei* (ATCC 9290) (356 bp); Lane 5, *uidA* for *E. coli* (ATCC 25922) (1103 bp); Lane 6, triplex for *gyrB, int,* and *uidA;* Lane 7, DNA 100 bp ruler (Thermo Scientific); Lane 8, *lacZ* for *E. coli* (ATCC 25922) (600 bp); Lane 9, *invA* for *Salmonella typhimurium* (ATCC 14028) (500 bp); Lane 10, *ompW* for *Vibrio cholera* (ATCC 14035) (345 bp), and Lane 11, triplex for *invA*, *ompW,* and *lacZ;* Lane 12, blank (nothing loaded).

Table 3 | Specificity of the primer sets

Reference strains	PCR results					
	E	C	Sh	Sa	V	P
Enterococcus faecalis ATCC 29212 (PTCC 1778)	–	–	–	–	–	–
Enterococcus faecalis ATCC 19433 (PTCC 1774)	–	–	–	–	–	–
Proteus vulgaris PTCC 1079	–	–	–	–	–	–
Pseudomonas putida ATCC 12633 (PTCC 1694)	–	–	–	–	–	–
Serratia marcescens ATCC 13880 (PTCC 1621)	–	–	–	–	–	–
Staphylococcus aureus ATCC 25923 (PTCC 1431)	–	–	–	–	–	–
Streptococcus pyogenes ATCC 19615 (PTCC 1762)	–	–	–	–	–	–
Bacillus cereus ATCC 11778 (PTCC 1015)	–	–	–	–	–	–
Salmonella typhi PTCC 1609	–	–	–	+	–	–
Enterobacter aerogenes ATCC 13048 (PTCC 1221)	–	+	–	–	–	–
E. coli NCIMB 11032 (PTCC 1395)	+	+	–	–	–	–
Vibrio cholerae PTCC 1611	–	–	–	–	+	–

PTCC: Persian Type Culture Collection.
E: *E. coli*-specific primers.
C: Coliform-specific primers.
Sh: *Shigella* spp.-specific primers.
Sa: *Salmonella* spp.-specific primers.
V: *Vibrio cholerae*-specific primers.
P: *Pseudomonas aeruginosa*-specific primers.
+, Positive PCR result.
–, Negative PCR result.

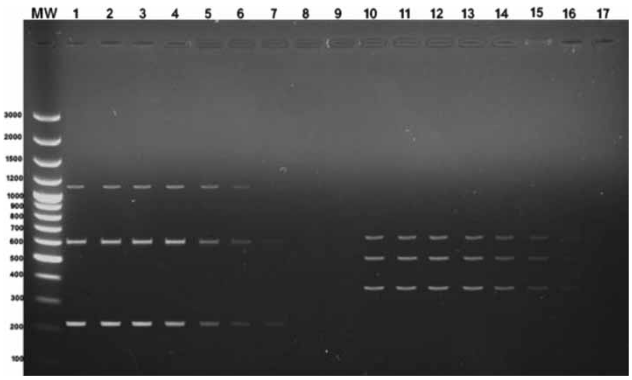

Figure 2 | Gel agarose analysis of two multiplex PCRs with a serial 10-fold dilution of mixed bacterial suspensions. Lane MW, DNA 100 bp ruler (Thermo Scientific); Lanes 1 to 8, first triplex PCR with DNA concentration from 3×10^8 to 3×10^1 CFU/ml. Lane 9, blank (nothing loaded). Lanes 10 to 17, triplex PCR with DNA concentration from 3×10^8 to 3×10^1 CFU/ml. As shown in this figure, a detection limit of 3×10^2–3×10^3 CFU/ml was noted for mixtures of all primers in two multiplex PCR protocols.

Application to environmental and spiked samples

As described earlier, we applied both multiplex PCR assays and culture-based tests for 52 environmental samples. In this regard, multiplex PCR assays showed that all of the Karaj River samples contained coliforms, *E. coli*, *Shigella* spp., *Salmonella* spp., and *P. aeruginosa*. However, only two out of 52 samples were contaminated with *V. cholerae*. Results of culture-based examinations justified the PCR assays, since exactly the same results were obtained, which confirms the excellent and ideal consistency and association between these methods (Table 4). Furthermore, for the spiked sample, all target genes were detected (Figure 2). These promising results proved the possibility of assessing six different types of bacteria in actual and artificial water matrices within just 16 hours (12 hours for enrichment and 4 hours for PCR assay).

DISCUSSION

In this study, we demonstrated a good level of correlation between the results of culture-based tests and multiplex PCR assays for detection of water-borne pathogens. Currently, there is no unified method to collect, process, and analyse water samples for all pathogenic microorganisms of interest (Bitton 2014; WHO 2014; Zhao *et al.* 2014). In addition, a number of problems are encountered in detection of pathogens in water sources such as low concentration of pathogens; this usually requires enrichment of the samples prior to detection processing, and the presence of inhibitors in water samples, including humic

Table 4 | Number of samples and positive results

Strain	Number of samples	C	P
Coliforms	52	52	52
E. coli	52	52	52
Shigella spp.	52	52	52
Salmonella spp.	52	52	52
P. aeruginosa	52	52	52
V. cholerae	52	5	5

C: No. of positive results through culture-based examinations.
P: No. of positive results through PCR assays.

acids and metals (Bitton 2014). Furthermore, since some water-borne pathogens exist in a viable but non-culturable (VBNC) state, false negative results may arise from culture-based methods. Therefore, the number of pathogens in this state could be underestimated and, if all of the microorganisms in the sample are in the VBNC state, the sample may be regarded as pathogen-free due to non-detection (Li *et al.* 2014).

To overcome the limitations of culture-based methods, molecular approaches have been developed to identify the microorganisms. These approaches, including PCR, have the advantages of quick analysis and higher sensitivity compared to the traditional culture-based methods (Ramírez-Castillo *et al.* 2015). However, if a single primer set is used (uniplex PCR), a large number of individual PCR assays would be necessary to detect all bacteria of interest, which can be a relatively laborious, costly, and time-consuming process. Because of this, multiplex PCR has been developed for co-detection of pathogens. It involves the simultaneous amplification of more than one target gene per reaction by multiple primer sets with different specificities. The resulting PCR amplicons of different molecular weight can be separated by agarose gel electrophoresis. The expense in terms of reagents and preparation time is lower in multiplex PCR than in systems where several tubes of uniplex PCRs are used. Using multiplex PCR, it is possible to directly detect several pathogens at the same time in a single test, and existing problems associated with conventional culture methods can be avoided (Abd-El-Haleem *et al.* 2003).

These numerous advantages have made multiplex PCR the method of choice for researchers. For instance, Tsen *et al.* developed a multiplex PCR assay for detection of heat-labile toxin I and heat-stable toxin II genes of enterotoxigenic *E. coli* (ETEC) in skim milk and porcine stools (Tsen *et al.* 1998). Si Hong Park *et al.* designed and developed a protocol by which *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes would be detected in a single tube (Park *et al.* 2011). Park *et al.* showed such protocols would reduce the time of detection from 48 hours with culturing to 4 hours with the multiplex PCR. In another study, Wei *et al.* applied a multiplex PCR assay for rapid detection of *Pasteurella multocida*, *Salmonella enterica*, *Riemerella anatipestifer*, and *Escherichia coli* in ducks (Wei *et al.* 2013). Wei *et al.* believed that distinguishing these pathogens based on clinical signs would be difficult because these pathogens can cause similar clinical signs and co-infections can occur, and concluded that the best method to detect and differentiate these bacteria is by multiplex PCR. In addition to conventional PCR, real-time PCR-based approaches for accurate quantification of bacterial RNA targets in water have also been developed. In this regard, Fey *et al.* succeeded in quantifying DNA with high accuracy and sensitivity from environmental water samples (Fey *et al.* 2004). In another study conducted by Huang *et al.*, a quadruplex real-time PCR assay was developed for detection and identification of *Vibrio cholerae* O1 and O139 strains and determination of their toxigenic potential (Huang *et al.* 2009).

In the present study, six primer sets were designed to simultaneously detect six different water-borne bacterial indicators and pathogens by two separate multiplex PCR protocols. The corresponding primer sets showed significant affinities for their target genes. In order to facilitate PCR product detection, the primer sets were designed so that the expected size of the amplicons from each target gene would be different in gel electrophoresis. In our study, no products were observed from the negative controls and only specific and expected PCR amplicons from the positive controls were obtained, confirming the accuracy, sensitivity and specificity of the protocols. Furthermore, we confirmed the applicability of multiplex PCR not only for spiked water but also for actual water samples. Our findings are generally consistent with results of other studies in which the detection limit of the multiplex PCR for the bacterial targets was estimated at 10^2 CFU/ml (Kong *et al.* 2002; Fan *et al.* 2008). We also believe that it is possible to reduce bacterial detection time in water to less than 16 hours. This can be achieved by enriching the sample to increase the number of bacteria prior to analysis by multiplex PCR.

CONCLUSION

This study confirms the applicability of multiplex PCR for water quality monitoring. In conclusion, our findings showed that the developed multiplex PCR protocols represent a simple and efficient diagnostic for co-detection of

water-borne bacteria and have the potential to provide earlier warnings of possible public health threats and more accurate surveillance of these organisms.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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