# DEVELOPMENT OF MULTIPLEX PCR FOR THE DETECTION OF TOTAL COLIFORM BACTERIA FOR *ESCHERICHIA COLI* AND *CLOSTRIDIUM PERFRINGENS* IN DRINKING WATER

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Abstract. Multiplex PCR amplification of *lacZ*, *uidA* and *plc* genes was developed for the simultaneous detection of total coliform bacteria for *Escherichia coli* and *Clostridium perfringens*, in drinking water. Detection by agarose gel electrophoresis yielded a band of 876 bp for the *lacZ* gene of all coliform bacteria; a band of 147 bp for the *uidA* gene and a band of 876 bp for the *lacZ* gene of all strains of *E. coli*; a band of 280 bp for the *plc* gene for all strains of *C. perfringens*; and a negative result for all three genes when tested with other bacteria. The detection limit was 100 pg for *E. coli* and *C. perfringens*, and 1 ng for coliform bacteria when measured with purified DNA. This assay was applied to the detection of these bacteria in spiked water samples. Spiked water samples with 0-1,000 CFU/ml of coliform bacteria and/or *E. coli* and/or *C. perfringens* were detected by this multiplex PCR after a pre-enrichment step to increase the sensitivity and to ensure that the detection was based on the presence of cultivable bacteria. The result of bacterial detection from the multiplex PCR was comparable with that of a standard plate count on selective medium (p=0.62). When using standard plate counts as a gold standard, the sensitivity for this test was 99.1 % (95% CI 95.33, 99.98) and the specificity was 90.9 % (95% CI 75.67, 98.08). Multiplex PCR amplification with a pre-enrichment step was shown to be an effective, sensitive and rapid method for the simultaneous detection of these three microbiological parameters in drinking water.

## INTRODUCTION

Microbial indicators, in particular coliform bacteria and *Escherichia coli*, have been established for assessing the microbiological safety of drinking water. The presence of *E. coli* in water is an indicator of fecal contamination and implies that pathogenic bacteria, viruses, and protozoa may also be present (Environment Agency, 2002). These indicators, used to assess the potential public health risk of drinking water and their presence or absence, are key elements of most drinking water quality guidelines (WHO, 1997).

The traditional method used to examine water samples for the presence of coliform bacteria and other pathogens was to culture the water sample on nutrient media. This method is time consuming and unable to distinguish between the closely related pernicious and benign strains of bacteria (Water Research Commission, 2001). It fails to detect viable but non-culturable organisms, where the bacteria lose their ability to grow on agar plates but remain metabolically active (Singh and McFetess, 1992; Hickey and Harkin, 1998).

The polymerase chain reaction (PCR) technique, a molecular biology method, is an interesting alternative for detecting microbiological indicators in water samples based on the detection of genetic material from target bacteria (Mullis and Faloona, 1987). This technique allows rapid detection of pathogens in water by providing levels of sensitivity and specificity difficult to achieve with traditional culture-based assays. It can be used to identify specific bacterial strains within mixed populations. Several PCR-based methods have been reported, in-

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cluding multiplex PCR, which evaluates for more than one type of pathogen with each analysis. Multiplex PCR has been used for different purposes. Since its first description in 1988, this method has been successfully applied to many areas of DNA testing, including analyses of deletions, mutations, and polymorphisms (Henegariu *et al*, 1997). For diagnostic purposes, multiplex PCR can be used for the detection of internal controls as well as for the detection of multiple pathogens in a single specimen (Bej and Mahbubani, 1992). Useful applications of multiplex PCR in the detection of other pathogenic bacteria have been previously reported (Lindström *et al*, 2001; Ibekwe *et al*, 2002).

Since traditional methods for microbiological analysis of drinking water require one to two weeks for analysis and confirmation, we tried to develop a rapid and simple PCR- based protocol for the direct detection of microbiological parameters in drinking water. The application of multiplex PCR and gene probes for coliform bacteria and *E. coli* detection in water samples has been previously reported (Bej *et al*, 1991b). In our study, multiplex PCR was developed for the rapid detection of total coliform bacteria for *E. coli*, and *Clostridium perfringens* in drinking water, which are microbiological parameters indicated in Thailand's Bottled Drinking Water Quality Standards. Multiplex PCR may be a more useful, rapid, and safe alternative for the microbiological detection of drinking water comparing with the traditional method.

## MATERIALS AND METHODS

### Bacterial strains

A total of 143 strains of bacteria, including reference strains and clinical isolates of *E. coli*, *C. perfringens*, other coliform bacteria (*Klebsiella* spp, *Citrobacter* spp, and *Enterobacter* spp), and other bacteria used in this study, were kindly provided by the Institute of Public Health Research, Ministry of Public Health, Thailand; Faculty of Public Health; and Faculty of Tropical Medicine, Mahidol University, Thailand. Most of the bacterial strains, except *C. perfringens* were grown on nutrient agar (Merck, Darmstadt, Germany) at 37ºC for 24 hours. *C. perfringens* was grown on Tryptose-sulfite-cycloserine (TSC) agar (Schalau Chemie, SA, Barcelona, Spain) supplemented with 5% egg yolk and incubated under anaerobic conditions in anaerobic jars at 37ºC for 24 hours.

### Genomic DNA extraction

Genomic DNA was extracted by phenol/chloroform extraction followed by ethanol precipitation, according to the procedure described by Ausubel *et al* (1996). An overnight growth of bacteria on agar was suspended in 1.5-ml TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0) in a microcentrifuge tube and centrifuged at 16,000*g* for 10 minutes. The pellet was resuspended in 576 μl TE buffer followed by the addition of 30 μl of 10% sodium dodecyl sulfate and 3 μl of proteinase K (20 mg/ml). After incubation at 37ºC for 1 hour, 100 μl of 5 M NaCl and 80 μl of CTAB-NaCl (10% CTAB in 0.7 M NaCl) solution were added before further incubation at 65ºC for 10 minutes. DNA was extracted with phenol/chloroform/isoamyl alcohol solution (25:24:1) and precipitated with isopropanol overnight at -20ºC. DNA was harvested by centrifugation at 12,000*g* for 20 minutes, washed with 70% ethanol and the pellet was resuspended in 100 μl TE buffer. The concentration and the quality of the DNA were determined by a spectrophotometer (Shimadzu Model UV 160 A, Shimatzu, Kyoto, Japan) at the wavelengths of 260 and 280 nm. DNA was stored at -20ºC until used for PCR amplification.

### Primer selection and optimization of multiplex PCR

The target genes, oligonucleotide primers, melting temperature (Tm) and product sizes for each pathogen are shown in Table 1. All oligonucleotide primers were synthesized by Bio Basic (East Markham, Ontario, Canada).

For optimization of the multiplex PCR amplification, 1 μl of 10 mM dNTPs, 25 pmol of each primer, 1 U of DNA polymerase (MBI Fermentas Inc., Burlington, Ontario, Canada), 10 ng of each DNA template, and 1X PCR reaction buffer (10 mM Tris- HCl (pH 8.8), 50 mM (KCl), 0.08% Nonidet P 40) were used with various concentrations of $MgCl_2$ (1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM, 3.5 mM and 4.0 mM). The final volume of

Table 1
Oligonucleotide primers used for multiplex PCR amplification.

| Bacteria | Primer set | Target gene | Tm (ºC) | Product size (bp) |
|---|---|---|---|---|
| Coliform bacteria | LZL: ATGAAAGCTGGCTACAGGAAGGCC<br>LZR: CACCATGCCGTGGGTTTCAATATT<br>(Bej *et al*, 1990) | *lacZ* | 74<br>68 | 876 |
| *E. coli* | UAL: TGGTAATTACCGACGAAAACGGC<br>UAR: ACGCGTGGTTACAGTCTTGCG<br>(Bej *et al*, 1991) | *uidA* | 68<br>70 | 147 |
| *C. perfringens* | PLC1: ATAGATACTCCATATCATCCTGCT<br>PLC2: TTACCTTTGCTGCATAATCCC<br>(Tansuphasiri, 2001) | Phospholipase C<br>(*plc*: α-toxin gene) | 66<br>60 | 280 |

the reaction mixture was adjusted to 25 μl with sterile deionized distilled water. All multiplex PCR reactions were performed in a GeneAmp 2400 PCR System thermocycler (Perkin Elmer Cetus Corp, Norwalk, CT, USA) with the following PCR temperature cycling parameters: initial denaturation at 94ºC for 10 minutes followed by 36 cycles of denaturation at 94ºC for 1 minute, primer annealing at various temperatures (45º, 50º, 55º, 56º, 58º and 60ºC) for 1 minute, primer extension at 72ºC for 1 minute, and the final extension at 72ºC for 10 minutes. Besides concentration of each primer, dNTPs, and DNA polymerase were varied for optimal multiplex PCR results. In each PCR amplification, a buffer control, to which no DNA template was added, was included as an internal control and DNA templates of coliform bacteria, *E. coli*, and *C. perfringens* extracted from reference bacterial strains were included as positive controls.

Detection of amplified DNA

Ten μl of the amplified product (amplicon) was evaluated for expected products by electrophoresis on 2% agarose gel in 1X TBE buffer [0.089 M Tris-base, 0.089 M boric acid, and 0.002 M EDTA (pH 8.0)]. After electrophoresis, the gel was stained with gelstar® (Cambrex Bio Science Rockland Inc, Rockland, ME, USA), visualized by Dark Reader (Clare Chemical Research, CO, USA) and photographed with a digital camera. The M23 100 bp + 1.5 kb DNA ladder (SibEnzyme, Novosibirsk, Russia) was used as molecular marker to indicate the size of the amplicons.

Specificity and sensitivity of optimized multiplex PCR with purified DNA

The specificities and sensitivities of these three sets of primers have been separately studied (Bej *et al*, 1990, 1991b; Tansuphasiri, 2001). In contrast, in our study, the sensitivities and specificities of these three primers pairs were determined with the optimized multiplex PCR.

To determine the specificities of these primers with the optimized multiplex PCR, 10 ng of each genomic DNA were prepared from coliform bacteria, *E. coli*, *C. perfringens* and other bacteria (*ie*, *Staphylococcus aureus*, and *Salmonella* spp) and were used as DNA templates for the multiplex PCR. DNA amplification was performed in a thermocycler as previously mentioned.

The detection limits for coliform bacteria, *E. coli* and *C. perfringens* with the purified DNA in the assay were investigated by diluting the DNA of *K. pneumoniae* ATCC 27736, *E. coli* ATCC 25922 and *C. perfringens* ATCC 12916 from 100 ng to 1 pg (a 10 fold serial dilution) and used as templates for the optimized multiplex PCR.

Sample preparation, processing, enrichment, and evaluation of sensitivity with bacterial cell suspension

*K. pneumoniae* ATCC 27736, a representative of coliform bacteria, *E. coli*, ATCC 25922 and *C. perfringens* ATCC 12916 were used for sensitivity testing with bacterial cell suspension.

Each bacterial strain was prepared by suspending 5-10 isolated colonies in 0.1% peptone water and the solution was adjusted to 0.5 McFarland standard (~$10^8$ CFU/ml). A serial tenfold dilution with 0.1% peptone water of each bacterial strain was performed for six-consecutive concentration ranges from $10^7$ to $10^2$ CFU/ml.

One ml of any dilution of each bacterial strain was spiked into 100 ml of sterile deionized distilled water and 10 ml of the spiked water sample was filtered through a 0.22 μm nitrocellulose membrane with a Swinex® Filter Holder (Millipore, Bedford, MA, USA). The membrane was placed in a 15-ml test tube containing 10 ml of thioglycollate broth (Difco, Detroit, MI, USA) and incubated at 37ºC for 6 hours. The sample was then centrifuged at 4,000 rpm for 15 minutes and the pellet was transferred to a 2-ml microcentrifuge tube and further centrifuged at 14,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed twice with 1.5-ml TE buffer. The pellet was resuspended in 100 μl TE buffer, heated at 95ºC for 10 minutes, further centrifuged at 14,000 rpm for 5 minutes and 10 μl of the supernatant was used as the template for the multiplex PCR amplification. The PCR procedure was performed as previously described for multiplex PCR. A viable count was performed at the same time by spreading 0.1 ml of each sample on Endo agar (Schalau Chemie, SA, Barcelona, Spain), M7h FC agar (Schalau Chemie, SA, Barcelona, Spain), and TSC-EY agar, selective media for coliform bacteria, *E. coli*, and *C. perfringens*, respectively. After 24-hours incubation at 37 °C in aerobic conditions for the coliform bacteria and *E. coli* and anaerobic conditions for *C. perfringens*, the colonies were counted and the lowest number of bacterial cells that could be detected by PCR was recorded.

### Blind experiment

Fifty-bacteria-spiked water samples, blinded to the investigator, were used to test the developed methods. In addition, the results obtained were confirmed by plating for viable counts. For each sample, one ml of $10^5$ CFU/ml or $10^4$ CFU/ml or $10^3$ CFU/ml or none of *K. pneumoniae* and/or *E. coli* and/or *C. perfringens* were spiked into 100 ml of sterile deionized distilled water and processed as previously mentioned. The results of multiplex PCR assays were compared with the results of standard plate counts.

### Sequencing

The multiplex PCR products were purified using Nucleospin® Extract (Macherey-Nagel, Germany). Automated sequencing was performed with the BigDye Terminator 1.3 and PCR primers in accordance with the recommendations of the manufacturer. The sequence was analyzed on an ABI prism model 3100 automated DNA sequencer (Bioservice Unit, National Science and Technology Development Agency, Bangkok, Thailand). The sequence was aligned with the BLAST search of the GenBank database (National Center for Biotechnology Information, Washington, DC, USA).

## RESULTS

### Optimization of multiplex PCR reaction and cycling

Simultaneous amplification of all three target genes was achieved by comparing band intensities and least nonspecific bands for each target DNA using PCR cycling parameters with primer annealing at 45º, 50º, 55º, 56º, 58º and 60ºC. An increase in primer annealing temperature to 60ºC resulted in relatively weaker amplified DNA products of some of the target genes, whereas primer annealing below 55ºC resulted in the appearance of nonspecific bands. Use of PCR with various concentrations of $MgCl_2$ revealed the bands of all three target genes products. Various concentrations of each oligonucleotide revealed the intensities of multiplex PCR products (data not shown), whereas the various concentrations of dNTPs gave the same result for the multiplex PCR products (Fig 1).

After the optimization step, the multiplex PCR reaction was performed in a total volume of 25 μl containing 10 ng of DNA template, 3 mM $MgCl_2$, 5.0 pmol of LacZ primer, 7.5 pmol of UidA primer, 12.5 pmol of PLC primer, 0.5 μl of 10 mM dNTPs, 0.7 U of DNA polymerase and 1X reaction buffer. The reaction mixture was processed in a programmable thermocycler, which

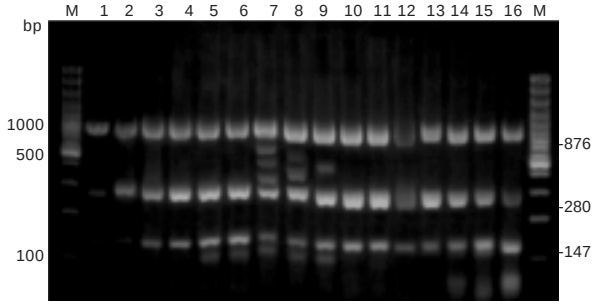


Fig 1–Agarose gel (2%) electrophoresis showing amplified multiplex PCR products by varying parameters. Lane M molecular size marker (100 bp DNA ladder); lanes 1 to 6 varying $MgCl_2$ concentrations of 1.5, 2, 2.5, 3, 3.5, and 4 mM $MgCl_2$, respectively, with an annealing temperature of 58ºC; lanes 7 to 12 varying annealing temperatures of 45º, 50º, 55º, 56º, 58º and 60ºC, respectively with $MgCl_2$ 3 mM; lane 13 multiplex PCR with an annealing temperature of 58ºC, 3 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, and 0.7 U of *Taq* DNA polymerase; lane 14 multiplex PCR with an annealing temperature of 58ºC, 3 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, and 1 U of *Taq* DNA polymerase; lane 15 multiplex PCR with an annealing temperature of 58ºC, 3 mM $MgCl_2$, 1 μl of 10 mM dNTPs, and 0.7 U of *Taq* DNA polymerase; lane 16 multiplex PCR with an annealing temperature of 58ºC, 3 mM $MgCl_2$, 1 μl of 10 mM dNTPs, and 1 U of *Taq* DNA polymerase.

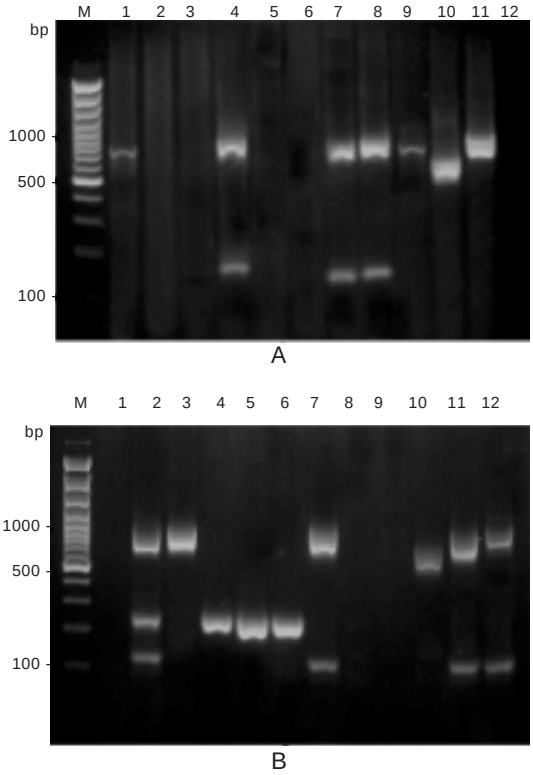


Fig 2–Agarose gel (2%) electrophoresis of PCR amplified products from various pure bacterial DNAs, using optimized multiplex PCR. A: Lane M: molecular size marker (100 bp DNA ladder); lanes 1 to 12, *K. pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, E. coli, Yersinia enterocolotica, Salmonell stanly, Shigella sonnei, Shigella flexneri, K. pneumoniae, Citrobacter freundii, Citrobacter diversus, and S. aureus*, respectively. B: Lane M: molecular size marker (100 bp DNA ladder); lanes 1 to 3, *Aeromonas* spp, positive control, *Enterobacter cloaceae*, lanes 4 to 6, *C. perfringens*, lanes 7 to 10, *E. coli, Leptospira sejroe, Leptospira pyrogenes* and *Citrobacter freundii*, respectively; lanes 11 and 12, *E. coli.*

carried out the following touchdown cycle parameters:

Initial denaturation at 94ºC for 10 minutes; 2 cycles of 94ºC for 1 minute, 62ºC for 1 minute, 72ºC for 1 minute; 2 cycles of 94ºC for 1 minute, 61ºC for 1 minute, 72ºC for 1 minute; 2 cycles of 94ºC for 1 minute, 60ºC for 1 minute, 72ºC for 1 minute; 2 cycles of 94ºC for 1 minute, 59ºC for 1 minute, 72ºC for 1 minute; 36 cycles of 94ºC for 1 minute, 58ºC for 1 minute, 72ºC for 1 minute; and a final extension at 72ºC for 10 minutes.

Evaluation of specificity and sensitivity of the optimized multiplex PCR

Specificity of the optimized multiplex PCR with purified target DNA. PCR amplification with three sets of oligonucleotide primers yielded a detectable DNA fragment of an expected molecular weight (876 bp for coliform bacteria; 876 bp and 147 bp for *E. coli*, and 280 bp for *C. perfringens*) only in the presence of their respective DNA templates. However, the amplified products of *K. pneumonaie* with the LacZ primer were larger than those of *E. coli*, *Citrobacter freundii*, and *Enterobacter* spp, indicating the difference between the target *lacZ* among these organisms. The use of LacZ and

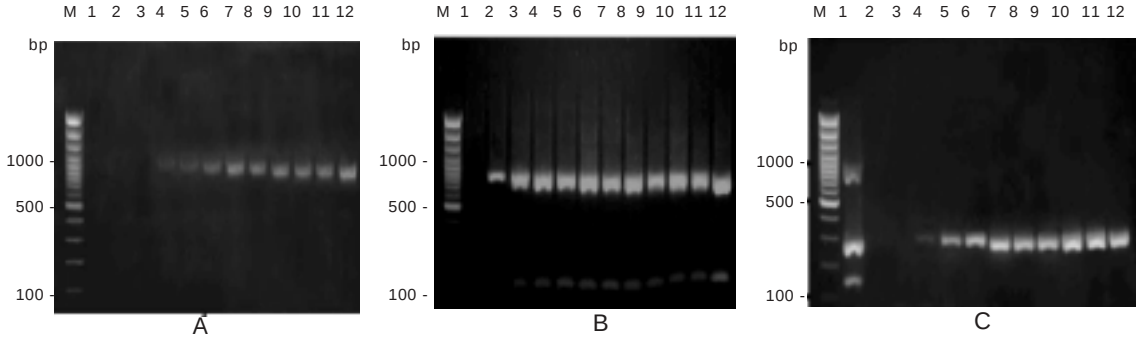


Fig 3–Agarose gel (2%) showing sensitivity of multiplex PCR with purified DNA. Genomic DNA was isolated from *K. pneumoniae* ATCC 27736 (A), *E. coli* ATCC 25922 (B), and *C. perfringens* ATCC 12916 (C), respectively. Lane M, molecular size marker (100 bp DNA ladder). Lanes 1 to 12 (both A and B), DNA per reaction, 1 pg, 10 pg, 100 pg, 1 ng, 2 ng, 3 ng, 4 ng, 5 ng, 10 ng, 50 ng, 100 ng and 1 μg, respectively. Lane 1 (C), positive DNA control. Lanes 2 to 12 (C), DNA per reaction, 1 pg, 10 pg, 100 pg, 1 ng, 2 ng, 3 ng, 4 ng, 5 ng, 10 ng, 50 ng, and 100 ng, respectively.

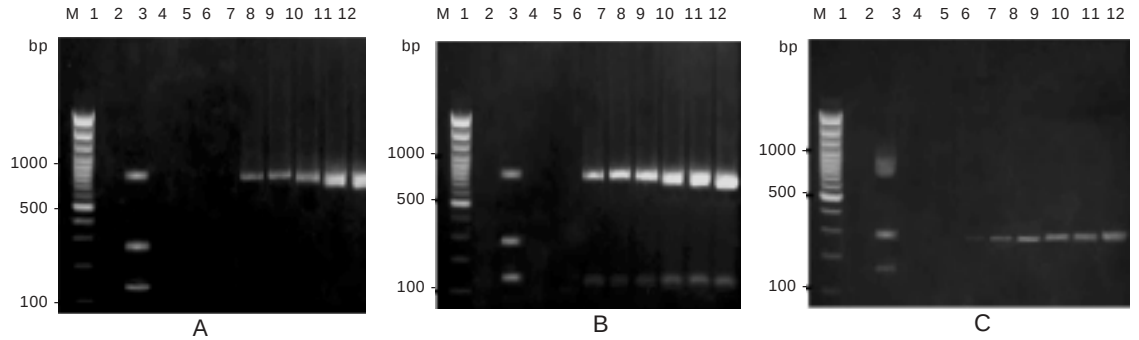


Fig 4-Agarose gel (2%) showing sensitivity of multiplex PCR with bacterial dilution from $10^0$ to $10^7$ CFU/ml of three bacterial strains: A. *K. pneumoniae* ATCC 27736, B. *E. coli* ATCC 25922, and C. *C. perfringens* ATCC 12916. Lane M molecular size marker (100 bp DNA ladder); lane 1, negative control; lane 2, positive control; lanes 3 to 10, bacterial dilution; $10^0$, $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$ CFU/ml, respectively.

UidA primers permitted the detection of *Shigella* spp as well as *E. coli* (Bej *et al*, 1991b) (Fig 2). These results indicate that each of the selected oligonucleotide primer sets for each of the target gene segments was specific for its respective target microbial pathogens, including *Shigella* spp.

Sensitivity of the optimized multiplex PCR with purified target DNA. The sensitivity of the optimized multiplex PCR for all three-target genes with purified target DNA was from 100 pg for *E. coli* and *C. perfringens* to 1 ng for *K. pneumoniae* (Fig 3).

Sensitivity of the optimized multiplex PCR with the bacterial cell suspension. The sensitivity of the optimized multiplex PCR for all three-target genes in the bacterial cell suspension spiked water was from 1 CFU/ml for *E. coli* and *C. perfringens* to 10 CFU/ml for *K. pneumoniae* (Fig 4).

Blinded experiment

The multiplex PCR method was used to detect coliform bacteria, *E. coli* and *C. perfringens* in 50 blinded spiked water samples and the results were compared to those from standard plate counts (Table 2). The multiplex PCR was

Table 2
Results of blinded spiked water samples tested by multiplex PCR and spread plate culture methods.

| | Spread plate culture method | | Total |
|---|---|---|---|
| | Positive | Negative | |
| PCR positive | 116 | 3 | 119 |
| PCR negative | 1 | 30 | 31 |
| Total | 117 | 33 | 150 |

recorded as positive if the amplified product of target gene appeared and negative if the amplified product of target gene was not present. For the standard plate count, the result was recorded as positive when the target bacteria formed a colony on the selective media and negative when target bacteria did not form colonies on the selective media after a 24-hours incubation period.

The number of specimens positive by multiplex PCR was 3-fold higher, but this was not statistically significant (McNemar's test, p = 0.62, OR = 3.0, 95% CI 0.24, 157.5). The multiplex PCR method, using the standard plate count as reference, had a sensitivity and specificity of 99.1% (95% CI 95.33, 99.98) and 90.9% (95% CI 75.67, 98.08), respectively.

Five positive samples for both PCR and spread plate had their base pairs of 147, 280, and 876 confirmed by sequencing with primers UAR or UAL, PLC1 or PLC2, and LZR or LZL respectively. The results were compared to the sequence data obtained from GenBank. The identities of *E. coli* β glucuronidase gene (*uidA*), *C. perfringens* phospholipase C gene (*plc*) and *E. coli* β galactosidase gene (*lacZ*) were revealed.

## DISCUSSION

Multiplex PCR has been used successfully for rapid detection, with high specificity and sensitivity, of various pathogenic bacteria from environmental waters (Bej *et al*, 1991b) and various food products (Brasher *et al*, 1998; Vantarakis *et al*, 2000). In our study, multiplex PCR was used to detect coliform bacteria, *E. coli* and *C. perfringens* in drinking water. This assay significantly compared with the standard plate count method. Selection of appropriate target genes, oligonucleotide primers, PCR reaction, and cycling parameters resulted in the simultaneous amplification of three target genes in a single reaction with a sensitivity of detection of $10^0$-$10^1$ CFU/ml of spiked water after a six-hour pre-enrichment step.

The specificity of the multiplex PCR assay was determined with 142 bacterial strains, including coliform bacteria, *E. coli*, *C. perfringens,* and other bacteria. The results were the multiplex PCR assay yielded a detectable DNA fragment of expected molecular weight only in the presence of their respective DNA templates, and gave negative results when tested with other bacteria, except *Shigella* spp, which gave the result of *E. coli* amplification.

The sensitivity of the multiplex PCR assay with purified DNA was 1 ng for *K. pneumoniae* and 100 pg for *E. coli* and *C. perfringens*, which is higher than in previous studies using PCR and gene probes for the detection of coliform bacteria, *E. coli* (Bej *et al*, 1990, 1991a) and *C. perfringens* (Tansuphasiri, 2001). When applying this method to spiked water samples, the sensitivity of the method was rather low ($10^4$ CFU/ml), therefore, we included a pre-enrichment step in our testing procedure in order to enhance the detection of these three bacteria when present in very low numbers. By including the enrichment step, we were able to detect cultivable pathogens. Following incubation in thioglycollate broth for 6 hours, it was possible to detect as few as $10^0$-$10^1$ CFU/ml of bacteria in spiked water. The reliability of the test is comparable to existing culture-based techniques.

The major benefit of this study was the development of a PCR detection method for more than one bacterial species in drinking water. We believe that this method can be used for the simultaneous detection of other microorganisms by using appropriate primers. It can be used as an alternative method for the routine microbiological analysis of drinking water. The high sensitivity and specificity can make this an ideal test for screening possible contaminated water samples. More efficient assays may be achieved if the sizes of the amplified DNA products are close to each other (Fratamocio and Strobaugh,

1998). For future study, new primers to obtain a *lacZ* gene product smaller than 876 bp should be considered.

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