

INFLUENCE OF ENRICHMENT BROTHS ON MULTIPLEX PCR DETECTION OF TOTAL COLIFORM BACTERIA, *ESCHERICHIA COLI* AND *CLOSTRIDIUM PERFRINGENS*, IN SPIKED WATER SAMPLES

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Abstract. Although multiplex PCR amplification condition for simultaneous detection of total coliform bacteria, *Escherichia coli* and *Clostridium perfringens* in water sample has been developed, results with high sensitivity are obtained when amplifying purified DNA, but the sensitivity is low when applied to spiked water samples. An enrichment broth culture prior PCR analysis increases sensitivity of the test but the specific nature of enrichment broth can affect the PCR results. Three enrichment broths, lactose broth, reinforced clostridial medium and fluid thioglycollate broth, were compared for their influence on sensitivity and on time required with multiplex PCR assay. Fluid thioglycollate broth was the most effective with shortest enrichment time and lowest detection limit.

INTRODUCTION

Microbiological parameters have been used for safety evaluation of drinking water. Traditional methods to examine microbiological parameters in water samples rely mainly on culturing, which is time consuming (Hickey and Harkins, 1998). Polymerase chain reaction (PCR) provides an alternative method for the detection of microbiological organisms in water samples. Several PCR-based methods have been reported during the past decade, including multiplex PCR for simultaneously amplification of more than

one target sequence in a specimen (Chamberlain *et al*, 1988).

Multiplex PCR amplification conditions for simultaneous detection of total coliform bacteria, *Escherichia coli* and *Clostridium perfringens* have been developed (Tantawiwat *et al*, 2005). The problem found during method development is that, although the multiplex PCR obtains results with high sensitivity when amplifying purified DNA (1 ng for *K. pneumoniae* and 100 pg for *E. coli* and *C. perfringens*, respectively), when applying the method to spiked water sample, the sensitivity of the method is rather low (10^4 cfu/ml).

Culture enrichment prior to PCR analysis has been utilized in many studies (Juvonen *et al*, 1999; Sharma and Carlson, 2000; Ferretti *et al*, 2001; Löfström *et al*, 2004). This technique serves many purposes, including dilution of PCR inhibitory substances in the sample, multiplication of the

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target organisms to provide detectable concentrations, dilution of dead cells, and increase in the ability to isolate the target organism for complementary tests (Sharma and Carlson, 2000). However, the type of enrichment broth is one factor affecting the success of the test, due to its ability to enhance the growth of certain bacteria species, while inhibiting the development of unwanted microorganisms (Chang *et al*, 1999).

This study was performed to compare the influence of three enrichment broths on sensitivity and time required for obtaining results from multiplex PCR for the detection of total coliform bacteria, *E. coli* and *C. perfringens*, in spiked water samples.

MATERIALS AND METHODS

Organisms and culture conditions

Klebsiella pneumoniae ATCC 27736 (a representative of total coliform bacteria) and *E. coli* ATCC 25922, were grown on nutrient agar (Merck, Darmstadt, Germany) at 37°C for 24 hours. *C. perfringens* was grown on tryptose-sulfite-cycloserine agar (Scharlau Chemie SA, Barcelona, Spain), supplemented with 5% egg yolk (TSC-EY) and incubated under anaerobic conditions at 37°C for 24 hours. Each bacteria strain was used to prepare a bacterial suspension ranging from 10 to 10⁵ cfu/ml.

Enrichment broths

Lactose broth (LB) (Merck, Darmstadt, Germany), reinforced clostridial medium (RCM) (Scharlau Chemie SA, Barcelona, Spain) and fluid thioglycollate broth (FTG) (Difco, Detroit, MI) were prepared according to the manufacturers' instructions, and dispensed aseptically as 10 ml aliquots into sterile 15 ml test tubes.

Water sample preparation, processing and multiplex PCR analysis

Five spiked water samples containing

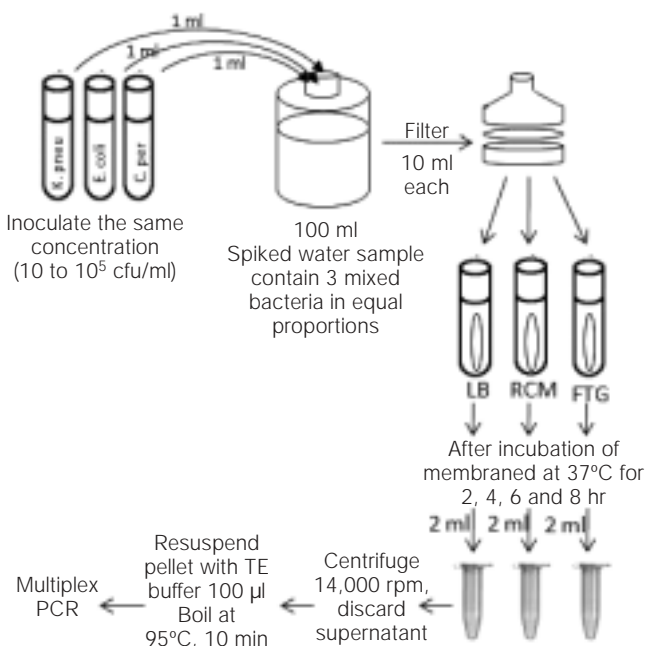


Fig 1—Spiked water sample preparation and processing.

all three bacteria strains in equal proportions (range from 0 to 10³ cfu/ml of each bacteria strain) were prepared and processed as shown in Fig 1.

The original cfu of each bacteria strain per ml of spiked water sample was estimated by spreading 0.1 ml of each spiked water sample on Endo agar (Scharlau Chemie SA, Barcelona, Spain), M7h FC agar (Scharlau Chemie SA, Barcelona, Spain), and TSC-EY agar, selective media for coliform bacteria, *E. coli* and *C. perfringens*, respectively. After 24 hours of incubation at 37°C in aerobic condition for coliform bacteria and *E. coli*, and anaerobic condition for *C. perfringens*, the numbers of colonies were counted.

Multiplex PCR amplification was performed as described previously (Tantawiwat *et al*, 2005). Ten ml of amplicons were separated by electrophoresis in 2% agarose gel in 1X TBE buffer (0.089 M Tris-base pH 8.0, 0.089 M boric acid and 0.002 M EDTA) and visualized by staining with Gelstar®

(Cambrex Bio Science Rockland, Rockland, ME) and Ddark Reader (Clare Chemical Research, Dolores, CO). The presence of amplicons of *lacZ* (876 bp), *uidA* (147bp) and *plc* (280 bp) was taken as positive results for the presence of coliform bacteria, *E. coli*, and *C. perfringens*, respectively.

RESULTS

Three enrichment broths namely, LB, RCM, and FTG, were compared for their influence on the results of multiplex PCR for the detection of total coliform bacteria, *E. coli* and *C. perfringens* in spiked water samples. The initial numbers of each bacteria strain in the spiked water samples were in the range 0-10³ cfu/ml.

Multiplex PCR assay, performed after enrichment of the samples in LB broth yielded (1) positive results for total coliform bacteria only after 4 and 6 hours of incubation with the original concentration of 10³ and 10¹ cfu/ml, respectively, (2) positive results for total coliform bacteria and *E. coli* after 6 hours of incubation with original concentrations of 10² to 10³ cfu/ml, (3) positive results for total coliform bacteria and *E. coli* after 8 hours of incubation with original concentrations of 10⁰ to 10³ cfu/ml, and (4) negative results for *C. perfringens* in all samples (Table 1).

For RCM broth, multiplex PCR assay yielded (1) positive results for all three bacteria after 8 hours of incubation with original concentrations of 10² to 10³ cfu/ml, (2) positive results for total coliform bacteria and *E. coli* after 8 hours of incubation with original concentration of 10¹ cfu/ml, and (3) positive results for only *C. perfringens* after 6 hours of incubation with original concentration of 10⁰ cfu/ml (Table 1). Water samples spiked with original concentrations of 10² and 10³ cfu/ml of each bacteria strain yielded negative results for *C. perfringens*.

After enrichment of samples in FTG broth, multiplex PCR assay yielded (1) positive results for total coliform bacteria, *E. coli*, and *C. perfringens* after 4 hours of incubation with the original concentration of 10³ cfu/ml, and (2) positive results for total coliform bacteria, *E. coli*, and *C. perfringens* after 6 and 8 hours of incubation with original concentrations of 10⁰ to 10³ cfu/ml (Table 1).

DISCUSSION

Culture methods have shown poor sensitivity to low level sample contaminations (D'Aoust *et al*, 1992). Several studies showed that PCR is the most promising method for rapid detection and identification of bacteria in a wide variety of samples (Aabo *et al*, 1993; Soumet *et al*, 1994; Wang and Yeh, 2002; Oliveira *et al*, 2003). However, small numbers of target microorganisms in a sample can still present a problem for PCR analysis.

Enrichment before PCR analysis can increase the numbers of target microorganisms. Inclusion of an enrichment step minimizes the risk of detecting DNA from dead cells (Sharma and Carlson, 2000). However, the specific nature of the enrichment broth can affect the results (Ryser *et al*, 1996; Nannapaneni *et al*, 1998). Similar to this study, these results showed that multiplex PCR could detect total coliform bacteria, *E. coli*, and *C. perfringens* in spiked water samples when FTG and RCM are used as enrichment broths, and FTG yields better results than RCM.

FTG contains sodium thioglycollate, thioglycollic acid and L-cystine, which reduces oxygen in water, while agar helps retard oxygen diffusion and helps maintain the stratification of organisms growing in different layers of the broth. Obligate anaerobes, which require an anaerobic environment, only grow in the lower areas of the tube. Microaerophiles, which prefer environments

Table 1
 Multiplex PCR detection of total coliform bacteria, *E. coli* and *C. perfringens* in spiked water samples after enrichment in different broths and enrichment time intervals.

Sample no.	Bacteria strains artificially inoculated in equal proportions in spiked water samples	Original number of each bacteria strain in spiked water samples (cfu/ml) ^a	Target gene	PCR product size (bp)	Multiplex PCR detection												
					LB			RCM			FTG						
					2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	
1	Total coliform bacteria	0	lacZ	876	-	-	-	-	-	-	-	-	-	-	-	-	-
	<i>E. coli</i>	0	uidA	147	-	-	-	-	-	-	-	-	-	-	-	-	-
	<i>C. perfringens</i>	0	plc	280	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Total coliform bacteria	10 ⁰	lacZ	876	-	-	+	-	-	-	-	-	-	+	-	-	+
	<i>E. coli</i>	10 ⁰	uidA	147	-	-	+	-	-	-	-	-	-	-	-	-	+
	<i>C. perfringens</i>	10 ⁰	plc	280	-	-	-	-	-	-	+	-	-	-	-	-	+
3	Total coliform bacteria	10 ¹	lacZ	876	-	-	+	-	-	-	-	-	-	+	-	-	+
	<i>E. coli</i>	10 ¹	uidA	147	-	-	+	-	-	-	-	-	-	-	-	-	+
	<i>C. perfringens</i>	10 ¹	plc	280	-	-	-	-	-	-	+	-	-	-	-	-	+
4	Total coliform bacteria	10 ²	lacZ	876	-	-	+	-	-	+	+	+	+	+	+	+	+
	<i>E. coli</i>	10 ²	uidA	147	-	-	+	-	-	+	+	+	+	-	+	+	+
	<i>C. perfringens</i>	10 ²	plc	280	-	-	-	-	-	-	-	-	-	-	-	-	+
5	Total coliform bacteria	10 ³	lacZ	876	-	+	+	+	-	+	+	+	+	+	+	+	+
	<i>E. coli</i>	10 ³	uidA	147	-	+	+	+	-	+	+	+	+	-	+	+	+
	<i>C. perfringens</i>	10 ³	plc	280	-	-	-	-	-	-	-	-	-	-	-	-	+

^a Estimated by spreading 0.1 ml of each spiked water sample on selective media and the numbers of colonies were counted after incubation for 24 hours.

LB, Lactose broth; RCM, Reinforced clostridial medium; FTG, Fluid thioglycollate broth; +, PCR positive; -, PCR negative

containing 10% or more carbon dioxide, grow in a thin layer below the richly-oxygenated layer. Facultative or aerotolerant anaerobes can grow throughout the medium, but primarily grow in the middle of the tube, between the oxygen-rich and oxygen-free zones (Austin Community College, 2007). Therefore, FTG can enhance the growth of both aerobic and anaerobic bacteria in the same sample.

RCM, which is proposed for the cultivation and enumeration of clostridia, anaerobes and facultative microorganisms in foodstuffs and other materials, is recommended as a nonselective enrichment medium for growing various anaerobic and facultative bacteria when incubated anaerobically (Merck, 2007). It can also be used as an enrichment broth under aerobic incubation conditions, in order to increase the numbers of total coliform bacteria, *E. coli* and *C. perfringens* in a water sample. However, after enrichment in RCM, this study showed that sensitivity of multiplex PCR analysis was less sensitive and enrichment took longer than in FTG.

LB enhanced the growth of total coliform bacteria and *E. coli*, leading to positive results for these two bacteria by multiplex PCR assay, and negative results for *C. perfringens* in all samples. Thus, lactose broth cannot be used to enrich samples containing both aerobic and anaerobic bacteria, because it does not support an appropriate environment for the growth of anaerobic bacteria.

In summary, when the influence of enrichment broths on the results obtained by multiplex PCR was compared, FTG and RCM could be recommended as enrichment broths prior the detection of total coliform bacteria, *E. coli*, and *C. perfringens* in spiked water samples. However, the performance of FTG was superior to that of RCM both in terms of sensitivity and time required.

ACKNOWLEDGEMENTS

The authors thank Prof Unchalee Tunsuphasiri, Faculty of Public Health, Mahidol University, and the Institute of Public Health Research, Ministry of Public Health, Thailand for providing the bacteria strain.

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