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Multiplex PCR (Polymerase Chain Reaction) Assay for Detection of *E. coli* O157:H7, *Salmonella* sp., *Vibrio cholerae* and *Vibrio parahaemolyticus* in Spiked Shrimps (*Penaeus monodon*)

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Abstract: The coastal aquaculture mainly shrimps constitute major export sector in Bangladesh and is increasingly shaped by international trade conditions and by national responses to those stringent quality and safety standards. PCR based validated methods for detection of major bacterial pathogens in shrimp might be very useful tool for ensuring quality and safety standards of exportable shrimps. The objective of this study was to evaluate overall performance (sensitivity and specificity) of the multiplex PCR assay for detection of *Vibrio cholerae, Vibrio parahaemolyticus, Salmonella* sp. and *Escherichia coli* O157:H7 from spiked shrimp samples. The targeted genes were *ompW* for *V. cholerae*, *tdh* for *V. parahaemolyticus*, *sefA* for *Salmonella* spp. and *hlyEHEC* for *E. coli* O157:H7. The genomic DNA was extracted by using standard method and amplified accordingly. Sensitivity of the assay was tested by inoculating the shrimp homogenate with viable cells of laboratory references strains (target pathogens). The genes were amplified individually both from culture homogenate and spiked samples. Twenty different uniplex and multiplex PCR assay were performed; the results showed that the sensitivity and specificity of multiplex PCR are comparable to that of the results of uniplex PCR for the samples. DNA extracted from shrimp samples spiked with non-target pathogen (*Bacillus cereus*, *Shigella flexneri* and *Staphylococcus aureus*) yielded negative results.

Key words: Multiplex PCR assay, spiked shrimp, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Salmonella* spp., *E. coli* O157:H7

INTRODUCTION

Shrimp is a very popular and common food item worldwide especially in western countries and is an important export comodity for many countries like Bangladesh. Presently Bangladesh contributes around 5% to the total global cultured shrimp production. There are nearly 1 million people employed in the shrimp sector in Bangladesh and generating over US$1.2 billion annually (Yunus, 2009). Shrimp is usually consumed uncooked and/or minimally processed, which makes it potential health risk food (Ahmed and Anwar, 2007). Hence, quality of shrimp is a major concern for both consumers and exporters as microbial pathogens from shrimp may be transmitted to humans when they are eaten undercooked or minimally processed or when they transfer the pathogen to other food products through cross-contamination (Khan *et al*., 2007). According to the guideline of ICMSF (International Commission on Microbiological Specification for Foods) and EU Standard, shrimp samples must be free of pathogens like *E. coli* O157:H7, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Salmonella* sp., Coagulase-Positive *Staphylococcus aureus* etc. (Ahmed and Anwar, 2007). The availability of reliable detection methods is lengthy and critical for identifying these pathogenic bacteria in shrimp. Selective media based methods for detection and identification of *E. coli* O157:H7, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Salmonella* sp. in shrimp are generally time-consuming and thus creates limitation for quick inspection and certification. Furthermore, the pathogens are often present in very low numbers among a background of indigenous microflora and stressed deep frozen matrix, thus rendering recovery of target organisms difficult (Jofre *et al*., 2005).

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Development of PCR based modified methods have been published for rapid, sensitive detection of food-borne pathogens in many previous studies (Hoorfar and Cook, 2003; Rijpens and Herman, 2002; Ahari *et al*., 2009; AlHaj *et al*., 2007; Malkawi and Gharaibeh, 2004). Since PCR can target unique genetic sequences such as virulence genes of microorganisms, it also has the potential advantage of being an extremely specific assay (Malorny *et al*., 2003). On the other hand, conventional methods generally require the use of media containing selective agents for pathogen detection and isolation, it may be difficult to detect injured cells that have the potential to recover and grow when the food is stored/preserved and consumed (Kawasaki *et al*., 2009). The use of DNA-based techniques enables the detection of pathogenic microbes exposed to stress conditions with greater sensitivity and reliability than conventional culture methods (Mandal *et al*., 2011). Thus, a sensitive, specific and rapid method that would allow detection of multiple pathogens simultaneously from foods will be useful for food processors and regulatory bodies (Bai *et al*., 2010).

PCR is the method of choice for detection of microorganisms in food (Sherfi *et al*., 2006; Seidavi *et al*., 2008). Simplicity, rapidity, reliability, reproducibility, sensitivity and specificity of PCR make it most competitive (Cheng *et al*., 2012). Uniplex PCR method amplify single gene at a time while multiplex PCR method simultaneously amplify more than one target sequence (Seidavi *et al*., 2008). Multiplex PCR method saved considerable time and effort in detection of pathogens. It is now proved that multiplex PCR method have compatible or even superior sensitivity over conventional cultural detection methods (Kawasaki *et al*., 2005; Yasmin *et al*., 2007).

The current study is aimed to develop a multiplex PCR method for qualitative detection of *Salmonella* sp., *Vibrio parahaemolyticus*, *Vibrio cholerae* and *E. coli* O157:H7 directly from spiked shrimp samples and to evaluate the sensitivity of the multiplex PCR method. Based on the results obtained further study will be designed for enhancement of sensitivity, specificity and validation of the method.

MATERIALS AND METHODS

Shrimp samples (*Penaeus monodon*): Shrimp samples were collected from different shrimp farms located on south-western region of Bangladesh. Shrimps were collected from grow out ponds, brought to the laboratory in iced boxes and stored at -20°C till analysis.

Reference strains and cultures: The bacterial strains used in this experiment were obtained from culture collection pool of Industrial Microbiology Laboratory, IFST, BCSIR. Four reference strains were used for inoculation in shrimp homogenate as well as for spiking of shrimp samples; these are namely *Salmonella typhi* ATCC-65154, *Vibrio parahaemolyticus* ATCC-17802, *Vibrio cholerae* ATCC-15748 and *E. coli* O157:H7ATCC-12079. Bacterial strains were cultured in Trypticase Soy Broth (TSB,Oxoid) containing 0.6% yeast extract in a shaking incubator at 37°C overnight. The strains were maintained in Trypticase Soy Agar (TSA, Oxoid) slants at 4°C.

Chemicals: PCR reagents were purchased from Promega (Madison, WI, USA). PCR primers were synthesized by 1st Base (Singapore). Bacteriological media and broths were purchased from Oxoid (Hampshire, England). The rest of the materials and chemicals and reagents used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Selection of genes and primers: Genes selected for this study were *tdh* for *Vibrio parahaemolyticus, ompW* for *Vibrio cholerae, hly*$_{EHEC}$ for *E. coli* O157:H7 and *sefA* for *Salmonella* sp. Primer sequence and product size of the genes are given in Table 1.

DNA extraction from target organisms: DNA from target organisms was extracted by phenol/chloroform and ethanol precipitation method (Wilson, 1987). Bacterial cells were grown overnight in nutrient broth at 37°C, aerated by shaking at 120 rpm in a shaking incubator. Harvesting was then performed by centrifuging the culture at 10000 rpm for 5 minutes. The supernatant was then discarded and remaining cell pellet was subjected to treatment with DNA extraction solution I (Tris HCl+EDTA+sucrose) for 30 minutes at 37°C on a water bath in order to disrupt cells. Then de-proteinization was done using DNA extraction solution II (proteinase K+SDS+NaCl) at 55°C for 1 h on a water bath. Phenol:chloroform:isoamylalcohol (25:24:1) solvent was used to precipitate proteins. The cell extract was mixed gently with the solvent and the nucleic acids were separated in the aqueous layer by centrifugation at 10000 rpm for 5 min. The aqueous solution of DNA was then removed using micropipette. The DNA was then concentrated by ethanol precipitation in the presence of Sodium acetate. After centrifuging and washing with 70% ethanol solution the final pellet was taken and suspended in TE buffer. This suspension was then stored at 4°C for further use (Fakruddin *et al*., 2012).

Table 1: Primer sequences and product size of the genes targeted

Gene	Primers	Sequences	Product size (bp)	Reference
tdh	tdh D3	5'-CCA CTA CCA CTC TCA TAT GC-3'	250	Hara-Kudo *et al.* (2003)
	tdh D5	5'-GGT ACT AAA TGG CTG ACA TC-3'		
sefA	A058	5'-GAT ACT GCT GAA CGTAGAAGG-3'	470	Oliveira *et al.* (2003)
	A01	5'-GCG TAA ATC AGC ATC TGC AGT AGC-3'		
ompW	ompW F	5'-•CAC CAA GAA GGT GAC TTT ATT GTG-3'	588	Goel *et al.* (2007)
	ompWR	5'-GAA CTT ATA ACC ACC CGC G-3'		
hly_{EHEC}	hly F	5'-GAG CGA GCT AAG CAG CTT G-3'	889	Islam *et al.* (2007)
	hly R	5'-CCT GCT CCA GAA TAA ACC ACA-3'		

DNA extraction from spiked shrimp samples: Before spiking shrimps were peeled and autoclaved, the samples were then spiked with overnight grown bacterial culture (10^6 CFU mL^{-1}). Following incubation at 37°C for 24 h, spiked shrimps were homogenized with sterile ringer solution in a stomacher. Three milliliter of stomached samples were inoculated into nutrient broth and incubated for 24 h. The overnight grown culture was subjected to DNA extraction and PCR. For extraction of DNA from food samples, method described by Yasmin *et al.* (2007) was followed.

Quantification and purity of DNA: Quantification of genomic DNA was done using 1.0% agarose gel electrophoresis in 1X TAE buffer followed by staining with ethidium bromide. The concentration of extracted DNA was also estimated by visual comparison of the band with 100 bp marker DNA. The purity and concentration of the extracted DNA was checked by measuring absorbances on T60 UV-VIS spectrophotometer at 260 and 280 nm. Purity was analyzed by absorbance ratio of 260/280 nm (Sahasraundhe and Deodhar, 2010).

Uniplex PCR amplification of *tdh*, *ompW*, hly_{EHEC} and *sefA* gene: PCR amplification was performed in a 30 μL reaction volume containing 50 ng of DNA template, 3 μL 10X PCR reaction buffer without $MgCl_2$, 0.5 μL 20 mM$MgCl_2$, 1 μL of dNTP mixture, 1 μL each of forward and reverse primer and 1 unit of *Taq* polymerase. Same reaction mixture was used for all of the genes. Thermal cycling was done in a DNA engine, BIO-RAD (USA). PCR reactions were maintained for initial denaturation at 94°C for 3 min, followed by 30 cycles of 1 min at 94°C (denaturation), 1 min at 55°C for *tdh* and *ompw*, 58°C for *sefA* and hly_{EHEC} (annealing) and 1 min at 72°C (extension). The final extension for 9 min at 72°C and hold temperature was maintained at 4°C. PCR products were mixed with 3 μL of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose, w/v) and electrophoresis was carried on 1.5% agarose gel in 1X TAE buffer at 100v for 1.5 hr and stained with ethidium bromide (10 μg mL^{-1}). A 50/100 bp DNA ladder was used as a standard molecular weight marker. Reference band sizes for *Salmonella, V. parahaemolyticus, V. cholerae and E. coli* O157:H7 were 470, 250, 588 and 889 bp, respectively.

Multiplex PCR amplification of *tdh*, *ompW*, hly_{EHEC} and *sefA* gene: Multiplex PCR was performed in a total volume of 30 μL containing 2 μL of mixed template DNA and 28 μL of PCR master mix composed of 1x PCR buffer, 5.0 mM$MgCl_2$, 25 μM of each of *Salmonella* sp. detection primers (A058 and A01), 25 μM concentration of each of *V. parahaemolyticus* detection primers (*tdh* D3 and *tdh* D5), 25 μM concentration of each of *V. cholerae* detection primers (*ompW F* and *ompW R*), 25 μM concentration of each of *E. coli* O157:H7 detection primers (*HlyF* and *HlyR*), 200 μMdATP, dCTP, dGTP and dUTP, 0.5 U of Ampli *Taq* Gold DNA polymerase (Invitrogen, USA) with a DNA thermal cycler. The thermocycler was programmed as 50°C for 2 min for carryover treatment and initial denaturation at 95°C for 5 min. The samples were then subjected to 30 cycles of 95°C for 1 min, 58°C for 1 min, 55°C for 1 min, 54°C for 1 min, 72°C for 1 min and then 72°C for 9 min. The amplified products were then analyzed by 2.0% agarose gel electrophoresis. Reference band sizes for *Salmonella, V. parahaemolyticus, V. cholerae and E. coli* O157:H7 were 470, 250, 588 and 889 bp, respectively. Two different multiplex set up have been standardized, one for targeting *sefA* and *tdh* genes and another for all four genes.

Specificity of the multiplex PCR setting: To investigate the specificity of the multiplex PCR method and to observe that the method do not amplify closely related genes present in other related organisms, *Bacillus* cereus-ATCC 10876, *Shigella flexneri* ATCC-12022 and *Staphylococcus aureus* ATCC-25923 were included in the study. DNA extracted from these organisms was subjected to PCR according to the multiplex settings.

RESULTS

Uniplex amplification and electrophoresis of PCR products: The PCR product of shrimp samples spiked with *Vibrio parahaemolyticus* ATCC-17802 gave a clear distinct band of 251 bp in size which confirms *tdh* gene of inoculated *V. parahaemolyticus* ATCC-17802 (Fig. 1). Samples spiked with *Salmonella* sp. (*SefA gene*) showed a distinct PCR product band of 470 bp (Fig. 2) and gave confirmation of *sefA* gene in the inoculated

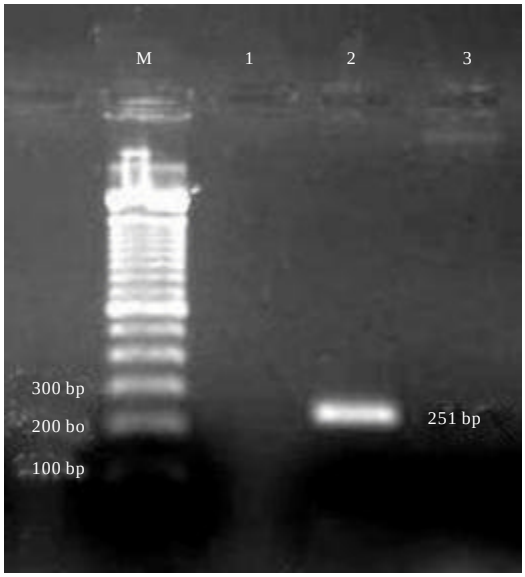

Fig. 1: Agarose gel electrophoretic analysis of PCR product of *tdh* gene of *V. parahemolyticus* (Lane M: 100 bp DNA ladder , Lane 1: Negative control, Lane 2: *tdh* gene product (251bp))

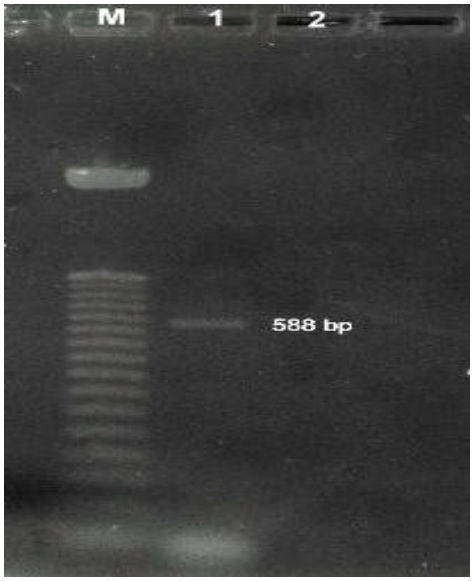

Fig. 3: Agarose Gel electrophoretic analysis of PCR product of *ompW* gene of *V. cholerae*. (Lane M: 50 bp DNA ladder, Lane 1: *ompW* gene product (588 bp), Lane 2: Negative control)

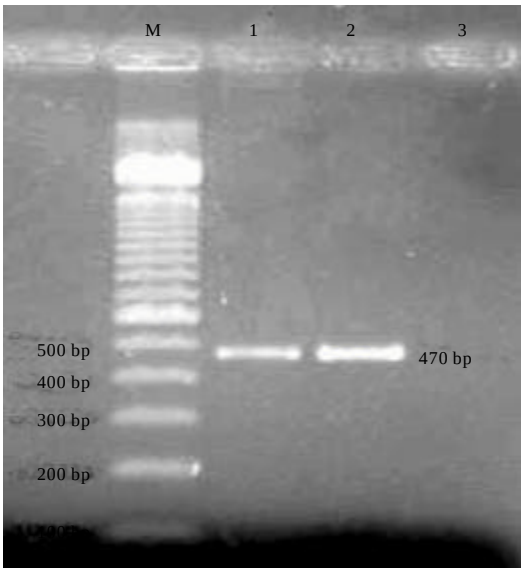

Fig. 2: Agarose Gel electrophoretic analysis of PCR product of *sefA* gene of *Salmonella spp.* (Lane M: 100 bp DNA ladder, Lane 1 and 2: *sefA* gene product (470 bp) , Lane 3: Negative control)

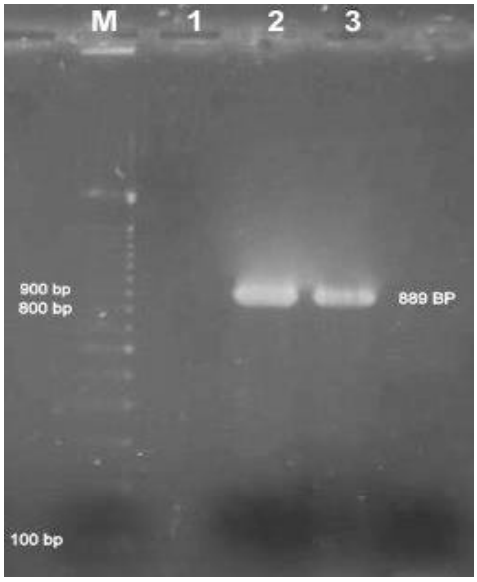

Fig. 4: Agarose Gel electrophoretic analysis of PCR product of hly_{EHEC} gene of *E.coli* O157:H7. (Lane M: 100 bp DNA ladder, Lane 1: Negative control, Lane 2: hly_{EHEC} gene product (889 bp))

Salmonella sp. Agarose gel electrophoresis results for both spiked samples with *V. cholerae* and *E. coli* O157:H7 showed distinct PCR product band of 588 and 889 bp, respectively (Fig. 3, 4). The results confirm amplification

Table 2: Comparison of multiplex and single PCR for the detection of pathogens in spiked shrimp samples

	Single PCR				Multiplex PCR	
Sample cohort	*tdh*	*sefA*	*ompW*	hly_{EHEC}	*Tdh+sefA*	*Tdh+sefA+ompW+*hly_{EHEC}
Spiked samples (n = 20)	16	15	13	12	15	9
	80%	75%	70%	75%	75%	55%

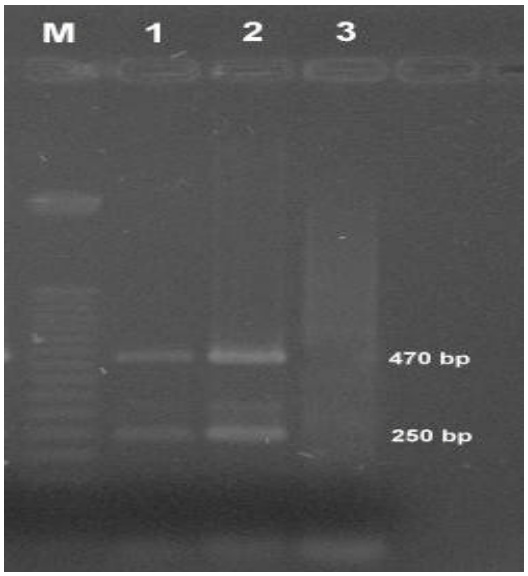

Fig. 5: Agarose gel electrophoretic analysis of products of multiplex PCR (Lane M: 50 bp DNA ladder, Lane 1 and 2: Multiplex PCR product (470 bp confirms *sefA* and 250 bp confirms *tdh*) Lane 3: Negative control)

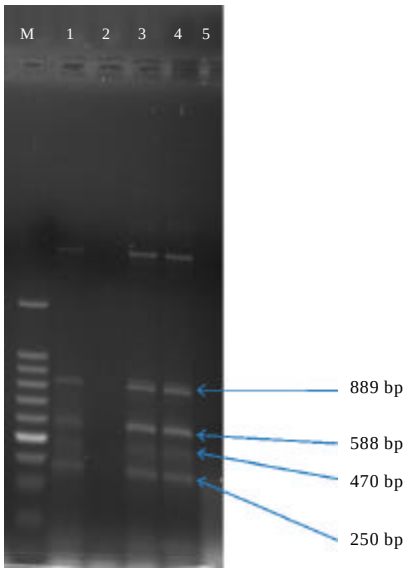

Fig. 6: Agarose gel electrophoretic analysis of product of multiplex PCR set up for simultaneous detection of four target genes from spiked shrimp sample, Lane M: 100 bp ladder, Lane 1, 2, 3, 4 Multiplex PCR product: 470 bp confirms *sefA*, 250 bp confirms *tdh*, 588 bp confirms *ompW* and 889 bp confirms hly_{EHEC} Lane 5: Negative control

of *ompW* and *hylEHEC* genes in shrimp samples spiked with *Vibrio* and *E.coli* strains.

Electrophoresis of multiplex PCR product of *sefA* and *tdh* genes: Multiplex PCR products were subjected to electrophoresis and two distinct PCR product bands were obtained and they were measured as 470 bp and 250 bp (Fig. 5). The results indicate that the designed multiplex set up was able to amplify *sefA* gene of *Salmonella* sp. and *tdh* gene of *V. parahaemolyticus* in a single reaction mixture.

Electrophoresis of multiplex PCR product of *sefA*, *tdh*, *ompW* and hly_{EHEC} genes: DNA samples extracted from spiked shrimp samples were investigated in multiplex PCR set up for simultaneous detection of all the target pathogens from shrimp sample. The PCR products were subjected to electrophoresis and results showed that all the four genes were amplified in multiplex set up (Fig. 6) as similar to that they were amplified in case of multiplex set up designed for *tdh* and *sefA* (Fig. 5).

Evaluation of multiplex PCR with spiked and pure DNA: For comparative analysis of uniplex and multiplex PCR set ups, results of different combinations uniplex and multiplex PCR has been summarized in Table 2. Uniplex amplifications yielded positive result in 70-80% spiked shrimp samples. Multiplex PCR setup with two genes yielded positive result in 75% of spiked shrimp samples whereas, multiplex PCR setup targeting four genes can detect the genes in 55% spiked shrimp samples.

DISCUSSION

Foods contaminated with pathogens play an important role in the dissemination of the pathogens to humans (Porteen *et al*., 2007) thus causes foodborne outbreak and concerns for public health. Development of PCR based modified detection methods are of huge interest because of more sensitivity, less time requirement and easy to handle large sample volume in the testing

laboratories. In a previous study (Fakruddin *et al*., 2012), multiplex PCR method for simultaneous detection of *Salmonella* spp. and *Vibrio parahaemolyticus* from enrichment cultures and spiked shrimp amples were reported.. The objective of this study was to extend the method for simultaneous detection of four important food pathogens (*Escherichia coli* O157:H7, *Salmonella* sp., *Vibrio parahaemolyticus* and *Vibrio cholerae*) from shrimp samples and to compare the multiplex PCR performance with uniplex PCR results in terms of sensitivity and specificity. The targeted genes were *ompW* for *V. cholerae*, *tdh* for *V. parahaemolyticus*, hly_{EHEC} for *E. coli* O157:H7 and *sefA* for *Salmonella* sp.

All the four targeted genes were amplified successfully, mixed DNA and DNA extracted from co-culture were subjected to a number of multiplex settings designed to detect the pathogens simultaneously. In some of the multiplex settings there was no amplified product indicating the setting was not suitable for amplification. In 3 multiplex settings (multiplex setting 1-3), only two of the genes (*tdh* and *sef*A) were detected. The limitation of the PCR settings to detect hly_{EHEC} and ompW may be due to primer incompatibility, difference in annealing temperature and lack of suitable PCR mixture. Again, difference in annealing temperature and product length (899, 588, 470 and 250 bp) may also be factors for the failure to detect 4 genes at a time. A negative PCR results does not necessarily indicate that no template DNA was present in the sample. Inhibitory substances present in a sample may influence the outcome of the PCR by lowering or completely preventing the amplification (Lund and Madsen, 2006). A false-negative PCR result is a major concern to the food industry where PCR is being used for pathogen detection; therefore, internal standards or controls have to be included in the PCR to avoid false-negatives (Jones *et al*., 2000).

The presence of certain components in shrimp samples can prevent recovery of injured cells and may require a longer enrichment phase prior to PCR detection. Optimization of methods for detection of different pathogens is necessary, in terms of both the DNA extraction and PCR amplification steps. Furthermore, different pathogens may require different enrichment media and procedures. However, we have overcome some of these limitations through the use of a nonselective enrichment step followed by a multiplex PCR assay for simultaneous detection of these important pathogens, reducing the total analysis time without loss of sensitivity.

A total of 20 shrimp samples were collected and spiked for the study. Spiked DNA samples were subjected to both uniplex and multiplex PCR and results were recorded. Two multiplex setup was applied, one aiming to detect two genes (*tdh* and *sefA*) and another aiming detection of all four genes (*tdh, sefA, ompW* and hly_{EHEC}). PCR with spiked DNA showed considerable variation in result. In uniplex PCR, *tdh* was detected in 16 (80%) sample, whereas *sefA* was detected in 15 (75%), ompW was detected in 13 (65%), $hly_{EHEC}$was detected in 12 (60%) samples (Table 1). In multiplex PCR with spiked DNA targeting two gene (*tdh* and *sefA*) resulted 75% amplification of both genes on the other hand multiplex PCR targetting all four genes resulted amplification of 9 out of 20 samples (45%). Some non-specific products were also produced in this set up (Fig. 6), this may be due to non-specific binding of any primer to shrimp DNA. To estimate the specificity of the method, DNA from shrimps spiked with non-target pathogens such as *Bacillus* cereus, *Shigella flexneri* and *Staphylococcus aureus* were subjected to multiplex PCR and found no amplification, indicates the specificity of the multiplex PCR method.

Kim *et al*. (2007) has developed a multiplex PCR assay for simultaneous detection of five foodborne pathogen such as *E. coli* O157:H7, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Vibrio parahaemolyticus*. Kawasaki *et al*. (2009) developed another multiplex PCR method for detection of *Salmonella* spp., *Listeria monocytogenes* and *E. coli* O157:H7 from various types of artificially inoculated food samples including shrimp. Labreuche *et al*. (2012) developed a quantitative multiplex PCR method with an internal amplification control for simultaneous detection of *Vibrio penaecida* and *Vibrio nigripulchritudo* from shrimp samples with high specificity and sensitivity (200 CFU mL^{-1}).

Development of multiplex PCR method with amplification of all targeted genes will be a valuable alternative screening and detection method for shrimp to be exported in foreign countries. Further investigation for amplification of *ompW* and hyl_{EHEC} genes as well as enhancement of sensitivity, specificity and generation of data for validation are required.

CONCLUSION

Based on the results, it can be concluded that the Multiplex PCR method targeted for two genes i.e. *tdh* and *sef*A is suitable for screening and detection of *V. parahaemoliticus* and *Salmonella* sp. from shrimp samples but before that proper validations of the method are required. On the other hand multiplex PCR method targeted for all four genes are yet to develop for screening

and detection. Detail investigation and root cause analysis for failure are needed and further research to be adopted for development of the method with amplification of all genes with high sensitivity and specificity.

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