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Development of Multiplex PCR (Polymerase Chain Reaction) Method for Detection of *Salmonella* spp. and *Vibrio parahaemolyticus* from Shrimp Samples of Bangladesh

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ABSTRACT

Lack of reliable and rapid method for detection of pathogens from shrimp in Bangladesh is an obstacle in ensuring the microbiological quality of the shrimp before export. *Salmonella* sp. and *Vibrio parahaemolyticus* are two potential pathogen for shrimp export value chain in Bangladesh. The objective of this study was to establish a multiplex polymerase chain reaction method for rapid and simultaneous detection of *Salmonella* spp. and *Vibrio parahaemolyticus* from export oriented shrimp samples. The targeted genes were *tdh* for *V. parahaemolyticus* and *sefA* for *Salmonella* spp. The genomic DNA was extracted and amplified for subsequent profiling. Validity of the multiplex PCR assay was tested by artificially inoculating the shrimp homogenate with viable cells of target pathogens. The genes were successfully amplified individually and simultaneously both from natural and spiked samples. Sensitivity of the assay was determined to be as low as 10^4 CFU mL^{-1}. Amplification of DNA extracted from other bacterial pathogens viz. *Bacillus cereus*, *Shigella flexneri* and *Staphylococcus aureus* yielded negative results. This multiplex PCR assay will provide specific, rapid and reliable results and allow for the cost effective detection of target pathogens in a single reaction tube in mixed bacterial communities that are prevalent in shrimp products.

Key words: Multiplex PCR, shrimp, validation, *Vibrio parahaemolyticus*, *Salmonella typhi*

INTRODUCTION

In western and developed countries, shrimp is considered as a potential health risk food for consumer due to its intended uncooked/minimally processed direct consumption. Contamination of uncooked raw shrimps by *Salmonella* was reported in many previous studies (Ahmed and Anwar, 2007). Pathogens from shrimp may be transmitted to humans when they are eaten undercooked or minimally processed or when other foods, which have been cross-contaminated by pathogens from shrimp, are eaten (Khan *et al*., 2007). *Vibrio parahaemolyticus* is a common food borne pathogen in Japan where infections have been linked to the consumption of aquaculture fish such as shrimp (Khan *et al*., 2007).

Salmonella sp. is fecal origin and is not indigenous to the aquatic environments (Akintola and Bakare, 2011). *Salmonella* is considered among the most important enteric food borne pathogen

whose presence in the food constitutes a severe health hazard (Fadel and Ismail, 2009; Malkawi and Gharaibeh, 2004). There are many reports on food-borne illness due to *Salmonella* contamination (Ahari *et al*., 2009). *Vibrio parahaemolyticus* is an autochthonous bacterium in the aquatic ecosystem and is known to be associated with infections in humans and aquatic animals (Khan *et al*., 2007).

Now-a-day PCR based molecular detection methods for analysis of food and water are gaining global interest for their rapidity and high sensitivity. PCR-based methods have better sensitivity and specificity for rapid detection of food borne pathogens (Yasmin *et al*., 2007). PCR has become the method of choice in recent times for monitoring of microorganisms from food samples because of its simplicity, rapidity, reliability, reproducibility, sensitivity and specificity (Seidavi *et al*., 2008).

On the other hand, conventional selective media culture and biochemical confirmation based methods for detection of pathogens are time consuming. These methods take several days to make the results available, thus increasing the risk of uptake and/or transmitting pathogens. Very often pathogens are present in food in very low numbers against high background of indigenous microflora and complex matrix system rendering recovery of low level of target organisms difficult (Sherfi *et al*., 2006).

Unlike uniplex (one target sequence based) PCR methods, multiplex PCR allows the simultaneous amplification of more than one target sequence in a single PCR reaction. Multiplex PCR saves considerable time and effort and decrease the number of reactions to be performed in order to assess the possible presence of microorganisms (Seidavi *et al*., 2008). Multiplex PCR system has been confirmed to be able to detect food pathogens with compatible or superior sensitivity to the conventional method over the same time period (Kawasaki *et al*., 2005).

The current study is aimed at development of a multiplex PCR method for simultaneous detection of *Salmonella* spp. and *Vibrio parahaemolyticus* present in shrimp samples.

MATERIALS AND METHODS

This study was carried out from March, 2011 to January, 2012 in Industrial Microbiology Laboratory of Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Bangladesh.

Reagents and media: 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) and Hi-Media, India. The rest of the materials and chemicals and reagents used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Bacterial strains and culture maintenance: The bacterial strains namely *Bacillus* cereus ATCC 10876, *Shigella flexneri* ATCC 12022, *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* strain ATCC 65154 and *Vibrio parahaemolyticus* strain ATCC 17802 were used in this experiment and were collected from master culture of Industrial Microbiology Laboratory, IFST, BCSIR, Dhaka. The working cultures were maintained in trypticase soy agar (TSA; Oxoid) slants at 4°C. Bacterial strains were usually grown in trypticase soy broth (TSB; Oxoid) containing 0.6% yeast extract in a shaking incubator at 37°C overnight. For viable cell determination, serial decimal dilutions of samples with phosphate buffered saline (PBS, pH 7.2) were made and plated on Bismuth Sulphide agar (Hi-Media, India) for *Salmonella typhi* and on thiosulphate citrate bile salt agar (Hi-Media, India) for *Vibrio parahaemolyticus*. The plates were then incubated at 37°C for 24 to 48 h before the colonies were counted.

Selection of genes and primers: Genes selected for this study were *tdh* for *Vibrio parahaemolyticus* and *sefA* for *Salmonella typhi*. Primer sequence for *tdh* was *tdh* D3 (5'CCACTACCACTCTCATATGC3') and *tdh* D5 (5'GGTACTAAATGGCTGACATC 3') (Hara-Kudo *et al*., 2003). Primer sequence for *sefA* was A058 (5'GATACTGCTGAACGTAGAAGG3') and A01 (5'GCGTAAATCAGCATCTGCAGTAGG 3') (Oliveira *et al*., 2003). The product size for *tdh* gene amplification product is 250 bp and for *sefA* gene is 470 bp.

DNA extraction from target organisms: DNA from working cultures of target organisms were 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. Bacterial cells were harvested by centrifuging the culture at 10000 rpm for 5 min. The supernatant was discarded and cell pellet was taken. The cell pellet was washed twice with sterile physiological saline for removing residual culture medium from the cells and was subjected to treatment with DNA extraction solution I (tris HCl+EDTA+sucrose) for 30 min 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 mixture was used to precipitate proteins. The cell extract was mixed gently with the solvent. 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.

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 also checked by measuring absorbances on T60 UVVIS spectrophotometer at 260 and 280 nm. Purity was analyzed by absorbances ratios i.e., 260/280 nm (Sahasrabudhe and Deodhar, 2010).

Uniplex amplification of *tdh* 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 both gene. 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 58°C for *sefA* (annealing) and 1 min at 72°C (extension). The final extension for 9 min at 72°C and holding temperature was maintained at 4°C. After amplification, PCR products were stored at 4°C till electrophoresis done. 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 100 V for 1.5 h and stained with ethidium bromide (10 μg mL^{-1}). A 100 bp DNA ladder was used as a standard molecular weight marker.

Multiplex PCR settings: Multiplex PCR was performed in a total volume of 30 μL containing 2 μL of template DNA and 28 μL of PCR master mix composed of 1X PCR buffer, 5.0 mM $MgCl_2$, 25 μM concentration 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), 200 μM dATP, dCTP, dGTP and dUTP, 0.5 U of AmpliTaq Gold DNA polymerase (Invitrogen, USA) with a DNA thermal cycler (DNA Engine, Bio Rad, USA). 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, 72°C for 1 min and then 72°C for 9 min. The amplified products were then analyzed by 2.0% agarose gel electrophoresis. Expected size for *Salmonella* and *V. parahaemolyticus* were 470 and 250 bp, respectively.

Sensitivity of the multiplex PCR: Overnight culture of the test organisms were used to prepare serial dilutions in sterile buffer solution, with bacterial concentrations 10^4, 10^5, 10^6 and 10^7 cells mL^{-1}. Cells were pelleted by centrifugation and DNA was extracted using the method described earlier. PCR was performed with the successful multiplex setting stated earlier and amplified products were then analyzed by 2.0% agarose gel electrophoresis.

Specificity of the multiplex PCR: To test the specificity as well as the validity of the mPCR method and to ensure 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 stated above.

Spiking of shrimp samples: Shrimp samples were peeled and sterilized by autoclaving at 121°C for 15 min. The cooled down sterilized shrimps 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. 3 mL of stomached samples were inoculated into nutrient broth and incubated for 24 h at 37°C. The overnight grown culture was subjected to DNA extraction and PCR.

DNA extraction from spiked shrimp samples: For extraction of DNA in food samples, method described by Yasmin *et al.* (2007) was followed.

Multiplex PCR with the inoculated shrimp samples: DNA extracted from spiked shrimp samples were subjected to both the uniplex and multiplex PCR setting stated earlier for the detection of the pathogens concerned. The amplified products were then analyzed by 2.0% agarose gel electrophoresis. Expected size for *Salmonella* and *V. parahaemolyticus* were 470 and 250 bp, respectively.

Multiplex PCR with the mixed culture (without incubation): The sensitivity of the multiplex PCR system for simultaneous detection of *Salmonella* sp. and *V. parahaemolyticus* was evaluated with the mixed cultures prepared. Each pathogen was grown to the late logarithmic phase in TSB+0.6% yeast extract. The cells were collected by centrifugation and resuspended in the original volume of PBS. Serial decimal dilutions were made with PBS to make 10^6 CFU mL^{-1}. One mL of the cell dilution was mixed with 9 mL of shrimp culture extract (25 g of shrimp was stomached with

225 mL of TSB+0.6% yeast extract and incubated at 35°C for 24 h) and immediately proceeds for DNA isolation according to method described by Yasmin *et al*. (2007).

RESULTS

Amplification of *sefA* gene of *Salmonella* sp.: The PCR product was separated by 1.5% agarose gel electrophoresis and visualized by UV transilluminator (Alpha Innotech). After electrophoresis, a 470 bp size distinct PCR product band was observed. The distinct size (470 bp) band confirms *sefA* gene in the reference *Salmonella* sp. ATCC 65154 (Fig. 1).

Amplification of *tdh* gene of *V. parahaemolyticus*: The PCR product was separated by 1.5% agarose gel electrophoresis and visualized by UV transilluminator (Alpha Innotech). After electrophoresis, a 250 bp size distinct PCR product band was observed. The distinct size band (250 bp) confirms *tdh* gene in the reference *Vibrio parahaemolyticus* ATCC 17802 (Fig. 2).

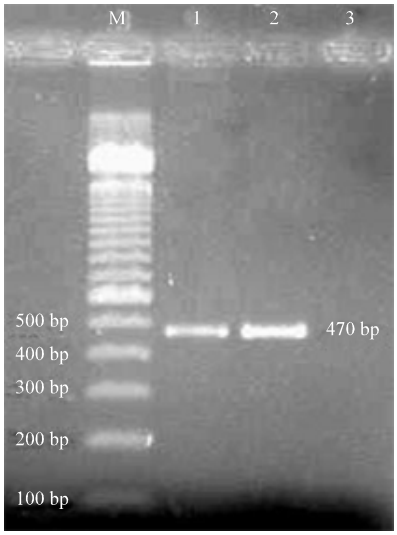

Fig. 1: Agarose gel electrophoretic analysis of PCR product of *sefA* gene of *Salmonella* spp., Lane M: 100 bp DNA ladder, Lane 1 and 2: *sefA* gene product (470 bp), Lane 3: Negative control

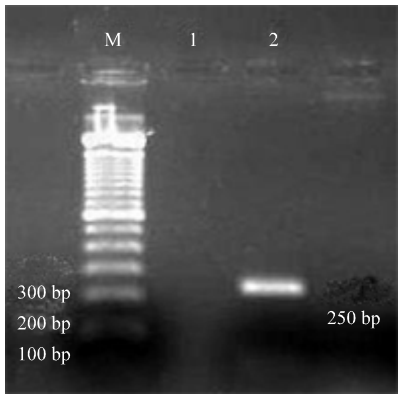

Fig. 2: Agarose gel electrophoretic analysis of PCR product of *tdh* gene of *V. parahaemolyticus*, Lane M: 100 bp DNA ladder, Lane 1: Negative control, Lane 2: *tdh* gene product (250 bp)

Multiplex amplification of *sefA* and *tdh* genes: After electrophoresis of PCR product, two distinct PCR product band was observed and their size was measured as 470 and 250 bp. From the amplification results of the multiplex PCR, it is evident that the designed set-up is able to amplify *sefA* gene of *Salmonella* sp. and *tdh* gene of *V. parahaemolyticus* in a single reaction mixture (Fig. 3).

Sensitivity of the multiplex PCR: Although the multiplex setting amplified the genes in all four DNA samples extracted (10^4, 10^5, 10^6 and 10^7 cells mL^{-1}) but the intensity of the PCR bands gradually decreased with decreasing cell concentration in the samples (Fig. 4).

Specificity of the multiplex PCR: No PCR band was found in case of *Bacillus cereus* ATCC 10876, *Shigella flexneri* ATCC 12022 and *Staphylococcus aureus* ATCC 25923. The results indicate that the method is specific for the target organisms *Salmonella* sp. and *V. parahaemolyticus* (Fig. 4). The result of the amplification reaction is shown in Fig. 5.

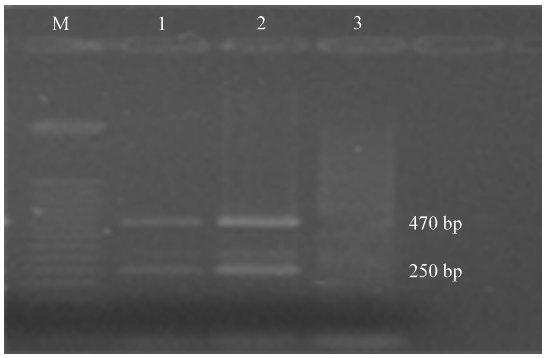

Fig. 3: Agarose gel electrophoretic analysis of products of multiplex PCR (Lane M: 50 bp DNA ladder, Lane 1 and 2: Multiplex PCR product (470 bp confirms *sefA* and 250 bp confirms *tdh*), Lane 3: Negative control)

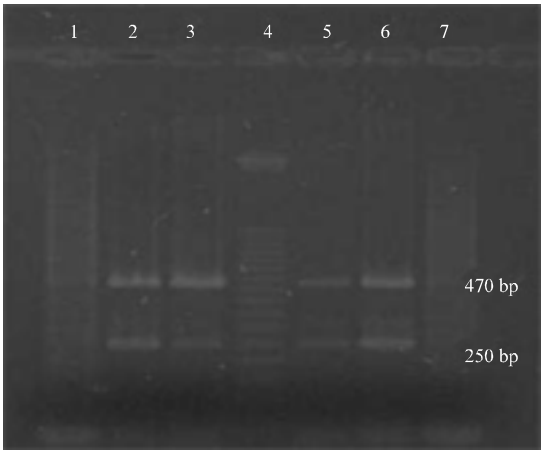

Fig. 4: Agarose gel electrophoretic analysis of products of multiplex PCR (Lane 2: 10^6 CFU mL^{-1}, Lane 3: 10^7 CFU mL^{-1}, Lane 4: 50 bp marker, lane 5: 10^4 CFU mL^{-1}, Lane 6: 10^5 CFU mL^{-1}, Lane 1 and 7: Negative control)

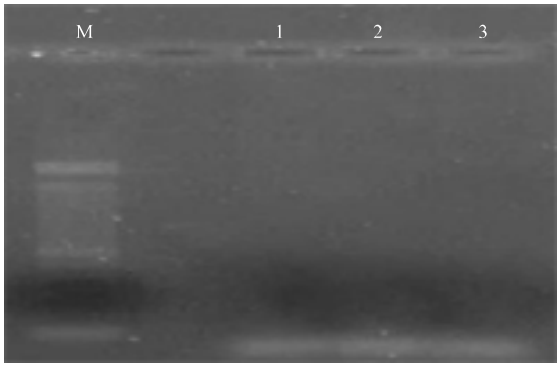

Fig. 5: Agarose gel electrophoresis of amplification with non target DNA (Lane M: 100 bp DNA ladder, Lane 1: *Bacillus cereus* ATCC 10876, Lane 2: *Shigella flexneri* ATCC 12022, Lane 3: *Staphylococcus aureus* ATCC 25923)

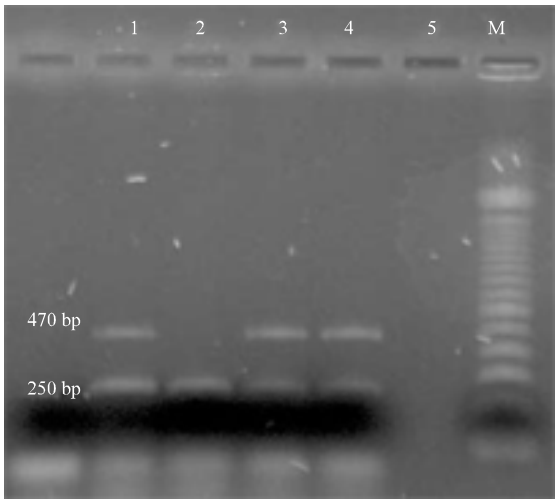

Fig. 6: Agarose gel electrophoretic analysis of products of multiplex PCR setup for simultaneous detection of the target pathogens from shrimp sample. Lane M: 100 bp ladder, Lane 1, 2, 3, 4: Multiplex PCR product (470 bp confirms *sefA* and 250 bp confirms *tdh*) Lane 5: Negative control

Multiplex amplification of DNA extracted from spiked shrimp samples: DNA extracted from shrimp samples spiked with *Salmonella typhi* ATCC 65154 and *V. parahaemolyticus* ATCC 17892 were subjected to PCR amplification with the designed multiplex set up. The multiplex set up able to amplify both of the genes (*sefA* and *tdh*) from all the samples (Fig. 6).

Multiplex PCR evaluated with the mixed culture (without incubation): DNA extracted from shrimp samples spiked with the target organisms without incubation were subjected to PCR amplification with the designed multiplex set up. The multiplex set up amplified both of the genes from all the samples. The results are shown in Fig. 7.

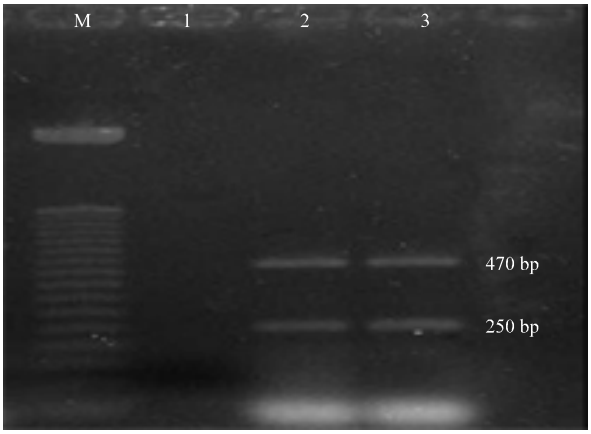

Fig. 7: Agarose gel electrophoretic analysis of products of multiplex PCR, Lane M: 50 bp DNA ladder, Lane 1: Negative control, Lane 2 and 3: Multiplex PCR product (470 bp confirms *sefA* and 250 bp confirms *tdh*)

DISCUSSION

In Bangladesh, shrimp cultivation and processing is one of the major sectors that earn a lot of foreign exchange every year and the sector is developing gradually. As it is mentioned earlier, export of shrimp is an important contributor to economic growth of the country. The shrimp sector is the second largest export earner for Bangladesh, accounting for an annual export of USD 445 million in 2007-08 (BBS, 2010). Export market of shrimp could be threatened if contaminated with pathogenic bacteria such as *Vibrio* spp., *Salmonella* spp., *E. coli* O157:H7 etc. (Porteen *et al.*, 2007). Bangladesh frozen shrimp exporters continue to have both real and perceived problems with buyers having stringent food regulation and standard in the U.S., the EU and Japan, concerning the safety and quality of their products (Ahmed and Anwar, 2007).

In most of the laboratories, microbiological examination and detection of food pathogens are selective media and biochemical characteristics based. These methods involve lengthy procedure and in some cases Level of Detection (LOD) is not satisfactory. Modern biotechnology has enabled us to use more reliable and sensitive detection methods. PCR-based methods have potential for rapid and sensitive detection of food borne pathogens. Multiplex PCR is a variant of PCR which enables amplification of many targets in one reaction by using more than one pair of primers (Farag *et al.*, 2010). Multiplex assays can be tedious and time-consuming and it requires lengthy optimization procedures, though it is a cost saving technique in many diagnostic laboratories (Elnifro *et al.*, 2000). The technique is subject to certain difficulties related in principal to the availability of primers for various pathogens (Martin *et al.*, 2000); the formation of primer dimers (Jannine *et al.*, 1997) and the carryover of certain inhibitory factors in the samples (Wilson, 1997; Parekh and Subhash, 2008). Thus an optimization of multiplex PCR conditions is necessary before use in detection of more than one pathogen targets concurrently (Ozdemir, 2005). The application of multiplex PCR will provide the ease, speed and economic advantage over the single PCR reaction for detecting multiple pathogens (AlHaj *et al.*, 2007).

In this study, we targeted to detect simultaneously *V. parahaemolyticus* and *Salmonella* sp. from shrimp samples. The targeted genes (*tdh* and *sefA*) were amplified individually and then by multiplex settings. Sensitivity of the multiplex assay was good as it amplified the genes from

10^4 CFU mL^{-1} sample. Similar results were found in the study of Seidavi *et al.* (2008) and AlHaj *et al.* (2007). The method is also specific for the target organisms as it did not produced any product during amplification with non-target organisms.

The performance of the method was checked by using DNA extracted from spiked and overnight incubated shrimp samples. The method successfully amplified both genes from all the samples. DNA extracted from spiked and not incubated shrimp samples were also subjected to PCR amplification and the method also amplified the genes from all the samples. Similar results were found by Malkawi and Gharaibeh (2004) in simultaneous detection of multiple *Salmonella* serotypes from chicken and meat products.

CONCLUSION

This multiplex assay will be valuable as a screening method for shrimp contaminated with these pathogens and will also be useful for identifying the sources of contamination. The main problem still to be solved in this study is the detection limit of the pathogens when inoculated in lower number along with other pathogens. Further study will help resolving this problem.

ACKNOWLEDGMENT

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