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Full Length Research Paper

Multiplex polymerase chain reaction assay for the detection of *Salmonella enterica* serovars in shrimps in 4 h

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A rapid and sensitive multiplex polymerase chain reaction (MPCR) based assay was developed for the detection of *Salmonella enterica* serovars such as Typhi (ATCC 122235), Paratyphi A (MTCC 735), Typhimurium (MTCC 98), Enteritidis (ATCC 13065), Weltevreden (MTCC 1169) Bovismorbificians (MTCC 1162), Brunei (MTCC 1168), Arizonae (MTCC 660) and Infantis (MTCC 1167) in shrimps within 4 h of pre-enrichment. The *Salmonella* genus specific gene of *himA* gene was selected and 16S-23S internal transcribed spacer region was used as an internal amplification control (IAC). The genomic DNA was extracted by using boiling and centrifugation method. Sensitivity of the assay was tested by artificially inoculating the shrimp homogenate with viable cells of *Salmonella*. The MPCR assay could detect up to 5 cells within 4 h of pre-enrichment. Amplification of DNA extracted from other bacterial pathogens viz. *Vibrio cholerae* (NICED 16582), *Escherichia coli* (ATCC 9637) and *Staphylococcus aureus* (ATCC 12598) yielded negative results. This MPCR assay provides specific, rapid and reliable results and allows for the cost effective detection of serovars of *S. enterica* in one reaction tube in mixed bacterial communities that are prevalent in shrimp products.

Key words: Multiplex polymerase chain reaction (MPCR), *Salmonella enteric*, *himA,* 16S-23S spacer region, 4 h assay.

INTRODUCTION

Salmonella is one of the major bacterial pathogens of food borne disease outbreaks. It has been classified into more than 2,000 serovars. Most of the human diseases caused by *Salmonella* have been associated with *Salmonella enterica* serotypes Typhi and Paratyphi. The most common contaminated foods associated with human salmonellosis include poultry, beef, pork, eggs, milk and seafood. The prevalence of *Salmonella* in Indian seafoods has been reported by few workers (Nambiar and Iyer, 1991; Hatta and Lakshmanaperumalsamy, 1997; Saroj et al., 2008). A zero tolerance has been prescribed for *Salmonella* in seafood for export trade (Liston et al., 1971). Among the *S. enterica* serovars, the most commonly implicated serovars in major outbreaks are Enteritidis, Anatum, Newport, Cerro, Montevideo, Infantis and Saintpaul (Perch et al., 2003). The incidence of *Salmonella* in fish and fishery products has also been reported from other countries including Japan (Saheki et al., 1989), Thailand (Rattagool et al., 1990), Hong Kong (Yam et al., 1999) and Spain (Martinez-Urtaza et al., 2003).

The ubiquitous distribution of *Salmonella* in the natural environment and its prevalence in many food types has made inspection of *Salmonella* in foods a mandatory requirement worldwide. Conventional methods that rely

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Table 1. Primers used in the MPCR assay.

Gene name	Primer sequences	Annealing temperature (°C)	Product size (bp)	References
himA	F-CGT GCT CTG GAA AAC GGT GAG R-CGT GCT GTA ATA GGA ATA TCT TCA	60	123	Bej et al., 1994
ITS	F-TAT AGC CCC ATC GTG TAG TCA GAA C R-TGC GGC TGG ATC ACC TCC TT	58	312	Chiu et al., 2005

on phenotypic characterization often take several days for detection and identification of this pathogen. There are reports on the PCR detection of this pathogen in foods including seafood (Liu and Li, 2001; Sanathkumar et al., 2003; Jeyasekaran et al., 2010). 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 non-amplification of target DNA and the amplification of non-target internal amplification control (IAC) when IAC is added to the PCR sample indicate that the IAC signal eliminates the possibility of false-negatives (Hoorfer et al., 2003). All the PCR based methods so far reported required about 12 to 24 h of pre-enrichment for the detection of *Salmonella* (Fach et al., 1999). The present study was aimed at developing a multiplex PCR that allowed the detection of *Salmonella* in food products by including 16S-23S spacer region as non target DNA (IAC) along with the *Salmonella* genus specific *himA* gene as a target DNA.

MATERIAL AND METHODS

Bacterial strains, media and reagents

Type cultures of *Salmonella* used in this work included *S. enterica* serovar Typhi (ATCC 122235), serovar Paratyphi A (MTCC 735), serovar Typhimurium *(*MTCC 98), serovar Enteritidis (ATCC 13065), serovar Weltevreden (MTCC 1169) serovar Bovismorbificians (MTCC 1162), serovar Brunei (MTCC 1168), serovar Arizonae (MTCC 660) and serovar Infantis (MTCC 1167). The other bacterial pathogens viz. *Vibrio cholerae (*NICED 16582), *Escherichia coli* (ATCC 9637) and *Staphylococcus aureus* (ATCC 12598) were used to check the specificity of the assay. The cultures were grown on Trypticase Soy broth (TSB) and incubated at 37°C for 18 to 24 h to obtain young culture prior to the extraction of DNA. Molecular grade water (Sartorius Stedim Biotech, Gottingen, Germany) was used for the preparation of buffers, chemicals and reagents. Media and their ingredients were purchased from Hi-Media Laboratories Pvt. Ltd, Mumbai, India.

Bacterial DNA extraction

The genomic DNA was extracted from young cultures (10^7 CFU/ml) by boiling- centrifugation method (Trkov and Avgustin, 1998). One milliliter aliquot of enrichment broth was centrifuged at 13,000xg for 10 min. The pellet was resuspended in 100 µl of molecular grade water, heated to 105°C in a dry bath (Bangalore Genei, Bangalore, India) for 10 min, cooled in ice and centrifuged at 10,000xg for 10 min. The supernatant was used for the PCR assay. The extracted genomic DNA was directly quantified in a Biophotometer (Eppendorf AG, Hamburg, Germany).

Primers

Two sets of primers belonging to *Salmonella* genus specific gene *himA* (Bej et al., 1994) and 16S-23S (Chiu et al., 2005) internal transcribed spacer region (ITS) were selected for this study. The IAC was incorporated into the PCR without impairing the amplification of *Salmonella* and without the loss of specificity and sensitivity on the detection of *Salmonella* in food products. All the primer sequences were synthesized by Ocimum Biosolutions Inc. Indianapolis, USA. The oligonucleotide primer sequences and their expected product sizes are listed in Table 1.

Uniplex PCR amplification and detection of *S. enterica*

The uniplex PCR for the detection of *Salmonella* using two sets of primers belonging to genus specific genes were performed in a Gradient Master Cycler (Eppendorf AG, Hamburg, Germany). All the PCR mixtures were handled in the Bio-Safety Cabinet Type II (Clean Air Systems, Chennai, India). The PCR amplification was performed in 25 µl of reaction mixture containing template DNA of *S. enterica* serovar Typhimurium (MTCC 98), 10X PCR buffer (100 mM Tris (pH 9.0), 500 mM KCl,15 mM $MgCl_2$ and 0.1% gelatin), 100 mM of each dNTP, 0.4 µg of forward and reverse primers of selected genes and 1.25 U of Taq DNA polymerase. The optimized uniplex PCR conditions consisted of initial denaturation at 94°C for 3 min. followed by denaturation at 94°C for 60 s; annealing temperature 60°C for *himA* gene and 58°C for 16S-23S internal transcribed spacer region (ITS) for 60 s; extension at 72°C for 90 s; for 35 cycles and final extension at 72°C for 3 min. The PCR products were run on 2% agarose gel containing ethidium bromide (5 mg/ml) in Tris Acetate EDTA buffer (TAE, pH 8.5) using submarine electrophoresis system (GE HealthCare Bio-Science Ltd., HongKong) and observed under UV Transilluminator using Gel Documentation System (Alpha Innotech, California, USA). The products were identified in comparison with the 100 bp DNA ladder (Real Biotech Corporation, Ohio, USA).

Optimization of multiplex PCR

Optimization was done by performing PCR in a gradient master cycler with different annealing temperatures. The MPCR conditions consisted of initial denaturation at 94°C for 3 min, followed by denaturation at 94°C for 60 s, annealing temperatures ranging from 57 to 63°C for 60 s; extension at 72°C for 90 s for 35 cycles; and final extension at 72°C for 3 min.

Multiplex PCR for *Salmonella*

With the optimized MPCR conditions, the multiplex PCR was performed for the selected serovars of *S. enterica* viz., Typhi (ATCC 122235), Paratyphi A (MTCC 735), Typhimurium (MTCC 98), Enteritidis (ATCC 13065), Weltevreden (MTCC 1169) Bovismorbificians (MTCC 1162), Brunei (MTCC 1168), Arizonae (MTCC 660) and Infantis (MTCC 1167). The optimized MPCR condition consisted of initial denaturation at 94°C for 3 min, followed by denaturation at 94°C for 60 s; annealing at 62°C for 60 s; extension at 72°C for 90 s; for 35 cycles and final extension at 72°C for 3 min. The amplified MPCR product was analyzed in 2% agarose gel containing ethidium bromide as described earlier.

Specificity of MPCR assay

The MPCR assay was performed with the other bacterial pathogens like *V. cholerae* (NICED 16582), *E. coli* (ATCC 9637) and *S. aureus* (ATCC 12598) to test for the specificity of the developed MPCR for *Salmonella*.

Detection limit of MPCR assay in shrimps

Shrimp homogenate (150 g) was sterilized at 100°C for 3 min. and then divided into six lots of 25 g and transferred into the sterile petriplates. Aliquots of 0.1 ml of tenfold serial dilution (10^{-2} to 10^{-7}) of an overnight grown culture of *S. enterica* serovar Typhimurium (MTCC 98) containing $1.00 \cdot 10^{7}$ CFU/ml was inoculated into 25 g of homogenized sample. About 100 µl of each dilution was plated on to BSA plates and incubated at 37°C for 48 h for enumerating the counts. The homogenized sample containing inoculum of *S. enterica* serovar Typhimurium (MTCC 98) was enriched with 225 ml of lactose broth (Hi-Media Laboratories, Mumbai, India) and incubated for 0, 2, 4, 6, and 8 h at 37°C. Uninoculated shrimp homogenate was used as a control. Immediately after the inoculation and after 2, 4, 6, and 8 h of pre-enrichment, 1.5 ml enriched sample was used to extract the genomic DNA and performed the PCR as described earlier. The experiment was independently conducted in triplicates.

Validation of MPCR assay

The seafood samples (10 Nos.) comprising crab, finfish and shrimp collected from the Fishing Harbour, Tuticorin, India were screened for the presence of *Salmonella* and clinical isolates (10 Nos.) of *Salmonella* received from Bose Clinical Laboratory, Madurai, India were checked for its confirmation with the developed method to validate the MPCR assay.

RESULTS

DNA extraction

The genomic DNA extracted by using the SDS-NaOH method had a DNA level of 85.6 ng/µl. The SDS-NaOH method easily extracted genomic DNA from bacterial cultures in less than 30 min (Trkov and Avgustin, 1998).

Uniplex PCR for *Salmonella*

The uniplex PCR performed using the genus specific *himA* gene and 16S-23S internal transcribed spacer region (ITS) of *Salmonella* produced an intense band of the expected product size (Figure 1). The product size of *himA* gene was 123 bp and 16S-23S internal transcribed spacer region (ITS) was 312 bp. The repeated PCR amplification of selected genes gave the reproducible results.

Optimization of multiplex PCR

The selected genes got amplified in all the annealing temperatures (57 to 63°C) tested (data not shown). Since the product yield was more at 62°C, it was selected as the optimum annealing temperature for the amplification of selected genes of *Salmonella*.

Development of MPCR

The *himA* and 16S-23S internal transcribed spacer region genes were found to be specific only for the genus *Salmonella* when they were tested with the optimized annealing temperature in the MPCR and got amplified for all the selected serovars of *S. enterica* viz., Typhi, Paratyphi A, Typhimurium, Enteritidis, Weltevreden, Bovismorbificians, Brunei, Arizonae and Infantis (Figure 2).

Sensitivity of detection of *Salmonella* in shrimps by MPCR

Even though the *Salmonella* genes were undetectable immediately after the inoculation in lactose broth (data not shown), the 225 ml homogenates inoculated with 10^{-2} and 10^{-3} dilutions of *Salmonella* equivalent to 1,06,753 and 20,184 cells showed the amplification of genus specific *himA* gene and 16S-23S internal transcribed spacer region after 2 h of enrichment (Figure 3). No amplification was observed in the lower dilutions. After 4 h of pre-enrichment, *Salmonella* genus specific *himA* gene and 16S-23S internal transcribed spacer region (ITS) were detectable even in the lower inoculum levels of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} equivalent to 1,125, 246, 64 and 5 cells, respectively (Table 2). The degree of specificity and sensitivity of the 4 h MPCR assay was very significant and virtually detected *Salmonella* from shrimp homogenates even with 5 cells (Figure 4). Further pre-enrichment for 6 and 8 h also yielded the same

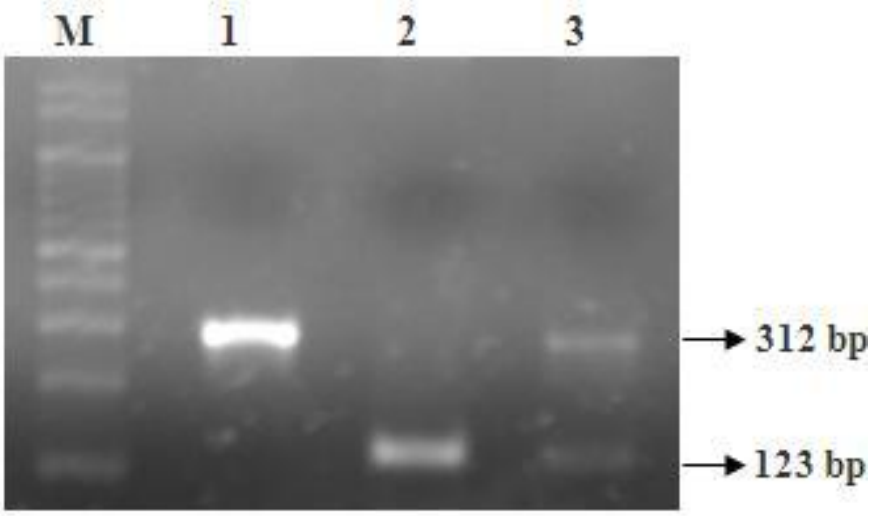

Figure 1. Ethidium bromide stained 2% agarose gel showing results of electrophoresis products of the PCR reaction for the detection of *S. enterica* serovar Typhimurium using genus specific *himA* and 16S-23S internal transcribed spacer regions (*ITS*) genes. Lane M: 100 bp DNA marker; lane 1: 16S-23S internal transcribed spacer region; lane 2: *himA*; lane 3: MPCR amplification of the selected two target genes.

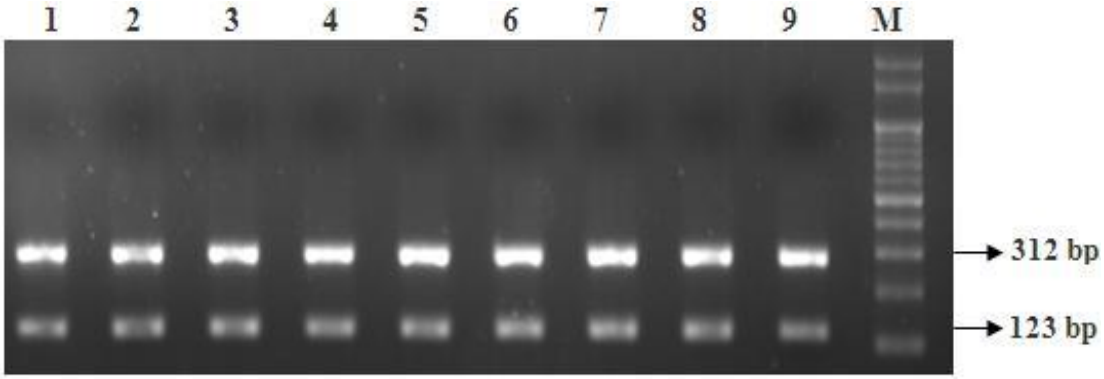

Figure 2. Ethidium bromide stained 2% agarose gel showing results of electrophoretic products of the MPCR reaction for the detection of *S. enterica* serovars using genus specific *himA* gene and 16S-23S spacer region. Lane 1: *S.* Typhi (ATCC 122235); lane 2: *S.* Paratyphi A (MTCC 735); lane 3: *S.* Typhimurium (MTCC 98); lane 4: *S.* Enteritidis (ATCC 13065); lane 5: *S.* Weltevreden (MTCC 1169); lane 6: *S.* Bovismorbificians (MTCC 1162); lane 7: *S.* Brunei (MTCC 1168); lane 8: *S.* Arizonae (MTCC 660); lane 9: *S.* Infantis (MTCC 1167); M: 100 bp DNA marker.

sensitivity. Hence, 4 h pre-enrichment was sufficient to detect the *Salmonella* in seafoods.

Validation of MPCR assay

The *Salmonella* genus specific *himA* gene and 16S-23S spacer region gave the positive results in 10% of the seafood samples screened for *Salmonella* and 70% of clinical isolates of *Salmonella* checked for confirmation.

DISCUSSION

The *himA* and *ITS* genes in this study were successfully used for the detection of *Salmonella* by PCR (Figure 1). Several uniplex PCR based methods for the specific detection of *S. enteric* in foods were earlier reported

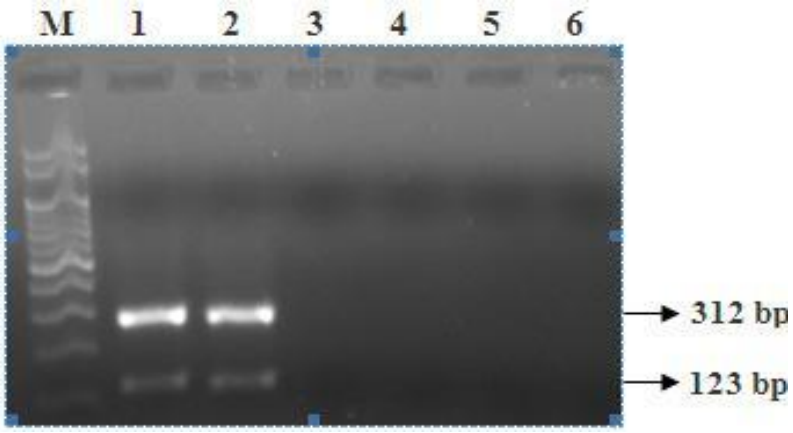

Figure 3. Agarose gel electrophoresis of the MPCR products obtained when testing the artificially contaminated shrimp muscle samples with *S. enterica* serovar Typhimurium (MTCC 98) using the genus specific *himA* gene and 16S-23S *ITS* region after 2 h of pre-enrichment. Lane M: 100 bp- DNA
-2 -3 -4 -5 -6 marker; Lane 1: 10 ; lane 2: 10 ; lane 3: 10 ; lane 4: 10 ; lane 5: 10 ; lane

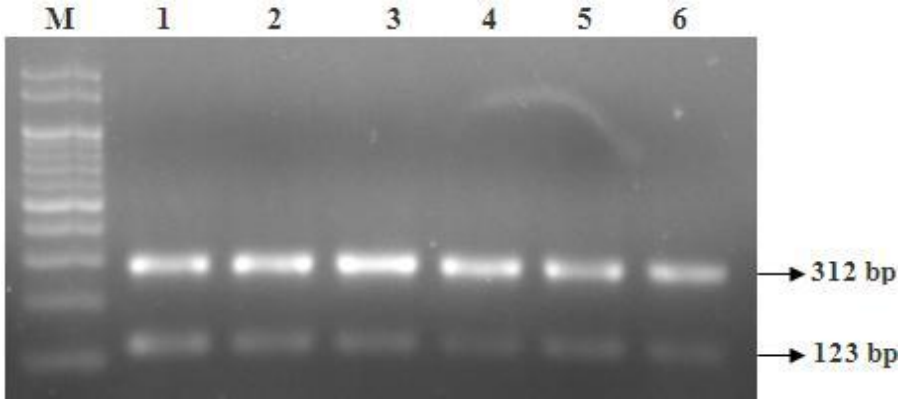

Figure 4. Agarose gel electrophoresis of the MPCR products obtained when testing the artificially contaminated shrimp muscle samples with *S. enterica* serovar Typhimurium (MTCC 98) using the genus specific *himA* gene and 16S-23S *ITS* region after 4 h of pre-enrichment. M: 100 bp DNA marker; Lane 1: 10^{-2}; lane 2: 10^{-3}; lane 3: 10^{-4}; lane 4: 10^{-5}; lane 5: 10^{-6}; lane 6: 10^{-7}.

Table 2. Detection of *S. enterica* serovar Typhimurium (MTCC 98) in artificially contaminated shrimps by MPCR assay.

Duration of pre-enrichment (in h)	Bacterial dilution					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
2	+	+	-	-	-	-
4	+	+	+	+	+	+
6	+	+	+	+	+	+
8	+	+	+	+	+	+

employing *invA* (Rahn et al., 1992), *hns* (Jones et al., 1993), *fimA* (Cohen et al., 1996), *histidine transport operon gene* (Cohen et al., 1993) and *hilA* (Pathmanathan et al., 2003).

It has been demonstrated that the detection of the genus *Salmonella* possessing two different genes by multiplex PCR with high specificity was possible in a relatively short period of time (Figure 2). This MPCR method

therefore could be very useful in the diagnosis of different serovars of *S. enterica*, though some serovars lack any one of the conserved DNA sequences. Targeting multiple microbial pathogens simultaneously in a single PCR reaction was reported to be more time-efficient and cost effective (Brasher et al., 1998). When the specificity of the developed assay was checked, the other bacterial pathogens viz., *V. cholerae*, *E. coli* and *S. aureus* gave negative results.

The amplification of the internal amplification control (IAC) and genus specific genes in the inoculation studies at the 4 h pre-enrichment with lower inoculum levels without any mismatching indicated that the developed multiplex PCR assay was highly sensitive to detect the *Salmonella* (Figure 4 and Table 2). However, it has been earlier reported that *Salmonella* was detected at a level of 10^6 cfu per 25 g of food samples with a uniplex PCR-based commercial Kit after 18 h enrichment period (Fach et al., 1999). Kumar et al. (2008) has also found that *Salmonella* was detected by uniplex PCR in seafoods spiked with viable *Salmonella* cells to a level of 10^6 to 2 cfu per 25 g in 250 ml after 8 h pre-enrichment. Another report indicated the detection limit of optimized uniplex PCR using the *invA* gene as $2.60 \cdot 10^4$ cfu / ml (Moganedi et al., 2007). Incorporation of a 6 h non-selective pre-enrichment step further increased the detection limit to 26 cfu / ml. Similar results have been previously reported by other workers (Bej et al., 1994; Fratamico, 2003; Myint et al., 2006) who observed that at least 10^3 to 10^5 CFU / ml must be present to give positive results by uniplex PCR without a pre-enrichment step and that 1 to 10 CFU / ml could be detected after a pre-enrichment step. All the reported pre-enrichment methods were based on uniplex method and without the IAC.

Of the seafood samples tested for the validation of developed MPCR assay, 10% of samples were found to be positive for *Salmonella*. However, the MPCR method confirmed 70% of the clinical isolates of *Salmonella* and it showed that the method is more accurate and rapid than the conventional identification method. Our findings are supported by D'Aoust (1992), who observed that conventional methods show poor sensitivity and sometimes false-positives.

Based on the results, it can be concluded that the developed MPCR assay was highly specific, sensitive, rapid and reliable for the detection of serovars of *S. enterica* from seafood and clinical samples.

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