

Research Paper

A novel multiplex PCR for the simultaneous detection of *Salmonella enterica* and *Shigella* species

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Submitted: January 9, 2013; Approved: September 9, 2013.

Abstract

Salmonella enterica and *Shigella* species are commonly associated with food and water borne infections leading to gastrointestinal diseases. The present work was undertaken to develop a sensitive and reliable PCR based detection system for simultaneous detection of *Salmonella enterica* and *Shigella* at species level. For this the conserved regions of specific genes namely *ipaH1*, *ipaH*, *wbgZ*, *wzy* and *invA* were targeted for detection of *Shigella* genus, *S. flexneri*, *S. sonnei*, *S. boydii* and *Salmonella enterica* respectively along with an internal amplification control (IAC). The results showed that twenty *Salmonella* and eleven *Shigella* spp., were accurately identified by the assay without showing non-specificity against closely related other Enterobacteriaceae organisms and also against other pathogens. Further evaluation of multiplex PCR was undertaken on 50 natural samples of chicken, eggs and poultry litter and results compared with conventional culture isolation and identification procedure. The multiplex PCR identified the presence of *Salmonella* and *Shigella* strains with a short pre-enrichment step of 5 h in peptone water and the same samples were processed by conventional procedures for comparison. Therefore, this reported multiplex PCR can serve as an alternative to the tedious time-consuming procedure of culture and identification in food safety laboratories.

Key words: *Salmonella*, *Shigella*, multiplex PCR, spiking.

Introduction

Diarrhoeal disease is an important public health problem in developing countries and also is an important cause of morbidity and mortality throughout the world. Globally *Salmonella enterica* and *Shigella* species remain major contributors to acute enteric infections (Jousilahti *et al.*, 1997). They are closely related enteric pathogens belonging to the family Enterobacteriaceae and are found naturally in the environment, humans and food (Jun *et al.*, 2004). Bacillary dysentery or Shigellosis caused by *Shigella* spp., is endemic throughout the world and it is among the most common causes of bacterial diarrhoeal diseases. It is responsible for approximately 165 million cases annually, of which 163 million are in developing countries and 1.5 million in industrialized ones (Kumar *et al.*, 2010). *Shigella* spp., are subdivided into four serogroups - *S. sonnei*, *S. boydii*, *S. flexneri* and *S. dysenteriae* and humans are the principal reservoir of infection. The infectious dose

of *Shigella* is as low as 10 bacterial cells (Germani and Sansonetti, 2006) and the transmission occurs person to person through the faecal-oral pathway, and this feature of shigellosis gives the pathogen epidemic outbreak potential and can rapidly be disseminated through contaminated food and water (Niyogi, 2005). The symptoms of *Shigella* infection range from mild watery diarrhoea normally in case of *S. sonnei* to severe bacillary dysentery with fever, abdominal pain, blood and mucus in stool samples caused mainly by strains of *S. dysenteriae* 1 (Kumar *et al.*, 2006). *S. flexneri* and *S. boydii* can cause either mild or severe illnesses.

Salmonella enterica are a major cause of foodborne illness leading to acute gastroenteritis throughout the world. *Salmonella* infections (Salmonellosis) are transmitted from animals to humans through food and occasionally from person to person through the fecal-oral route (D'Aoust, 1989). Although gastroenteritis often occurs in large epidemics among individuals who have eaten con-

taminated food, family outbreaks and sporadic cases are even more common (Moffat *et al.*, 2012). Salmonellosis in the human occurs in a variety of forms, presenting a broad clinical spectrum. *Salmonella* infections of humans and animals continue to remain a major public health hazard worldwide. This also has a large negative economic impact on food production.

With the continuing impact of these pathogens on human and animal health, the need for rapid and accurate detection methods for these organisms both in environmental and clinical samples remains high. Moreover, identification of *Salmonella enterica* and *Shigella* species is important for surveillance, prevention and control of food-borne diseases (Li *et al.*, 2005). An accurate and rapid procedure for identification would help identify the sources, reservoirs and or their transfer through food chain. Conventional methods recommended for the detection of these pathogens are generally cumbersome, inefficient, labor intensive and time consuming (Miescier *et al.*, 1992; D'Aoust, 1989). DNA-based methods are better for identification of food-borne pathogens because these methods rely on the nucleic acid composition of the bacterium instead of their phenotypic expressions that may be variable under culture conditions (Hill *et al.*, 1985; Olsen *et al.*, 1995; Batt, 1997). The PCR represents a rapid procedure with both high sensitivity and specificity for the immediate detection, identification of specific pathogenic bacteria from various food matrices (Lantz *et al.*, 1994; Hill, 1996) and multiplex PCR; has been used to detect several pathogens in a single reaction (Way *et al.*, 1993; Brasher *et al.*, 1998; Soumet *et al.*, 1999).

Multiplex PCRs have been reported where both *Salmonella enterica* and *Shigella* genus could be identified together but not with the individual species (Vantarakis *et al.*, 2000; Kong *et al.*, 2002; Li and Mustafa, 2004). In few multiplex PCR reports the targeted genes such as *Vir A* and *invA* (Vantarakis *et al.*, 2000; Villalobo and Torres, 1998), *ipaH* (Kong *et al.*, 2002), *phoP/phoQ* (Li and Mustafa, 2004), were not totally specific as some *E. coli* genes were also getting amplified. Therefore, it was planned to work out on a multiplex PCR that can conclusively differentiate all four *Shigella* spp., along with *Salmonella enterica* and that could be directly applied for the food samples. The new multiplex PCR system standardized in this study could detect target organisms directly from food and environmental samples with 5 h preenrichment step and without exhibiting non specific amplification even among closely related organisms. The genomic DNA could be extracted by boiling method in order to save time and also to reduce the cost. The sensitivity of the newly developed multiplex PCR is better when compared with the reported (Vantarakis *et al.*, 2000) multiplex PCR.

For this multiplex PCR two genus specific genes *invA* (Invasion protein gene 'A') of *Salmonella enterica* (Rahn *et al.*, 1992) and *ipaH1* (invasion plasmid antigen H) gene

of *Shigella* (Brigittle *et al.*, 1998) were selected. For species differentiation of *Shigella*, species specific genes *wzy* (O-antigen polymerase), *wbgZ* (putative epimerase/dehydratase) and *ipaH1* (invasion plasmid antigen H) of *S. boydii* (Tao *et al.*, 2004), *S. sonnei* (James *et al.*, 2000) and *S. flexneri* (Kenia *et al.*, 2010), respectively were selected.

Moreover, an IAC, which has now become almost mandatory for diagnostic PCR's, was also incorporated to account for the false negative results during the PCR reaction. Following standardization, the multiplex PCR was evaluated with *Salmonella enterica* and *Shigella* strains individually and in combination to assess the sensitivity and the specificity. Spiking studies were also conducted in food samples to evaluate the efficacy of the system. The detection of all these pathogenic bacteria simultaneously from food samples could be completed within 8-10 h.

Materials and Methods

Bacterial strains and cultural methods

The bacterial strains selected to test the diagnostic specificity of PCR are listed in Table 1. These included reference strains, human clinical isolates and food and environmental isolates. Standard strains and isolates were grown in Nutrient broth and then streaked on selective agars procured from Himedia (Mumbai, India). Presumptive colonies selected on Deoxycholate Citrate Agar (DCA), *Salmonella Shigella* Agar (SSA) and Xylose Lysine Deoxycholate Agar (XLD) were further subjected to biochemical tests viz., Lysine Decarboxylation, Hydrogen di sulphide (H₂S) production, carbohydrate fermentation and Urease production and motility test (Food and Drug Administration, 1995). The bacterial strains were periodically subcultured and were used for DNA extraction.

DNA Extraction

Each tube containing 5 mL of Luria Bertani broth (LB) was inoculated with each culture and incubated (37 °C, 24 h) in shaking conditions. Template DNA from each bacterial strain was extracted by boiling lysis method (Theron *et al.*, 2001) or by commercial DNA extraction kit (Qiagen, Germany). One milliliter of cells was removed and centrifuged at 10,000 rpm for 3 min. The pellet was resuspended in 50 mL of distilled water, boiled for 12 min, and centrifuged again for 3 min at 12,000 rpm. The supernatant was transferred to a new microfuge tube and the DNA concentration was estimated by NanoDrop-ND-1000 spectrophotometer (Thermo scientific, India) and the DNA was stored at -20 °C until further use.

Primers and Internal amplification control

Five sets of primers were designed to detect *invA*, *ipaH*, *ipaH1* and *wbgZ* and *wzy* genes using Gene Bank database sequences. Conserved regions were selected and

primers were designed with Gene runner software (<http://www.generunner.com>). The gene bank accession numbers of the gene sequences used to design the primers are given in the Table 2. Sequences of all primers (Table 2) were evaluated using the Primer-BLAST and BLASTn tools (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to identify any non-specific targets and to anticipate the specificity of the PCR assay. PCR products ranged from 254 bp to 866 bp in length. Appropriate care was taken to ensure that the primer pairs had similar annealing temperature and the resulting amplicons had a minimum 50 bp difference among themselves (Table 2). The primer pairs were custom synthesized from Eurofins Pvt Ltd., Bangalore (Table 2).

To check the presence of inhibitors within PCR mixture, an IAC was constructed. The primers used in this reaction had 5' overhanging ends, which were identical to the primer *wbgZ* (factor essential for putative epimerase/dehydratase protein) whereas 3' ends were complementary to a DNA sequence of pUC 19 (Table 2). The PCR reaction mixture for generation of IAC DNA contained 6 picomolar of each primer, 100µM each dNTPs (Sigma), 0.5 units of *Taq* polymerase, 2.0 millimolar MgCl₂ in 1x PCR buffer (MBI Fermentas, Canada) with 175 ng pUC 19 plasmid DNA was used as template. The reaction procedure consisted of 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 57 °C for 1 min and extension at 72 °C for 1 min. The DNA was denatured for 4 min in the beginning and finally extended for 10 min at 72 °C (Eppendorf master cycler gradient, Hamburg Germany). PCR product was purified using commercially available kit (Qiagen, Hilden, Germany). The concentration of IAC DNA was determined spectrophotometrically at 260 nm and was stored in DDW at -20 °C. The following equation was used to calculate the copy number of the PCR product concentration: $\text{weight of PCR fragment (in g } \mu\text{L}^{-1}) \times (6.023 \times 10^{23}) / (660 \text{ g mol}^{-1} \times \text{number of base$

pairs of PCR fragment) = the number of genomic copy per microlitre (Mahesh *et al.*, 2009).

Standardization of multiplex PCR

Initially, presence of all genes in the standard strains was confirmed by performing monoplex PCRs. Multiplex PCR was standardized by empirically varying critical factors that affect multiplexing, primer concentrations, amount of MgCl₂, annealing time and temperature. Multiplex PCR was optimized in an Eppendorf master thermal cycler (Hamburg, Germany) with a reaction volume of 30 µL reaction containing 2.5 picomolar each of *IpaH1* F and *IpaH1* R primers, 4.0 picomolar each of *wzy* F and *wzy* R primers, 5.0 picomolar each of *IpaH* F and *IpaH* R primers and 6.0 picomolar each of *wbgZ* and *invA* F and *wbgZ* and *invA* R primers, 200 µM of each dNTP, 7000 copies of IAC DNA, 1 unit of *Taq* polymerase, 2 mM MgCl₂ in 1x PCR buffer with 1.2 µL of mixed template DNA from bacterial samples. Amplification consisted of initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 57 °C for 1 min and extension at 72 °C for 1 min and followed by a final extension at 72 °C for 10 min. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and visualized under UV transilluminator (G-Box, syngene, India).

Determination of specificity and sensitivity of multiplex PCR

The specificity of the multiplex PCR primers was determined against different organisms as shown in Table 1 by taking 1.5 µL of template DNA from each of the organism (approx. 10⁷ cfu). The procedure for PCR was essentially the same as described earlier. To assess sensitivity, minimum amount of DNA detectable by multiplex PCR,

Table 2 - List of targeted genes, primer sequences and expected product size.

Primer	Sequence (5' - 3')	Amplicon size (bp)	Genes targeted	Accession Number
<i>ipaH1</i>	For: GTTACCTGTACTCCCTGCTT Rev: CTAGCCTTCCTTGTGCAA	254	<i>IpaH1</i>	NC_008258
<i>wzy</i>	For: ACCAAGGAGTTGTTTCATGA Rev: GAAGCCCTGGTAAAGTGC	305	<i>wzy</i>	AY369140
<i>wbgZ</i>	For: ATGTTGCTAATACCAGTTGG Rev: TAGAGAGAACTTCACACGGT	460	<i>wbgZ</i>	NC_007385
<i>ipaH</i>	For: TGAGAATTTGCCTCCACA Rev: CTAGCCTTCCTTGTGCAA	595	<i>ipaH</i>	NC_008258
<i>invA</i>	For: GAGGAAAAAGAAGGGTCCG Rev: CTCAACTTCAGCAGATACCA	780	<i>invA</i>	NC_006511
IAC	For: ATGTTGCTAATACCAGTTGGATGACGGTGAAAACCTCT Rev: TAGAGAGAACTTCACACGGTCCTGGTATCTTTATAGTCCTG	866	IAC	

IAC primers are flanked by *wbgZ* primers on 5' end.

10-fold serial dilutions of overnight growth of *S. flexneri* ATCC 9199, *S. sonnei* ATCC 9290, *S. boydii* ATCC 9207 and *S. typhimurium* MTCC 98 were serially diluted in sterile saline (0.8%) to achieve concentrations of 10^8 - 10^0 cfu/mL. DNA was prepared by boiling method and multiplex PCR assay was performed on the boiled lysates of each diluted culture in order to detect the minimum number of cells. *Escherichia coli* genomic DNA was used as a negative control.

Analysis of experimentally spiked food sample

In order to estimate the interference of food matrices on the sensitivity, detectable concentration of target organisms (10^8 cfu/mL) were ten-fold serially diluted in 10 mL saline. Retort processed *chicken pulav* rinse was prepared by macerating 10 g of sample in 90 mL of peptone water using stomacher. The rinse was centrifuged at 1,500 g to get rid of the particulate debris and supernatant was stored in aliquots of 10 mL at -20 °C for further use. Ten milliliter each of *chicken pulav* rinse were spiked with *S. flexneri* ATCC 9199, *S. sonnei* ATCC 9290, *S. boydii* ATCC 9207 and *S. typhimurium* MTCC 98 culture with cell concentration ranging from 10^8 to 10^1 cfu mL⁻¹ and incubated overnight (18 h) at 37 °C. One milliliter was taken at the end of the incubation period from all the samples and processed for DNA extraction by boiling method. The DNA (1.5 µL) was used as template in PCR assay. Simultaneously 100 µL sample from each dilution was plated to assess the cfu's. Similar sampling and analysis were done after 5 h incubation also.

Investigation of *Salmonella enterica* and *Shigella* spp., from food and environmental sources

Fifty food and environmental samples obtained from various localities in Mysore region were assessed for the presence of *Salmonella enterica* and *Shigella* species following standard enrichment and isolation procedures and also by multiplex PCR method (Table 3). For this 25 g of sample was aseptically cultured in 225 mL of Lactose broth and incubated at 37 °C for 24 h. About 1 mL of sample was transferred to 9 mL of tetrathionate broth (Himedia, India) and incubated at 37 °C for 24 h. Subsequently, a loopful of

broth was streaked on surface of SDA, SSA and XLD agar (Himedia, India) and incubated at 37 °C for 24 h. Further, biochemical tests were carried out by conventional methods for suspected isolates (Wallace and Thomas, 1998). Genomic DNA (1.5 µL) prepared from 1 mL enrichment broth (short enrichment incubation step of 5 h in peptone water) by boiling lysis method was used in multiplex PCR.

Results

Bacterial strains and cultural methods

All the cultures as listed in Table 1 were characterized by conventional method and colonies selected on DCA, SSA and XLD agar were further confirmed by biochemical tests. *Salmonella* cultures were found to be positive for fermentation of Mannitol, Dextrose, Lysine decarboxylation, H₂S production, and negative for fermentation of Lactose, Sucrose and urease whereas *Shigella* cultures were non-motile, positive for glucose fermentation, negative for H₂S, gas production, lysine decarboxylation and urease as shown in (Table 1).

Standardization of Multiplex PCR for the detection of selected genes:

The reaction conditions for the multiplex PCR assay were optimized to ensure that all of the target gene sequences were satisfactorily amplified. Care was taken to avoid areas of homology with other organisms. The concentration of primers was optimized after standardizing individual monoplex PCRs (Figure 1) with their respective genes and had almost equal annealing temperature, which reduced the non-specific amplification. The annealing temperature of 57 °C was selected because at this temperature adequate resolution of all the amplified products could be seen. Serial dilution of IAC incorporated in the multiplex assay revealed that 3000 copies of the same resulted in unhindered amplification of *wbgZ* gene for which the IAC primer pair was competitive besides meticulously amplifying other multiplex genes (Figure 2) and the detection limit of the assay was found to be 5 pg. The ability of the multiplex PCR to identify *Salmonella enterica* and *Shigella* spp.,

Table 3 - Comparison of biochemical and mPCR testing on field samples.

Sample	Total no. of samples analysed	No. of positive samples for <i>Salmonella enterica</i>		No. of positive samples for <i>Shigella</i> spp.			
		Conventional method	Multiplex PCR	Conventional method	Multiplex PCR		
					<i>S. flexneri</i>	<i>S. sonnei</i>	<i>S. boydii</i>
Spoiled egg	15	1	1	Nil	Nil	Nil	Nil
Poultry litter	10	1	1	1 (<i>Shigella</i> spp.)	1	Nil	Nil
Chicken	25	1	1	Nil	Nil	Nil	Nil
Total	50	3	3	1	1	Nil	Nil

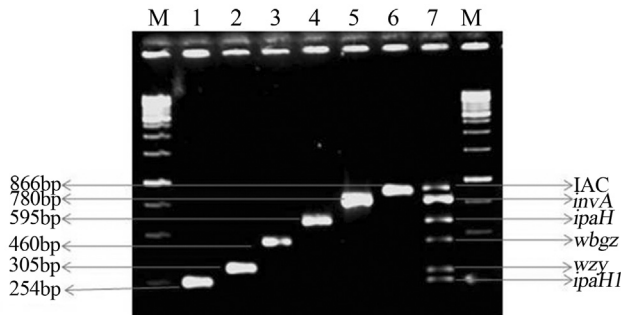


Figure 1 - Agarose gel showing monoplex and multiplex PCR format.

was validated using *Salmonella* and *Shigella* spp., and other strains listed in Table 1.

All the cultures were identified correctly by the standardized multiplex PCR (Figure 1 and Table 1). The standardized multiplex PCR revealed the presence of five bands after agarose gel electrophoresis. On amplification, multiplex PCR yielded a band of 780 bp *invA* gene for *Salmonella enterica*, 254 bp *ipaHI* gene for *Shigella* genus, 305 bp *wzy* gene for *S. boydii*, 460 bp *wbgz* gene for *S. sonnei* and 595 bp *ipaH* gene for *S. flexneri* on agarose gel electrophoresis. IAC, was coamplified with *wbgz* primers and had the amplicon size of 866 bp. As a negative control multiplex PCR was tested with water, no amplicons were observed except IAC. This internal control is highly recommended when PCR-based methods are used to detect bacterial pathogens (Hoorfar *et al.*, 2004). All these results were compared with the conventional culture, isolation and biochemical identification procedures. In the multiplex PCR described here, 1.2×10^3 copies of IAC DNA appeared suitable in avoiding competition between target DNA and IAC DNA for *wbgz* primers particularly at low concentration of target DNA. In PCR with an IAC, a control signal will be produced even when there is no target sequence present. This helps in revealing failures of PCR reactions (Hoorfar *et al.*, 2004).

Robustness

There was no significant loss in the visibility of bands at less (10%, 20%) or more (10%, 20%) concentrations of

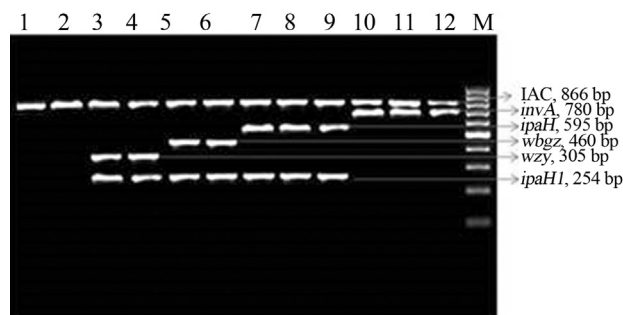


Figure 2 - Agarose gel showing individual genes with IAC.

PCR reagent and IAC DNA. Temperature variation of ± 2 °C also did not make any appreciable change in the profile.

Specificity and sensitivity of multiplex PCR

There was no amplified product obtained except that of IAC with non *Salmonella* and *Shigella* strains (Table 1). All the genes specific for *Shigella* spp., were detected until 10^{-8} dilution which was equivalent to 4×10^2 cfu/mL. In case of *Salmonella enterica*, *invA* gene was detected until 10^{-7} dilution which was equivalent to 2×10^3 cfu/mL.

Analysis of artificially contaminated chicken samples:

The detection limit was found to be 200 cfu/g, 10 cfu/g, and 30 cfu/g and 20 cfu/g of *S. typhimurium*, *S. flexneri*, *S. sonnei* and *S. boydii* respectively in chicken pulav rinse after 5 h of enrichment (Table 3).

Analysis of field samples

Further evaluation of multiplex PCR was undertaken on 50 natural samples (chicken, eggs and poultry litter) and the comparison done with conventional culture isolation and identification procedure. The multiplex PCR could identify the presence of *Salmonella* and *Shigella* in three and one samples respectively and the same samples also yielded target organisms by the conventional method.

Discussion

Salmonella and *Shigella* spp. are frequently encountered as food borne outbreaks or occurring as sporadic cases (Alejandro *et al.*, 2012). The conventional methods of isolation and identification of these pathogens primarily depends on selective media followed by a battery of biochemical tests that are laborious, costly and time consuming (Radji *et al.*, 2010). Generally, other molecular-based methods like PCRs in monoplex and multiplex formats (Liu *et al.*, 2008), PCR non radioactive labelling, PCR-RFLP PCR-ELISA (Ojha *et al.*, 2013) and DNA microarray have a well grounded potential to overcome certain insufficiencies of the conventional methods and is found to be sensitive and reproducible but its application in diagnostic field is difficult due to its high cost and the requirement of skilled person (Rahn *et al.*, 1992). Several reported PCR do not discriminate between *Shigella* at the genus and species levels, nor did they differentiate *Shigella* from closely related pathogens such as *Salmonella*, *Citrobacter* and enteroinvasive *E. coli* (Germani and Sansonetti, 2006; Liu *et al.*, 2008, Vantarakis *et al.*, 2000).

In some multiplex PCR reports, the targeted genes such as *Vir A* and *invA* (Vantarakis *et al.*, 2000; Villalobo and Torres, 1998), *ipaH* (Kong *et al.*, 2002), *phoP/phoQ* (Li and Mustafa, 2004), were not totally specific as some *E. coli* genes were also getting amplified. According to the ohja *et al.* (2013) the primers *invC*, *rfc*, *wbgz* and *rfpB* designed for the identification of *Shigella* spp., *S. flexneri*, *S.*

sonnei and *S. dysenteriae* were specific however according to NCBI blast analysis the primers showed cross reactivity with other members of enterobacteriaceae family. Most of the reported multiplex PCRs lack the inclusion of an IAC, to rule out false negative reactions (Liu et al., 2008; Vantarakis et al., 2000). The false negative PCR reaction might occur due to variation in performance of PCR thermal cyclers, incorrect PCR mixture, inefficiency in Taq polymerase, personnel and the presence of PCR inhibitors in the sample matrices, therefore inclusion of an IAC in the multiplex PCR becomes necessary (Malorny et al., 2003).

In the current study, we developed a novel multiplex PCR by combining genus specific and species specific genes and also the conserved genes of respective organisms which has enabled the system to identify *Salmonella enterica* and differentiate *Shigella* species. In addition, to make the multiplex PCR assay acceptable to the present norms of a diagnostic PCR, IAC was also included. The proficiency of individual primers to yield specific amplification for the target bacterial species was confirmed initially by monoplex PCR's and then adapted into multiplex format. Primers designed for this multiplex PCR were chosen to maintain a near uniform annealing temperature and also care was taken to maintain 50 bp differences among different PCR products for clear resolution. The selected primers were tested to examine the possible cross-reactions of primers by homology searches using the basic local alignment searching tool (BLAST) program. The IAC was incorporated in pUC 19 plasmid DNA flanked by *wbgz* primers. Optimum number of copies of IAC DNA was selected to give a good visible band in order to avoid competition between target DNA and IAC DNA for *wbgz* primers. Another important criterion for a diagnostic PCR is robustness. No significant loss in the visibility of bands could be observed even with the variation of PCR reagents as well as IAC and DNA concentration.

The *invA* gene of *Salmonella enterica* selected in the study has already been proven to be an important diagnostic tool for detection as it contains sequence unique to this genus (Rahn et al., 1992; Shanmugasamy et al., 2011). Similarly *ipaH* gene which is present in multiple copies on both the plasmid and chromosome of *Shigella spp* is considered to be a good and specific diagnostic tool for the detection of *Shigella* from clinical and food samples (Kenia et al., 2010; Thong et al., 2005). James et al. (2000) sequenced and compared the O antigen gene cluster with other Gram negative bacteria and it showed that the *wbgZ* gene was conserved for *S. sonnei*. *WbgZ* gene was used for the molecular cloning of *S. sonnei* (Zou et al., 2001) and there was no report where this gene is used for PCR based detection. Gene *wzy* encoding for O-antigen polymerase is highly specific for identification of individual O antigens in case of *S. boydii* (Jiang et al., 2004). All the four *Shigella* species (*S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae*) could be identified correctly with this standardized multiplex PCR

using species specific primers in case of *S. flexneri* (*ipaH*), *S. boydii* (*wzy*) and *S. sonnei* (*wbgz*). *S. dysenteriae* was not included in our study. The standardized multiplex PCR has *Shigella* genus specific primer (*ipaH1*) which was helpful in identifying *S. dysenteriae* where amplification of the *ipaH1* gene and IAC and absence of other amplicons confirmed the presence of *S. dysenteriae*. The selectivity and specificity of *invA* gene for *Salmonella enterica* and *ipaH1* gene for *Shigella* genus, as observed in our study confirms the earlier observation of Jasmid et al. (2008).

The multiplex PCR had a reasonably high level of sensitivity in experimentally spiked chicken samples. Detection of very low levels of bacterial contamination in food necessitates that these samples to be cultured for a few hours in peptone water for providing conditions for growth and multiplication of bacterial pathogens to a detectable level, dilution of inhibitory substances present in food and dilution of dead target cells, which provides some assurance that the detected DNA belongs from viable target cells. The multiplex PCR reported in our study had a reasonably high level of sensitivity in experimentally spiked chicken pulav samples and able to detect as low as 10^1 and 10^2 organisms per ml of *Shigella* and *Salmonella enterica* respectively following 5 h enrichment in peptone water thus the sensitivity was much better when compared to other reports (Babu et al., 2013; Ojha et al., 2013). According to Ohja et al. (2013) detection limit of mpcr after preincubation in GNB (Gram Negative Broth) was 5×10^4 cells and according to Babu et al. (2013) mpcr detection limit was 10 cfu/PCR after 10 h of incubation in BHI broth.

Epidemiological studies on *Shigella* have established that 10 cells are sufficient to be an infective dose (Wachsmuth and Morris, 1989). The results presented in this work showed that PCR with *ipaH1* primers could be a useful tool for the detection as its sensitivity is better when compared with the reported multiplex PCR (Vantarakis et al., 2000). This detection sensitivity was adequate enough to precisely pickup the presence of these pathogens from among the natural food samples. However, the detection sensitivity of spiked food samples is higher when compared with pure reference culture which may be due to the overnight enrichment of spiked food samples. When evaluated on a few naturally occurring food and environmental samples, the multiplex PCR detected three samples positive for *Salmonella*, one sample positive for *S. flexneri* with identical results obtained following the conventional culture, isolation and biochemical identification procedures. This strengthens the claim of the developed multiplex PCR as a viable and reliable alternative for simultaneous detection of these organisms within 5-8 h when compared to the weeklong cumbersome isolation and identification procedures. Considering the low cost involved and relatively much shorter time needed to detect these important organisms this tool is useful for investigation of food borne outbreaks where these organisms are involved. Both multiplex

PCR and conventional isolation and identification method showed good analytical and diagnostic accuracy. This means that non-target microorganisms tested were negative both by PCR and culture while all *Salmonella* and *Shigella* strains were positive by both methods. The multiplex PCR assay established in our study highlights the usefulness of the multiplex PCR for concurrent and rapid detection of *Salmonella enterica* and differentiation of *Shigella* spp from food and environmental samples.

Conclusion

The major contribution of this study was to develop a multiplex PCR to differentiate *Salmonella enterica* and *Shigella* spp. from other closely related members of Enterobacteriaceae family. The standardized multiplex PCR resulted in appropriate PCR amplicons while IAC alone amplified from non *Salmonella* and non *Shigella* species and in negative control where no template DNA was added. This protocol would help in detecting *Salmonella enterica* and *Shigella* genus and also differentiating *Shigella* spp., during routine investigations of food and clinical samples and also during the biological emergencies. The reported multiplex PCR in our study is simple, inexpensive and sensitive and enables the quick and precise detection of *Salmonella* and *Shigella* strains and could be used in the routine diagnostic laboratory.

Acknowledgments

Authors are thankful for DRDO for providing necessary facilities to carry out this work.

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