Performance Analysis of Multiplex-PCR based Detection of *Salmonella* sp. and *Salmonella* Typhimurium in Chicken Egg Samples

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**ABSTRACT**

Polymerase Chain Reaction (PCR) based molecular detection techniques of food pathogens are taking place of conventional media based biochemical methods due to more quick and reliable performances. Detection of *Salmonella* sp. from food matrixes requires several steps, culture media and at least four consecutive days of incubation period, therefore we designed primer based (*invA* and *flfC* genes) multiplex-PCR analysis for detection of *Salmonella* sp. and *Salmonella* Typhimurium from chicken egg samples of local market in Dhaka city. The prevalence of *Salmonella* spp. and *Salmonella* Typhimurium were analyzed by standard biochemical detection method as well as multiplex-PCR technique from a total of 200 egg samples. The primers were selected for the presence of *invA* and *flfC* genes of *Salmonella* sp. and *Salmonella* Typhimurium, respectively. Twenty four samples out of 200 egg samples were found positive for *Salmonella* sp. through standard culture technique, on the other hand among all the biochemically positive samples, only 16 samples (66%) offered excellent response in multiplex-PCR assay. The results indicate that the present multiplex-PCR technique requires further optimization for getting higher response for detection of *Salmonella* sp. and *Salmonella* Typhimurium from egg samples.

**Key words:** *Salmonella* sp., *Salmonella* Typhimurium, biochemical detection, primer based multiplex-PCR

**INTRODUCTION**

*Salmonella* have been regarded as one of the most prevalent food borne pathogens around the world (Gillespie et al., 2003; Malorny et al., 2003a). Individual attacks with *Salmonella* have been increasing globally since 1980 and have been proven to be related mainly to intake of egg and egg items (CDC, 1998; Khakhria et al., 1997).

There are three different types of *Salmonella* (*Salmonella typhi*, *S. choleraesuis* and *S. enteritidis*) and thousands of serovars that contaminate a wide range of different hosts (Hook, 1985). Some types and serovars are coordinator tailored (e.g., *S. typhi* and *S. gallinarum*),
while others can contaminate a wide range of hosts (e.g., *Salmonella* Typhimurium and *S. enteritidis*). Although attack of epithelial tissues seems to be a typical important virulence aspect of all salmonellae, it is not known whether all types and serovars socialize with eukaryotic tissues likewise. Actually, there is some proof to recommend that variations may be available. Difficult variations of *S. choleraesuis* and *S. typhi* are lacking in their capability to get into mammalian tissues (Finlay et al., 1988; Mroczenski-Wildey et al., 1989), while *Salmonella* Typhimurium rough variations are not (Kihlstrom and Edebo, 1976; Kihlstrom and Nilsson, 1977). However, *S. blockley, S. weltevreden* and *S. amsterdam* have been determined as typical serovars discovered in broilers, levels and dog breeder parent stock respectively and *Salmonella* has been recognized in egg from layers, according to a Thai review (Mead et al., 1999).

*Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis are the most frequently isolated serovars from food-borne outbreaks throughout the world (Horikstad et al., 2002). According to the antigenic profile of *Salmonella* species they show different disease syndromes and host specificity. Therefore, it is necessary and important to discriminate *Salmonella* serovars from each other in order to ensure that each pathogen and epidemiology is correctly recognized (Lim et al., 2003).

*Salmonella* isolation by conventional culture methods, are based on non-selective pre-enrichment followed by selective enrichment and plating on selective and differential agars. Suspected colonies are then confirmed by biochemical and serological methods (Van Kessel et al., 2003). Generally, these techniques take longer time, since they give only presumptive results after 3-4 days and definitive results after 5-6 days (Malorny et al., 2003b). Rapid detection methods, such as DNA or RNA probing, immuno-detection methods and nucleic acid hybridization have been developed but they do not have enough sensitivity and specificity (Zhu et al., 1996). Selective enrichment combined with PCR has been applied to the detection of many bacterial pathogens (Schrank et al., 2001) to improve sensitivity and dilution of PCR inhibitory substances (Fluit et al., 1993). *In vitro* amplification of DNA by the PCR method is a powerful tool in microbiological diagnostics (Malorny et al., 2003b). Several genes have been used to detect *Salmonella* in natural environmental samples as well as food and feces samples namely *invA, invE, himA, phoP, ipaB, iroB, lamb* and *fliC* etc. (Malorny et al., 2003a, b; Feder et al., 2001; Bej et al., 1994; Way et al., 1998; Kong et al., 2002; Szabo and Mackey, 1999; Bej et al., 1991; Ilo et al., 1997). The flagellin gene *fliC* encodes the major component of the flagellum in *Salmonella enterica* serovar Typhimurium (Aldridge et al., 2006). Due to high variability of its central region the *fliC* gene has also been used for molecular typing studies on *Salmonella* (Dauga et al., 1998). This structural gene encodes the phase 1 flagellar protein (H1 antigen) and is expressed alternately with the *fliB* gene which encodes the phase 2 flagellar protein (H2 antigen) (MacNab, 1996).

The *invA* gene of *Salmonella* is unique to this genus and has been demonstrated as an appropriate PCR focus on, with prospective analytical programs (Rahn et al., 1992). Boosting of this gene now has been acknowledged as a worldwide conventional method for recognition of *Salmonella* genus (Malorny et al., 2003a). This gene encodes amino acids in the inner tissue layer of bacteria which is accountable for attack to the epithelial tissues of the host (Darvin and Miller, 1999). The present study results suggest that the *invA* and *fliC* genes based multiplex-PCR assay appeared to be useful for detection of *Salmonella sp.* and *Salmonella* Typhimurium serovar from chicken egg samples.
MATERIALS AND METHODS
Collection and enrichment of samples: For the present study 10 local markets of Dhaka city such as Tezgaon poultry farm, New Market Kacha Bazar, Katabon Kacha Bazar, Kawran Bazar, Mohammadpur, Kazipara Mirpur, Azimpur Kacha Bazar, Shewrapara Mirpur, Anandabazar, Matijheel Kacha Bazar were selected for sample collection. The whole egg samples were inoculated in 100 mL sterile buffered peptone water (HI-MEDIA, India) containing beaker. The inoculated buffered peptone water was incubated at 35°C for 24 h. For selective enrichment, 1 mL portions from pre-enriched BPW broth was transferred into two tubes containing 10 mL of Selenite-Cystine Broth (HI-MEDIA, India) and Tetrathionate Broth (HI-MEDIA, India), respectively. The tubes were vortexed well and incubated at 35°C for 24 h.

Biochemical detection of *Salmonella* sp.: Selectively enriched samples from both the Selenite-Cystine and Tetrathionate Broth were streaked onto the surface of Xylose Lysine Deoxycholate (XLD) Agar, *Salmonella-Shigella* (SS) Agar and Bismuth Sulfite Agar (BSA). All microbiological media were obtained from HI-MEDIA, India. The Petri-dishes were incubated at 35°C for 24 h. After incubation, typical or suspicious *salmonella* colonies were confirmed by biochemical tests including Urease test, motility test, gram staining, sugar (lactose, glucose and esculin) fermentation test, MR-VP reaction, TSI test, LIA test, Indole reaction, oxidase reaction and catalase reaction. Biochemically confirmed isolates were then transferred to nutrient agar slant for further analysis (Ahmed *et al.*, 2011).

Chromosomal-DNA extraction: Chromosomal-DNA of the culture was isolated using DNA-boiling method. One milliliter fresh culture (grown at 37°C for 18 h) was transferred to a micro-centrifuge tube and centrifuged for 8 min at 8000 rpm. The supernatant was discarded and the pellet was re-suspended in Phosphate Buffer Saline. Then the suspension was boiled in water bath (100°C) for 10 min and immediately transferred into ice-cube. After cooling down, it was centrifuged at 3000 rpm for 10 min and the supernatant was transferred into a sterile 1.5 mL micro centrifuge tube. This DNA was used for multiplex-PCR (Table 1).

Multiplex-PCR amplification of extracted DNA: The purified DNA was used as template for the multiplex-PCR assay. Two primer pairs were used whose sequences are shown in Table 2. The 139 and 141 primers are specific for the *invA* gene of *Salmonella* sp. (Rahn *et al.*, 1992) and fliC-1 and fliC-2 primers are specific for the *fliC* gene of *Salmonella Typhimurium* (Soumet *et al.*, 1999). Reactions with these primers were carried out in a 25 μL amplification mixture consisting of 2.5 μL 10× PCR buffer (500 mM KCl, 200 mM Tris HCl), 1.25 μL dNTPs (10 mM), 1.5 μL MgCl₂ (50 mM), 0.5 μL of each primer, 0.5 μL of Taq DNA polymerase (fermentase) and 2 μL of extracted DNA (Soumet *et al.*, 1999). Amplification was performed in a gradient thermocycler (Biorad, DNA engine). The cycling conditions were as follows: An initial incubation at 95°C for 5 min, followed

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>GC%</th>
<th>MW</th>
<th>Target gene</th>
<th>Amplicon fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>139</td>
<td>GTC AAA TTA TCG CCA GGT TGC GGC AA</td>
<td>50.0</td>
<td>7995</td>
<td><em>invA</em></td>
<td>284</td>
</tr>
<tr>
<td>141</td>
<td>TGC TGC CAG CCT CAA AGG AAC C</td>
<td>54.5</td>
<td>6673</td>
<td></td>
<td></td>
</tr>
<tr>
<td>flic-1</td>
<td>CGG TGG TGC CCA GGT TGC TAA T</td>
<td>54.5</td>
<td>6797</td>
<td><em>fliC</em></td>
<td>559</td>
</tr>
<tr>
<td>flic-2</td>
<td>ACT CTT GCT GGC GGC GGT ACT T</td>
<td>43.8</td>
<td>4945</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
by 35 cycles of denaturation at 94°C for 60 sec, annealing at 56°C for 30 sec, elongation at 72°C for 30 sec and final extension period for 10 min at 72°C. Amplified products were electrophoresed in 2% agarose gel and a 50 bp DNA ladder (Promega) was used as a size reference.

**Agarose gel electrophoresis and gel documentation of the PCR product:** When the PCR amplification completed, 15 µL PCR products and 2 µL blue juice (invitrogen) were mixed and loaded on 2% agarose gel. Fifty bp DNA ladder (Promega) was used to determine the size of the PCR product. The buffer used for the gel preparation and electrophoresis was 1 times TBE solution and the running condition was 60 V for 2 h. After electrophoresis, the gel was stained with ethidium bromide solution (0.5 µg mL⁻¹) and washed with distilled water for 15 min each. Gel was visualized using a gel documentation system (Alpha Innotech).

**RESULT AND DISCUSSION**

All *Salmonella* isolates recovered from egg samples showed similar colony characteristics on BS agar, XLD agar and SS agar. The morphological characteristics of all isolates were same i.e., Gram negative, short rod and motile. All isolates fermented glucose producing acid and gas but did not ferment lactose and esculin. They were negative to Voges Proskauer test, indole test and oxidase test but all isolates showed positive result to methyl red test, catalase test with the exception of a few isolate.

On the basis of biochemical tests a total of 24 out of 200 (12%) samples found positive for *Salmonella* sp. Multiplex-PCR assay was done for all these 24 isolates using 139 and 141 primers that amplify a 284 bp sequence of the invA gene and fliC-1 and fliC-2 primers that amplify a 559 bp sequence of the fliC gene (Fig. 1). Among these isolates, eleven (isolate- 218, 238, 63S, 81S, 106S, 118S, 119S, 122S, 128S, 149S and 150Y) were found positive for gene invA confirmed *Salmonella* sp. and four (isolate-18S, 53Y, 96S, 109S) harboured fliC gene represented *Salmonella* Typhimurium, only the isolate 74Y was found positive for both invA and fliC genes (Table 2). Although all these 24 isolates were confirmed as *Salmonella* spp by biochemical tests but only 16 isolates out of 24 were positive in multiplex-PCR assay (Fig. 2). In a previous study the prevalence of *Salmonella* spp in egg samples in Bangladesh was found 8% while the detection method was biochemical (Ahmed *et al*., 2011).

![Fig. 1: Multiplex-PCR assay of all 24 biochemically positive isolates using two sets of primers. The 284 bp amplified product from invA gene specific for Salmonella sp. and 559 bp from fliC gene specific for Salmonella Typhimurium. Lanes 1, 3, 7, 10, 11, 16, 18, 19, 20, 21, 22, 23 show amplification of invA, lane 8 shows both invA and fliC amplified, lanes 12, 17, 20 show amplification of fliC. M Lanes contain 50 bp DNA ladder](attachment:image.png)
The predominant serotypes differ in different regions but *Salmonella* Typhimurium is not as prevalent as other serotypes (Uyttenbogaardt et al., 1998; Soumet et al., 1999; Joys, 1985) and our results also showed a very low prevalence of this serotype after detection using multiplex-PCR (4 out of 24 samples). Oliveira et al. (2002) reported that the multiplex-PCR assay using *invA* gene for detection of *Salmonella* and *fliC* gene for identification of *Salmonella* Typhimurium from poultry related samples was 100% specific.
In this study we processed 200 samples from 10 different markets of Dhaka city and the results determine the lower prevalence of Salmonella sp. and even lower in case of Salmonella Typhimurium in chicken eggs in Dhaka city. The results of this study highlight the usefulness of the multiplex-PCR for concurrent and rapid detection of Salmonella sp. and Salmonella Typhimurium from chicken eggs. The multiplex-PCR based detection can be an alternative method for detection of Salmonella sp. and Salmonella Typhimurium but more optimization of the PCR techniques are required for higher sensitivity and specificity.

REFERENCES
CDC, 1998. Standardized molecular sub typing of food borne bacterial pathogens by pulsed field gel electrophoresis. The National Molecular Sub typing Network for Food borne Disease Surveillance, Centers for Disease Control and Prevention (CDC), Atlanta.


