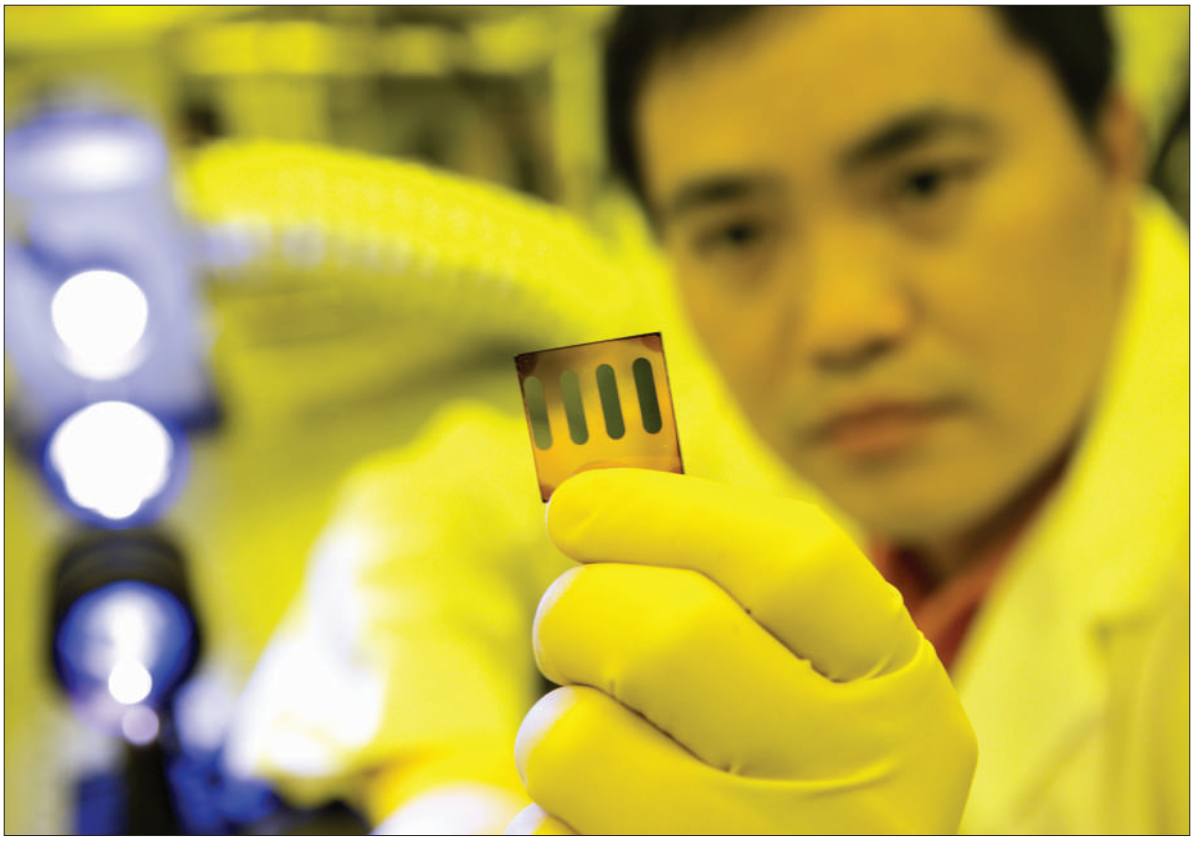

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Performance Analysis of Multiplex-PCR based Detection of *Salmonella* sp. and *Salmonella* Typhimurium in Chicken Egg Samples

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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 *fliC* 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 *fliC* 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 (Herikstad *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.*, 1993; Kong *et al.*, 2002; Szabo and Mackey, 1999; Bej *et al.*, 1991; Itoh *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 *fljB* 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 (Darwin 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 10x PCR buffer (500 mM KCl, 200 mM Tris HCl), 1.25 μL dNTPs (10 mM), 1.5 μL $MgCl_2$ (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 1: Primers used in the multiplex-PCR

Primer	Sequence (5' -3')	GC%	MW	Target gene	Amplicon fragment (bp)
139	GTG AAA TTA TCG CCA CGT TCG GGC AA	50.0	7995	*InvA*	284
141	TCA TCG CAC CGT CAA AGG AAC C	54.5	6673		
fliC-1	CGG TGT TGC CCA GGT TGG TAA T	54.5	6797	*fliC*	559
fliC-2	ACT CTT GCT GGC GGT GCG ACT T	43.8	4945		

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- 21S, 39S, 63S, 81S, 106S, 116S, 119S, 122S, 128S, 149S and 159Y) 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).

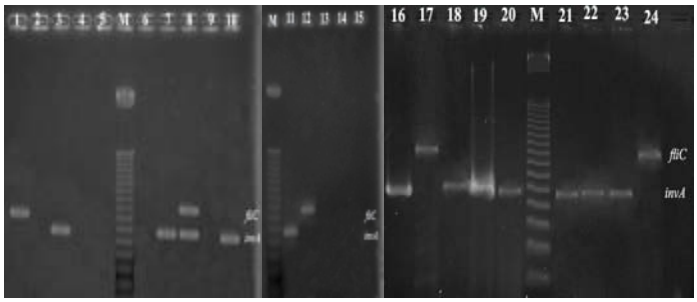

Fig. 1: Multiplex-PCR assay of all 24 biochemically positive isolates using two sets of primers. The 284 bp amplified product from *invA* gene specific for *Salmonella* sp. and 559 bp from *fliC* gene specific for *Salmonella* Typhimurium. Lanes 1, 3, 7, 10, 11, 16, 18, 19, 20, 21, 22, 23 show amplification of *invA*, lane 8 shows both *invA* and *fli*C amplified, lanes 12, 17, 20 show amplification of *fliC*. M Lanes contain 50 bp DNA ladder

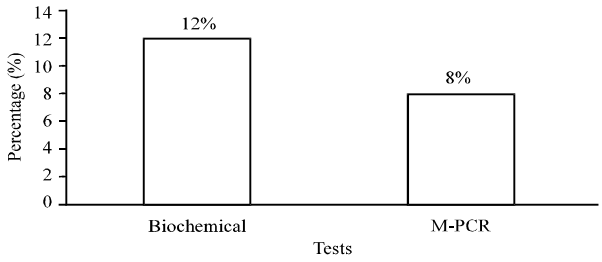

Fig. 2: Percentage of detection of *Salmonella* by both biochemical tests and multiplex-PCR (M-PCR) tests

Table 2: Comparison between biochemical detection and multiplex-PCR detection of isolates

	Detected isolate		
Isolate	Biochemical method	Multiplex-PCR assay	Gene amplified
2S	*Salmonella* sp.	ND	ND
18S	*Salmonella* sp.	*Salmonella* Typhimurium	*fliC*
19S	*Salmonella* sp.	ND	ND
20S	*Salmonella* sp.	ND	ND
21S	*Salmonella* sp.	*Salmonella*	*invA*
22S	*Salmonella* sp.	ND	ND
23S	*Salmonella* sp.	ND	ND
25Y	*Salmonella* sp.	ND	ND
28Y	*Salmonella* sp.	ND	ND
39S	*Salmonella* sp.	*Salmonella*	*invA*
41S	*Salmonella* sp.	ND	ND
53Y	*Salmonella* sp.	*Salmonella* Typhimurium	*fliC*
63S	*Salmonella* sp.	*Salmonella*	*invA*
74Y	*Salmonella* sp.	*Salmonella* Typhimurium	*fliC, invA*
81S	*Salmonella* sp.	*Salmonella*	*invA*
96S	*Salmonella* sp.	*Salmonella* Typhimurium	*fliC*
106S	*Salmonella* sp.	*Salmonella*	*invA*
109S	*Salmonella* sp.	*Salmonella* Typhimurium	*fliC*
116S	*Salmonella* sp.	*Salmonella*	*invA*
119S	*Salmonella* sp.	*Salmonella*	*invA*
122S	*Salmonella* sp.	*Salmonella*	*invA*
128S	*Salmonella* sp.	*Salmonella*	*invA*
149S	*Salmonella* sp.	*Salmonella*	*invA*
159Y	*Salmonella* sp.	*Salmonella*	*invA*

ND: Not detected

The predominant serotypes differ in different regions but *Salmonella* Typhimurium is not as prevalent as other serotypes (Uyttendaele *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.

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