

Molecular Screening of Virulence Genes from *Salmonella enterica* Isolated from Commercial Food Stuffs

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The present research work was carried out for the screening of virulence genes associated with the *Salmonella enterica* isolated from commercial food stuffs by polymerase chain reaction (PCR). A total of 134 samples of commercial food stuffs constituting of raw meats of poultry, pork, beef, raw eggs, dairy and bakery products were purchased from the departmental stores, supermarkets and local butcher shops of Salem, Erode and Coimbatore districts of Tamil Nadu, India. Samples were aseptically processed for the isolation of *S. enterica* through broth enrichment methods. PCR was performed with various virulence genes specific primers of *S. enterica*. Microbiological investigations resulted in *Salmonella* isolates in 35 (26.11%) samples. In PCR, invasive gene (*invA*; 244bp), *Salmonella* enterotoxin gene (*stn*; 617bp), plasmid encoded fimbriae (*pefA*; 700bp), *Salmonella* Enteritidis fimbriae (*sefC*; 1103bp) and *Salmonella* plasmid virulence C gene (*spvC*; 571bp) were detected in 100%, 100%, 51.42%, 25.71% and 42.85% isolates respectively. Present study suggested that *invA* and *stn* virulence genes are much conserved in *S. enterica* isolated from commercial food stuffs and could be used independently as a gene marker for the rapid detection of the virulent strains of *S. enterica*. The prevalence of *spvC* gene is restricted into the isolates of a few definite sources. The result emphasized the risk of transferring these zoonotic organisms to human via food chain is impending danger for the mankind.

Key words: *Salmonella enterica*, Commercial food stuffs, PCR, Virulence.

The genus *Salmonella* is an enteric, gram negative, rod shaped bacteria grouped in the family of Enterobacteriaceae and one of the most important food-borne pathogens associated with a hyperendemic diarrhoeal disease called

Salmonellosis around the world affecting both man and animal alike (Prakash *et al.*, 2005). Salmonellosis is a fatal disease and is spread to human being from eating (orally) of improperly cooked foods such as meat, eggs, unpasteurized milk, dairy products, bakery products and also by direct contact with faeces/diarrhoea from infected animals (Holt *et al.*, 1994).

Salmonella have several sub species among them *Salmonella enterica* subspecies *enterica* (subspecies 1) is responsible for 99.5% of food borne illness in humans and animals

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(Pignato *et al.*, 1998). *S. enterica* includes several virulence genes which encode products that assist the organisms to express its virulence in the host. Among the virulence genes, *inv*, *sef* and *pef* are considered for adhesion and invasion of the pathogen in the host system and *spv* gene for systemic disease state in the host cells. While *stn* virulence gene codes for enterotoxin production *sop* and *pip* genes are associated actual expressions of host pathogenic processes (Murugkar *et al.*, 2003). Hence, accurate and systematic method must be adopted for the screening of virulence genes from *S. enterica* isolates originated from the infected samples.

Commercial food products mainly constituted by poultry and poultry based products have been implicated as a major reservoir of *Salmonella* infections in humans which are predominantly considered under public health status and economic value (Salehi *et al.*, 2005). Therefore, the prevention of *Salmonella* infection via commercial food products should be given primary importance by the food processing industries (Mahé *et al.*, 2008). Present study was undertaken to isolate *S. enterica* from the commercial food stuffs and to screen its virulence genes present within the isolates by PCR for epidemiological study.

MATERIAL AND METHODS

Sample details

A total of 134 commercial food stuffs samples including raw meats of poultry meat(32), pork meat(18), beef meat(18), raw eggs(25), dairy products(26), and bakery products(15) were collected from randomly selected departmental stores, supermarkets and local butcher shops in Salem, Erode and Coimbatore districts, Tamil Nadu, India. The food stuffs were purchased in regular consumer bags and immediately transferred to the laboratory for microbial investigation.

Isolation of *Salmonella enterica* serovars

Food samples (1g) were placed in 10 ml of buffered peptone water (Hi-Media, Mumbai) as pre-enrichment media, and incubated at 37°C for 18 h. The incubated broth samples were enriched by transferring 0.1 ml of the broth into 1 ml tetrathionate (TT) broth (Hi-Media, Mumbai), incubated at 37°C for 24 hr. The cultured broths

were streaked onto xylose lysine deoxycholate agar (Hi-Media, Mumbai) and brilliant green agar (Hi-Media, Mumbai), incubated at 37°C for 24 hour. Suspected colonies of *Salmonella* were purified and identified by motility test, Gram and flagellar staining, indole test, methyl red test, Voges-Proskauer test, citrate test (growth on Simmon's citrate agar), urease test, gelatin hydrolysis, H₂S production, acid and gas production test from glucose, mannitol, maltose, sorbitol, adonitol, sucrose, salicin, and lactose.

Screening of virulence genes by duplex and single step PCR

Suspected bacteria were screened for the detection of virulence encoding genes like the invasion gene (*invA*), *Salmonella* plasmid virulence gene (*spvC*), plasmid encoded fimbriae (*pefA*), *Salmonella* Enteritidis fimbriae (*sefC*) and *Salmonella* enterotoxin gene (*stn*) by PCR. In this study, two sets of duplex PCR and one set of single step PCR were performed. The first set of dPCR was used for the amplification of *invA* and *spvC* genes, while, second set of dPCR performed for the detection of *pefA* and *sefC* genes. In single step PCR, amplification was carried out for the *stn* gene. The specific forward and reverse primer pairs (Table 1) were commercially synthesized (Eurofins Genomics India Pvt. Ltd., Bangalore). *S. enterica* serovar Typhimurium (MTCC 98) and *Aeromonas hydrophila* (MTCC 646) strains were used as positive and negative controls respectively.

Preparation of template DNA

Bacterial colonies were freshly grown in nutrient agar plates, suspended in 150µl of sterile distilled water in a micro centrifuge tube, gently vortexed and boiled for 10 min in a water bath. Micro tubes were then centrifuged at 10000 rpm for 5min at 4°C. Top supernatant were carefully collected and used as a source of template DNA.

PCR conditions

In every case, the PCR amplifications was carried out in 25µl reaction volume in Thermal Cycler (Eppendorf, Germany) containing 12.5µl of 2 × PCR master mix (Promega, USA) containing 4mM magnesium chloride, 0.4mM of deoxynucleotide triphosphates (dNTPs), 0.5U of *Taq* DNA polymerase, 150mM tris-hydrochloric acid, pH 8.5 (Promega, USA), 2.5µl of template DNA and a specific concentration of each forward and reverse primer pairs (Table 1).

Table 1. Details of virulence gene specific primers used in PCR

Virulence genes	Oligonucleotides (5' to 3')	Concentration (µM)	Fragment size (bp)	Reference
<i>invA</i>	For:acagtgcctgttacgacctgaat Rev:agacgactggtactgatcgataat	1	244	Chiu and Ou, (1996)
<i>spvC</i>	For:actcctgccaaccaaatgcgga Rev:tgtctcttgcatttgcaccatca		571	
<i>pefA</i>	For:tgtttccgggctgtgct Rev:cagggcatttgcattcttcc	0.5	700	Murugkar <i>et al.</i> , (2003)
<i>sefC</i>	For:gcaaaaccaatgcgactgta Rev:cccaccagaaacattcatccc		1103	
<i>stn</i>	For:ttgtgctctatcactggcaacc Rev:attcgtaacccgctctcttcc	1	617	

Table 2. Isolation and virulence gene detection of *S. enterica* isolates

Source of sample collection	No of samples collected from various geographical locations	No of <i>Salmonella</i> isolated from various geographical locations	No of <i>Salmonella</i> isolates / of sample tested (%)	No of isolates positive for virulence genes by PCR				
				<i>invA</i>	<i>spvC</i>	<i>pefA</i>	<i>sefC</i>	<i>stn</i>
Poultry meat	12 ^b	5 ^b	12/32 (37.5)	12	6	8	1	12
	10 ^a	2 ^a						
	10 ^c	5 ^c						
Pork meat	7 ^a	1 ^a	5/18 (27.77)	5	1	3	1	5
	6 ^b	2 ^b						
	5 ^c	2 ^c						
Beef meat	8 ^c	2 ^c	6/18 (33.33)	6	2	4	1	6
	6 ^b	3 ^b						
	4 ^a	1 ^a						
Raw eggs	10 ^c	3 ^c	6/25 (24.00)	6	6	1	5	6
	7 ^a	1 ^a						
	8 ^b	2 ^b						
Dairy products	10 ^c	3 ^c	5/26 (19.23)	5	0	2	1	5
	9 ^b	1 ^b						
	7 ^a	1 ^a						
Bakery products	6 ^c	1 ^c	1/16 (6.25)	1	0	0	0	1
	6 ^b	0 ^b						
	3 ^a	0 ^a						
Total (%)	134	35	35/134 (26.11)	35 (100)	15 (42.85)	18 (51.42)	9 (25.71)	35 (100)
	[38(28.35) ^a , 47(35.07) ^b , 49(36.56) ^c]	[6(17.14) ^a , 13(37.14) ^b , 16(45.71) ^c]						

a: Salem, b: Erode, c: Coimbatore

In each set, PCR had an initial denaturation for 4 min at 94°C and a final extension for 7 min at 72°C. The amplification cycles for the first set of dPCR undergone denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and

extension at 72°C for 2 min respectively. The second set of dPCR was run for the denaturation at 94°C for 55 sec, annealing at 55°C for 55 sec and extension at 72°C for 55 sec respectively. The single step PCR was exclusively run for the denaturation

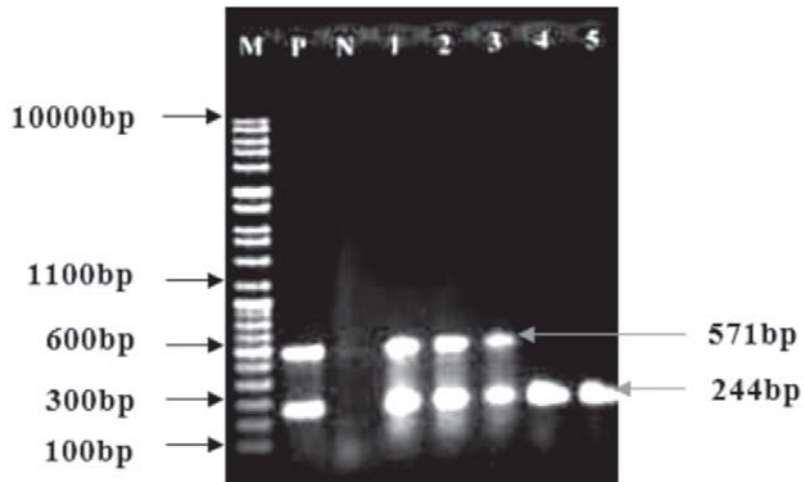


Fig 1. Detection of *invA* (244bp) and *spvC* (571bp) genes of *S. enterica* by duplex PCR
 Lane M: High range DNA ruler (GeNei, India)
 Lane P: Positive control [*S. enterica* serovar Typhimurium (MTCC 733)]
 Lane N: Negative control [*A. hydrophila* (MTCC 646)]
 Lanes 1-3: Field isolates positive for *invA* and *spvC* genes
 Lanes 4-5: Field isolates positive for *invA* gene

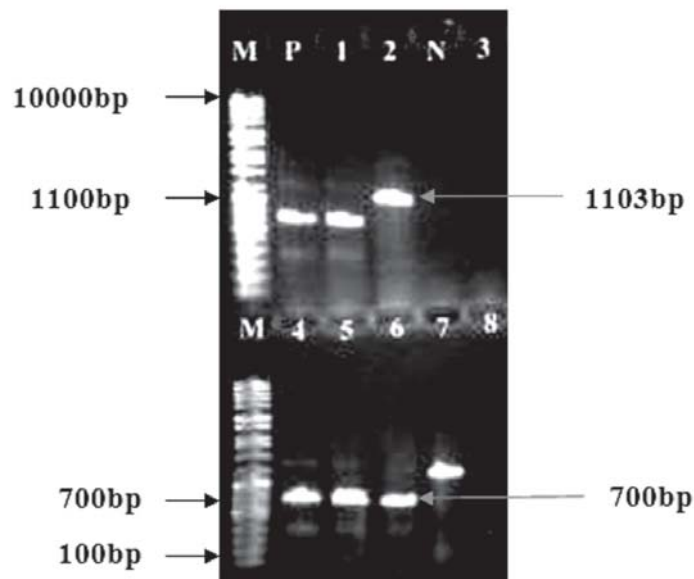


Fig 2. Detection of *pefA* (700bp) and *sefC* (1103bp) genes of *S. enterica* by duplex PCR
 Lane M: High range DNA ruler (GeNei, India)
 Lane P: Positive control [*S. enterica* serovar Typhimurium (MTCC 733)]
 Lane N: Negative control [*A. hydrophila* (MTCC 646)]
 Lanes 1, 4, 5 & 6: Field isolates positive for *pefA* gene
 Lanes 2 & 7: Field isolates positive for *sefC* gene
 Lanes 3 & 8: Field isolates negative for virulence genes

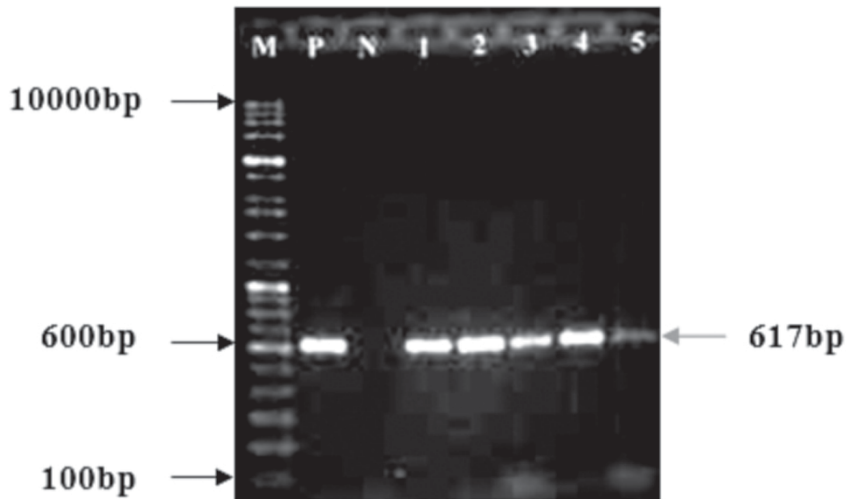


Fig 3. Detection of *stn* (617bp) gene of *S. enterica* by single step PCR
 Lane M: High range DNA ruler (GeNei, India)
 Lane P: Positive control [*S. enterica* serovar Typhimurium (MTCC 733)]
 Lane N: Negative control [*A. hydrophila* (MTCC 646)]
 Lanes 1-5: Field isolates positive for virulence genes

at 94°C for 60 sec, annealing at 59°C for 60 sec and extension at 72°C for 60 sec respectively.

Agarose gel electrophoresis

The PCR amplicons (5µl) were electrophoresed in 1.2 % agarose gel in TAE (Tris-acetate-EDTA, pH 8) buffer, stained with ethidium bromide (0.4 µg / ml) and observed under Gel Documentation system (Universal Hood, BIORAD, Italy).

RESULTS AND DISCUSSION

In the present study, prevalence of *Salmonella enterica* was made possible by broth enrichment method of isolation from various sources and geographical locations. A total of 35 (26.11%) *S. enterica* were isolated from 134 commercial food samples (Table 2). Among these 35 isolates, 12 (37.5%), 6 (33.33%), 5 (27.77%), 6 (24.00%), 5 (19.23%) and 1 (6.25%) were isolated from poultry meat, beef meat, pork meat, raw eggs, dairy products and bakery products respectively. The isolated *Salmonella* bacteria were found to be motile, Gram negative, flagellated, indole negative, methyl red positive, Voges-Proskauer negative, citrate positive, urease negative, gelatin hydrolysis negative, positive for H₂S production

and acid and gas production from glucose, mannitol, maltose, and sorbitol. No acid production was observed from adonitol, sucrose, salicin, and lactose. Similar biochemical characteristics from *S. enterica* serovar Typhi isolated from humans were observed (Ganeshkumar *et al.*, 2010). Microbiological investigation showed that the isolation frequencies of *S. enterica* were higher in meat samples than raw eggs, dairy and bakery products. This also implied that poultry, beef and pork meat, raw eggs and dairy products provide suitable environment for *S. enterica* to grow at an increasingly higher rate irrespective of its geographical locations. In other study, the prevalence of *Salmonella* in chickens and beef cattle was found to be 32.5% (39/120) and 4.6% (11/240) respectively and among the cattle isolates, the prevalence rate was found significantly higher in fasted cattle (7.46%), than in non-fasted cattle (0.94%) (Abouzeed *et al.*, 2000). Wegener *et al.*, (1997) also detected high frequencies of *Salmonella* isolates from poultry, poultry products, cattle and dairy products and suggested that these food products are the major sources of *Salmonella* infections in humans.

The virulence of *Salmonella* is linked to a number of virulence factors and in this study;

few of them were screened by PCR. In the first set of dPCR which run for *invA* (244bp fragment) and *spvC* (571bp fragment) genes resulted positive amplifications in 35 (100%) and 15 (42.85%) isolates respectively (Table 2, Fig. 1.), similarly, the second set which run for *pefA* (700bp fragment) and *sefC* (1103bp fragment) genes showed positive amplifications in 18 (51.42%) and 9 (25.71%) isolates respectively (Table 2, Fig. 2.). The single step PCR which performed for *stn* (617bp fragment) gene resulted positive amplifications in all the 35 (100%) isolates (Table 2, Fig. 3.). The detection of *invA*, *spvC*, *pefA*, *sefC* and *stn* genes from the isolates of *S. enterica* originated from food, faecal or clinical samples by PCR were investigated by various researchers (Swamy *et al.*, 1996 & Chiu and Ou, 1996 & Shome *et al.*, 2006).

In the present study, both *invA* and *spvC* genes were positive for 15 (42.85%) isolates, while only 20 (57.14%) isolates were positive for *invA* gene alone. In a study conducted with 38 isolates of *Salmonella* serovars, all 38 were found positive for *invA* gene whereas, 16 isolates produced additional *spvC* genes (Chiu and Ou, 1996). Swamy *et al.*, (1996) reported that out of 245 *Salmonella* isolates, *invA* genes were amplified by all and 37 (15.1%) isolates mostly originated from egg contents or from the egg production environment harbored *spvC* genes in addition to *invA* genes. Montenegro *et al.*, (1991) found that frequencies of detection of *spvC* gene vary from 48 to 87% within the *S. enterica* isolated from faecal, food or environmental samples. The detection frequency of *spvC* gene in this study was found to be 42.85% from the isolates originated from only meat and raw egg environments. This suggested that the prevalence of *Salmonella* virulence plasmids is restricted within the isolates of some defined origin of sources.

In this study, none of the *S. enterica* isolates amplified *pefA* and *sefC* genes together in dPCR, all the *pefA* and *sefC* genes were independently detected. Like *invA* gene, *stn* gene also demonstrated 100% amplification among the isolates. The PCR result suggested that the distribution of *pefC* gene was more in meat samples compared to the dairy products and raw eggs, but, *sefC* gene was amplified higher in isolates originated from raw eggs. There was only one isolate

originated from the bakery products and was found negative for *pefA*, *sefC* and *spvC* genes. In a comparable study for the distribution of *stn*, *pef* and *sef* genes among 95 *Salmonella* isolates originated from poultry, pig, cattle and humans samples, all 95 (100%) were found to harbour *stn* gene, whereas, 85 (89.47%) and 36 (37.89%) isolates were positive for *pef* and *sef* genes respectively (Murugkar *et al.*, 2003). In another study carried out with 23 *S. enterica* isolates originated from faecal samples of pigs, and poultry, all were found positive for *stn* genes, whereas, 11 and 4 isolates were positive for *pefA* and *sefC* genes respectively (Shome *et al.*, 2006).

The 100% detection rate of *inv* and *stn* genes by PCR suggested that these two genes are conserved among *S. enterica* isolates (De Oliveira *et al.*, 2003 & Murugkar *et al.*, 2003). The virulence genes screening by PCR also suggested that both *inv* and *stn* genes are predominant virulence genes necessary for the serovars of *S. enterica* to express virulence in the host. The PCR assays employing with either *invA* or *stn* virulence genes appeared to be rapid, sensitive, and specific means to identify *S. enterica* isolates from commercial food stuffs.

Although, commercial food samples in this study were collected randomly from Coimbatore, Erode and Salem Districts of Tamil Nadu, the number of *Salmonella* isolated from each geographical location varied. From Table 2, it is very clear that the isolation rate of *Salmonella* were much higher in the samples obtained from Coimbatore 16 (45.71%), than Erode 13 (37.14%) and Salem 6 (17.14%). This study gives a preliminary idea about the distribution of *S. enterica* isolates in the three neighbouring districts located in the Southwest of the Chennai (state capital), Tamil Nadu. The persistence of *Salmonella* in the commercial food environment was an important characteristic in its prevalence. The PCR detection of *invA*, *spvC*, *pefA*, *sefC* and *stn* genes among the isolates of *S. enterica* also signified the higher risk of food-borne infections caused by these zoonotic bacteria into humans. Therefore, aseptic storage practice and proper sterilization of food products needs to be improved in the departmental stores, supermarkets and even in local butcher shops in Tamil Nadu India.

CONCLUSION

Present study suggested that PCR is a sensitive, reliable tool for the rapid detection of *S. enterica* serovars from the commercial food products. High prevalence of virulent strains of *S. enterica* from poultry, beef and pork meat products, raw eggs and dairy products clearly indicated that consumption of improperly cooked commercial food stuffs are unsafe and may result in serious food-borne illness in humans. Further, molecular analysis need to be carried out to achieve the accurate distribution of the virulence genes among the various serovars of *S. enterica* and their protein products in the molecular pathogenesis in humans as well as animals which will help us to develop preventive measures against the deadly pathogens.

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