Detection and Virulence Gene Characterization of *Salmonella* Isolates from Fish by Conventional and Molecular Methods

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**ABSTRACT**

*Salmonella* isolates (n=9) used in this study were isolated from 216 fish samples collected from retail market of Anand city, Gujarat, India. *Salmonella* serovars from fish were isolated and identified as per BAM USFDA method and serotyped at National *Salmonella* and *Escherichia* Centre (NSEC), Kasauli (Himachal Pradesh, India). Virulence factors of *Salmonella* serovars have been characterized with *invA*, *spvR*, *spvC* and *stn* genes using the polymerase chain reaction (PCR). All *Salmonella* serovars possessed 284 bp *invA* gene and 260 bp enterotoxin (*stn*) gene but lacks *spvR* or *spvC* gene. This showed that all *Salmonella* serovars isolated from fish were capable of producing gastroenteric illness to humans but were unable to produce systemic infection. The *invA* and *stn* genes might be the specific targets for *Salmonella* identification.

**Keywords:** Enterotoxins, gastroenteritis, PCR, *Salmonella*, virulence

**Introduction**

*Salmonellae* are leading food borne pathogens that cause common enteric infections in man and animals. The changes that have occurred in the food industry over the last two decades viz., centralized production and large-scale distribution, have been accompanied by great increase in human salmonellosis incidences (Altekruse et al., 1997). Different genes like *inv*, *spv* and *stn* have been identified as major virulence genes responsible for salmonellosis. *InvA* gene has been found responsible for invasiveness in the gut epithelial tissue of human and animals and *stn* gene causes entero-toxic effect to epithelial cells leading to enteric disorder. The *spv* genes play role in systemic infection by enabling *Salmonella* survival in macrophages. The chromosomally located invasion gene *invA* thought to trigger the invasion of salmonellae into cultured epithelial cells (Hitchcock et al., 1986; Asten and Dijk, 2005). While an operon (*spvRABCD*), containing five genes, is present on plasmids commonly associated with some serotypes, the *spv* genes possibly have the ability to increase severity of enteritis and allow infection and persistence at extra-intestinal sites. The SpvR protein is a LysR-like transcription factor and an essential positive regulator of both the *spvR* gene and the *spvABCD* operon (Pullinger et al., 1989 and Guiney et al., 1995) while *spvC* is virulence related gene on plasmid required for survival within host cell (Chiu and Ou, 1996). Enterotoxin gene (*stn*) plays a role in causing gastroenteritis by producing enterotoxin.

The purpose of our study was to assess the potential virulence of *Salmonella* isolates from fish by detecting the presence of the *invA*, *spvR*, *spvC* and *stn* virulence genes using Polymerase Chain Reaction (PCR).

**Materials and Methods**

**Isolation and identification of *Salmonella***

*Salmonella* isolates (n=9) used in the present study were isolated from 216 fish samples collected from retail market during the period of June to December, 2010 in Anand city (Gujarat), India. Types of fishes included Surmai (*Scomberomorus commerson*), Jira (*Cypselurus comatus*), Tarali (*Sardinella longiceps*), Mrigal (*Cirrhinus cirrhosis*), Mangur (*Clarias batrachus*) and Halwa (*Apolectus niger*). All the *Salmonella* isolates from fish were identified as per standard protocol described in Bacteriological Analytical Manual (BAM), U.S. Food and Drug Administration (USFDA) (Andrews and Hammack, 2001). Culture media and antibiotic supplements used in the study were procured from Hi-Media Laboratories,

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Mumbai. Briefly, 25 g of each type of sample was thoroughly triturated in a sterile mortar and pestle and transferred to 225 ml pre-enrichment in lactose broth. Subsequently 0.1 and 1 ml of pre-enriched sample was transferred to enrichment broths i.e. Rappaport-Vassiliadis soybean meal (RVSM) broth and tetrahydrionate broth (TTB), respectively, followed by 24 h of incubation at 42°C and 37°C, respectively. The enrichments were streaked on brilliant green (BG) agar and xylose lysine deoxycholate (XLD) agar and incubated for 24 hour at 37°C. Typical pink colonies with or without black centers on XLD and colourless or pink or opaque-white colonies often surrounded by pink or red zone on BGA were picked and streaked further on BSA for purification. The pure cultures were streaked on triple sugar iron (TSI) agar and incubated at 37°C for 18 hours. Those producing typical reaction on TSI (red slant and yellow butt with H₂S production-blackening of agar) were further characterized by biochemical tests viz., catalase, oxidase, decarboxylation of lysine using lysine iron agar, production of indole, methyl red test, Voges Proskauer test, utilization of citrate and urease test. The colonies identified as Salmonella were preserved in 20 per cent glycerol broth at -20°C for further characterization.

Serotyping of Salmonella isolates

All biochemically typical Salmonella isolates were serotyped at National Salmonella and Escherichia Centre (NSEC), Central Research Institute (CRI), Kasauli (Himachal Pradesh), India.

DNA extraction and polymerase chain reaction

The DNA of isolates of Salmonella was separated by bacterial lysis method (Medici et al., 2003). A loopful of culture was dissolved into 100 µl of sterilized DNase and RNase-free milliQ water (Millipore, USA) and samples were heated at 95°C for 10 min, cell debris were removed by centrifugation at 10,000 rpm for 7 min and 3 µl of the supernatant was used as a DNA template in PCR reaction mixture. PCR was performed with four sets of primer pairs specific for the invasion gene invA, spoR gene, spoC gene and stn gene as shown in table 1. PCR amplifications were performed in a final volume of 25 µl containing DNA template (3 µl), 2x PCR master mix (MBI Fermentas) (12.5 µl), 10 pmol/µl of each primer (MWG-Biotech AG, Germany) (1 µl) and 7.5 µl nuclease free water. Amplification for invA gene was carried out as previously described by Kumar et al. (2008) with minor modifications. The reaction conditions involved initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s. A final extension of 5 min at 72°C was employed. The amplification for spoR, spoC and stn gene was carried out similarly by employing standardized annealing temperature (Table 1.) Amplification products were separated by electrophoreoses on 2% agarose gel stained with 0.5 µg/ml of ethidium bromide with a 100 bp DNA ladder as molecular weight marker (Gene RulerTM, 100 bp DNA ladder, MBI Fermentas).

Results and Discussion

In the present study, out of 216 fish samples collected only 9 (4.16%) were found positive for Salmonella spp. The distribution of recovered isolate among fish species were 3 from Surmai (Scomberomorus commerson), 2 from Mangur (Clarias batrachus) and one each from Jira (cypselurus comatus), Tarali (Sardinella longiceps), Mrigal (Cirrhinus cirrhosis), Halwa (Apoloectus niger). All the isolates of Salmonella revealed characteristic morphological features on the selective plates as described earlier. On preliminary biochemical characterization they revealed characteristic IMViC pattern. All nine Salmonella isolates (six of which belonged to serovar Weltevreden and remaining three were nontypable) contained the invasion gene invA, other studies having reported similar results (Swamy et al., 1996; Oliveira et al., 2003; Bhatta et al., 2007 and Maria Araque, 2009) which was expected since the invA is an invasion gene conserved among Salmonella serotypes. Kumar et al. (2014) also reported that all the Salmonella isolates showed positive reaction in invA LAMP assay, whereas non-Salmonella isolates did not amplify the target gene.

Table 1. Primer pairs used for virulence gene characterization of Salmonella isolates

<table>
<thead>
<tr>
<th>Primer pair target</th>
<th>Primer sequence (5’→3’)</th>
<th>Annealing (°C)</th>
<th>Length (base pair)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| invA               | F: GTG AAA TTA TCG CCA CGT TCG GGC AA  
R: TCA TCG CAC CGT CAA AGG AAC C | 63 | 284 | Kumar et al. (2008) |
| spoC               | F: ACT CCT TGC ACA ACC AAA TGC GGA  
R: TGT CTT CTG CAT TTC GGC ACC ACC ATC A | 63 | 571 | Oliveira et al. (2003) |
| spoR               | F: CAG GTT CCT TCA GTA TCA CA  
R: TTT GGC CGG AAA TGG TCA GT | 57 | 310 | Pasmans et al. (2005) |
| stn                | F: CTT TGG TCG TAA AAT AAG GCG  
R: TGC CCA AAG CAG AGA GAT TC | 55 | 260 | Makino et al. (1999) |
Fig. 1. Agarose gel showing PCR amplified product (284 bp) for invA gene in Salmonella isolates.

Fig. 2. Agarose gel showing PCR Amplified product (571 bp) for spvC gene in Salmonella isolates. Lane 1,2,3 (Negative) amplification results, S= standard, L=100 bp ladder

Fig. 3. Agarose gel showing PCR amplified product (310 bp) for spvR gene in Salmonella isolates. Lane 1,2,3 (Negative) amplification results. S= standard, L=100 bp ladder

Fig. 4. Agarose gel showing PCR amplified product (260 bp) for stn gene in Salmonella isolates.

Similar to invA gene, all isolates produced 260 bp DNA fragment specific for stn gene which was in agreement with Dinjus et al. (1997), Rahman (1999), Soto et al. (2006) and Rao et al. (2014). Thus all the Salmonella isolates were found highly invasive (invA positive) and enterotoxigenic (stn positive) (Fig. 1 and 4).

The spvR and spvC virulence genes were not detected in any of the Salmonella strains, as shown in Fig. 2 and 3, respectively. This shows that the strains lacked the plasmid borne virulence characters and thus the inability to cause the systemic infection. A further conclusion of our study is that the invA and stn genes can be used as specific targets for detection of Salmonella as they are conserved among the salmonellae irrespective of serotype and plasmid borne genes (spv) are not specific targets for the same.

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References


