Isolation and Molecular Characterization of Virulence-Associated Genes of *Salmonella* from Buffalo Meat Samples in Western Region of India

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ABSTRACT

*Salmonella* is found worldwide in cattle and is considered among the most important animal related zoonotic disease. *Salmonella* is a leading cause of foodborne illness viz. enteric illness. In the present investigation 16 (10.66%) of isolates of *Salmonella* spp. were found from 150 raw buffalo meat and offals viz. liver, lung, muscle, intestine and ground beef (30 each), collected from the retail meat market of Anand, (Gujarat) India. All the 16 isolates of *Salmonella* spp. were screened for the presence or absence of virulence associated genes by using the polymerase chain reaction (PCR). All the *Salmonella* isolates were subjected to serotyping and all the isolates of *Salmonella* spp. revealed the presence of *invA*, *str* and *fmaA* genes. Fourteen (87.5%) of the isolates showed the presence of *spvR* gene and 8 (50%) had the *spvC* gene. Serotyping of *Salmonella* isolates revealed that *Salmonella enterica* serovar Typhimurium was the only detected serovar. The presence of invasiveness and enterotoxicity of *salmonella* isolates in buffalo meat and offals showed their ability to cause systemic infections and which may appear to be threat to the public and a health concern.

Keywords: *Salmonella*; Buffalo Meat; Zoonotic Disease; Serotyping; PCR.

INTRODUCTION

Food borne diseases caused by nontyphoid salmonella represent an important public health problem worldwide. Underdeveloped and technologically developed countries are struggling with foodborne outbreaks which result in illness, death and large economic losses. In underdeveloped countries there are more than one billion cases of gastroenteritis and up to 5 million deaths annually (1). In the United States alone, an estimated 1.4 million cases of salmonellosis is thought to occur annually, of which about 200,000 cases are reported to the CDC. Salmonellosis is more common in the warmer months of the year and accounts for 30% (about 400 yearly) of deaths resulting from foodborne illnesses in USA and the most commonly isolated serovar are *Typhimurium* and *Enteritidis* (2, 3). A variety of foods have been implicated as vehicles transmitting salmonellosis to humans, including poultry, beef, pork, eggs, milk, cheese, fish, shellfish, fresh fruit and juice, and vegetables (4).

Young children, the elderly and patients with chronic illnesses or immunocompromised are particularly susceptible to salmonellosis. Infective dose of *Salmonella* bacterium required overcoming host defenses and cause disease varies, usually about $10^6$ to $10^8$ CFU. It has been reported that lower numbers of *S. enterica* may be capable of causing outbreaks, especially in cases involving foods with a high fat content (5, 6).

There are three syndromes observed following the consumption of *Salmonella* as salmonellosis, typhoid fever and paratyphoid fever. Although different *Salmonella* serovars may vary in their degree of virulence, it is presumed that all are pathogenic *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis have been implicated, in particular,
as causes of human salmonellosis. However, in South-East Asia, *S. enterica* serovar Weltevreden has been reported as a frequent and increasing cause of human infection (7).

Different virulence genes such as *inv, stn, fim* and *spv* have been identified as major genes responsible for virulence factors in *Salmonella*. The invasion (*invA*) gene found to be present in *Salmonella* pathogenicity islands (SPI) and responsible for invasion in the gut epithelial tissue of human and animals, whereas, *stn* gene causes enterotoxic effect to epithelial cells, leading to enteric disorder (8, 9).

There are only few reports of incidence of *Salmonella* in retail raw buffalo meat and offals so the present study was undertaken with the aim to isolate and identify *Salmonella* from raw buffalo meat and offals sold in retail market from Anand, (Gujarat) India. The recovered *Salmonella* isolates were subjected to biochemical characterization and detection of virulence genes by PCR.

**MATERIAL AND METHOD**

Samples

Altogether 150 raw buffalo meat and offals comprising of ground beef (keema), muscle, intestine, liver and lung (30 samples each) were collected into sterilized polyethylene bags in the morning hours as they are offered for public sale from different retail buffalo meat shops located in Anand, (Gujarat) India and transported to the P.G. research laboratory of the Veterinary Public Health & Epidemiology department in an icebox for further processing and microbiological analysis.

<table>
<thead>
<tr>
<th>Primer pair target</th>
<th>Primer sequence (5’→3’)*</th>
<th>Annealing a</th>
<th>Length b</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>invA</em></td>
<td>F:GTG AAA TTA TCG CCA CGT TCG GGCAA R:TCA TCG CAC CGT CAA AGG AAC C</td>
<td>64°C</td>
<td>284 bp</td>
<td>11</td>
</tr>
<tr>
<td><em>stn</em></td>
<td>F:CTT TGG TCG TAA AAT AAG GCG R:TGC CCA AAG CAG AGA GAT TC</td>
<td>55°C</td>
<td>260 bp</td>
<td>12</td>
</tr>
<tr>
<td><em>fimA</em></td>
<td>F:CCT TTC TCC ATC GTC CTG AA R:TGG TGT TAT CTG CCT GAC CA</td>
<td>56°C</td>
<td>85 bp</td>
<td>13</td>
</tr>
<tr>
<td><em>spvR</em></td>
<td>F:CAG GTT CCT TCA GTA TCG CA R:TTT GGC CGG AAA TGG TCA GT</td>
<td>57°C</td>
<td>310 bp</td>
<td>14</td>
</tr>
<tr>
<td><em>spvC</em></td>
<td>F: ACT CCT TGG ACA ACC AAA TGC GGA R:TGG CTT CGT CAT TTC GCC ACC ATC A</td>
<td>63°C</td>
<td>571 bp</td>
<td>15</td>
</tr>
</tbody>
</table>

* The Forward primer (F) listed first followed by the Reverse primer (R).

a Annealing temperature in °C.

b Length of amplification product in base pairs
Serotyping of Salmonella isolates

Cultures identified as *Salmonella* 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 prepared by bacterial lysis method. A loopful of culture was taken in a micro-centrifuge tube in 100µl of sterilized DNAse and RNAse-free Milli-Q water (Millipore, USA). After being vortexed the samples were heated at 95°C for 10 min; cell debris was removed by centrifugation and 3µl of the supernatant was used as a DNA template in PCR reaction mixture. All the *Salmonella* isolates were first screened for the presence or absence of virulence associated genes by using the PCR protocols separately standardized for the detection of different genes. The PCR was standardized for the detection of five genes viz. *invA*, *str*, *fimA*, *spvR* and *spvC* as per methodology with suitable modifications given in Table 1. Standardization of PCR was done by using standard strain of *S. Typhimurium* obtained from NICED, Kolkata.

All the PCR primers and molecular reagents were procured from Bangalore Genei, Bangalore, India. The reaction was carried out in thin walled PCR tubes in 25 µl reaction volume with different concentration of reactants under different annealing temperatures and cycling conditions.

Briefly, the reaction mixture was optimized to contain 12.5µl 2X PCR master mixes (MBI Fermentas), 10 pmol of each forward and reverse primer, 7.5µl nuclease free distilled water and 3µl of DNA template. The reaction was performed in the thermal cycler with pre-heated lid (Lid temperature 105°C). Reaction conditions employed were: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 58°C for 1.5 min, and 72°C for 1.5 min. A final extension of 7 min at 72°C was employed. Reaction condition was the same as earlier described except for the annealing temperature of respective primer used as shown in Table 1. On completion of the reaction the amplified products were analysed on agarose gel electrophoresis through 2% agarose gel stained with 5 µg/ml of ethidium bromide with a 100 bp DNA ladder as molecular weight markerand visualized under UV light.

RESULT AND DISCUSSION

In the present study out of 150 raw buffalo meat and offal samples, 16 (10.66%) were found positive for *Salmonella* spp. All the sixteen isolates revealed characteristic features of *Salmonella* producing pink colonies with or without black centers from Xylose Lysine Deoxycholate (XLD) and colourless or pink or opaque-white colonies often surrounded by pink or red zone from Brilliant Green (BG) agar. On preliminary biochemical characterization they revealed characteristic IMViC pattern as shown in Table 2.

Organ wise, a higher prevalence of (26.66%) was observed among the samples of ground beef tested followed by (20%) in intestine and (6.66%) in muscle, whereas no samples of lung and liver was found positive for *Salmonella* spp. Earlier studies indicated variable prevalence ranging from 0% (16) to 28.3% (17) in beef. In addition to this reported prevalence rates of 3.4%, 5.8%, 12%, 14.4%, 8.5%,...
Salmonella from Buffalo Meat and Offals in India

4.2%, 16.9% and 8.5%, respectively from beef samples (18, 19, 20, 21, 22, 23, 24, 25).

In the present investigation all the 16 isolates of Salmonella isolated from 150 retail market buffalo meat and offals were subjected to PCR assays for the detection of virulence-associated genes. All the 16 isolates of Salmonella yielded desired amplified product of approximately 284 bp, 260 bp and 85 bp similar to that of reference strain of Salmonella using the primer pairs for invA, stn and fimA respectively (Figure 1, 2 and 3). The virulence profile of Salmonella isolates is shown in Table 3.

The findings in this study are in agreement with reports on detection of these genes in Salmonella enteritidis (26, 27, 28, 29 and 30). Detection of these genes may indicate the virulence potential of the isolates.

Moreover, among 16 isolates of Salmonella 14 (87.5%) were positive for spvR gene yielding the desired amplified product of approximately 310 bp (Figure 4) and 8 (50%) were positive for spvC gene yielding the desired amplified product of approximately 571 bp (Figure 5). In contrast to our results higher prevalences of spvR gene and spvC gene was reported (31, 32) while (33, 34 and 35) reported a lower prevalence of these genes.

In the present study all the sixteen isolates belonged to serovar Typhimurium. The results of present investigation are in agreement with reports of (19, 20, 21, 25 and 36) for

<table>
<thead>
<tr>
<th>Test</th>
<th>Typical Salmonella reaction</th>
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<tbody>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Indole test</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red (MR) test</td>
<td>+</td>
</tr>
<tr>
<td>Voges Proskauer (VP)</td>
<td>-</td>
</tr>
<tr>
<td>Citrate test</td>
<td>+</td>
</tr>
<tr>
<td>Urease test</td>
<td>-</td>
</tr>
<tr>
<td>Lysine decarboxylation</td>
<td>+</td>
</tr>
<tr>
<td>Triple Sugar Iron agar slant</td>
<td>Red/Yellow/H₂S⁺</td>
</tr>
</tbody>
</table>

Table 2: Specific identification of Salmonella isolates

<table>
<thead>
<tr>
<th>Source of Sample</th>
<th>No. of sample analyzed</th>
<th>Total No. of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Beef</td>
<td>30</td>
<td>8 (26.66%)</td>
</tr>
<tr>
<td>Muscle</td>
<td>30</td>
<td>6 (20%)</td>
</tr>
<tr>
<td>Intestine</td>
<td>30</td>
<td>2 (6.66%)</td>
</tr>
<tr>
<td>Liver</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>16 (10.66%)</td>
</tr>
</tbody>
</table>

Table 3: The Organwise prevalence and virulence profile of Salmonella isolates

Figure 3: Agarose gel showing PCR Amplified product (85 bp) for fimA gene in Salmonella isolates

Figure 4: Agarose gel showing PCR Amplified product (310 bp) for spvR gene in Salmonella isolates

Figure 5: Agarose gel showing PCR Amplified product (571 bp) for spvC gene in Salmonella isolates
presence of \textit{S. Typhimurium} from buffalo meat samples. In conclusion, our study highlights the microbiological hazard of \textit{Salmonella} contamination of raw buffalo meat and offal products during storage and improper handling or cooking of buffalo meat which can lead to human food-borne illness. Hence, epidemiological data are needed to inform public health authorities about the nature and magnitude of the problem and to monitor trends over time.

**ACKNOWLEDGEMENTS**

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**REFERENCE**


