ISOLATION AND CHARACTERIZATION OF SALMONELLA SPP. FROM BUFFALO MEAT SAMPLES

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ABSTRACT

Salmonella is found worldwide in cattle and is considered as a most important animal related zoonotic disease. Salmonella is a leading cause of foodborne illness viz. enteric illness. In the present investigation 16 (10.66%) isolates of Salmonella spp. were isolated from 150 buffalo meat samples viz. liver, lung, muscle, intestine and ground beef (30 each), collected from the retail meat market of Anand city, Gujarat. All the 16 isolates of Salmonella spp. isolated from the 150 retail market buffalo meat samples 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 antibiotic susceptibility testing and serotyping. All the 16 isolates of Salmonella spp. revealed presence of the invA, stn and fimA genes. While, 14 (87.5%) of the isolates showed presence of the spvR gene and eight (50%) had the spvC gene. Serotyping of Salmonella isolates revealed that Salmonella enterica serovars Typhimurium was the only detected serovar. The presence of invasiveness and enterotoxicity of salmonella isolates in buffalo meat samples shows their ability to cause systemic infections and appeared to be a threat to public health.

Keywords: Salmonella, buffalo meat, zoonotic disease, Serotyping, PCR

INTRODUCTION

Foodborne diseases caused by nontyphoid salmonella represent an important public health problem worldwide. Underdeveloped and technologically developed countries are struggling with foodborne disease 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 (Gould and Russell, 2003). 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 (Lynch et al., 2006). Salmonella infection accounts for 30% (about 400 yearly) of deaths resulting from foodborne illnesses in the USA and the most commonly isolated serovars are Typhimurium and Enteritidis (CDC, 2007). Salmonellosis is more common in the warmer months of the year (CDC, 1995). 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 (Kariuki et al., 2006).

Young children, the elderly and patients with chronic illnesses or immunocompromised systems

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are particularly susceptible to salmonellosis (Bell and Kyriakides, 2002). 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 (Bell and Kyriakides, 2002).

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. enteric* serovars Typhimurium and *S. enteric* serovars Enteritidis have been implicated, in particular, as causes of human salmonellosis. However, in South-East Asia, *S. enteric* serovars Weltevreden has been reported as a frequent and increasing cause of human infection (Lunestad et al., 2007). Increasing antimicrobial resistance has become one of the most common concerns in relation to food borne Salmonellae. As more than 90% salmonellosis are food borne, antibiotic-resistant *Salmonella* in food-producing animals has become a focus of the debate on antibiotic use in food animals (Hohmann, 2001; Salyers, 2002; Su et al., 2004).

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 an enterotoxic effect on epithelial cells, leading to enteric disorder (Hitchcock et al., 1986; Asten and Dijk, 2005).

There are only few reports of incidence of *Salmonella* in retail raw buffalo meat so the present study was undertaken with the aim to isolate and identify *Salmonella* from raw buffalo meat sold in retail markets in Anand city and the recovered isolates were subjected to biochemical characterization, in vitro antimicrobial drug resistance pattern and detection of virulence genes associated by PCR.

**MATERIALS AND METHODS**

**Samples**

Altogether 150 buffalo meat samples comprising of ground beef (keema), muscle, intestine, liver and lung (30 each) were collected in sterilized polyethylene bags in the morning hours as they were offered for sale to the public at different retail buffalo beef meat shops located in Anand city, and transported to the P.G. Research Laboratory of the Veterinary Public Health and Epidemiology Department in an icebox for further processing and microbiological analysis.

**Isolation and identification of *Salmonella***

The standard protocol described in the *Bacteriological Analytical Manual* (BAM), U.S. Food and Drug Administration (USFDA), the method of Andrews and Hammack (2001), was adopted for the isolation of *Salmonella* spp. from buffalo meat samples. Briefly, 25 g of each type of sample was thoroughly triturated with 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 225 ml pre-enrichment in lactose broth. Subsequently 0.1 and 1 ml of pre-enriched sample was transferred to enrichment in Rappaport-Vassiliadis Soybean Meal (RVSM) broth and Tetrathionate Broth (TTB), respectively, followed by 24h of incubation at 42 and 37°C, respectively. The enrichments were streaked on Brilliant Green (BG) agar and xylose
lysine deoxycholate (XLD) agar and incubated for 24 h at 37°C. Typical colonies on XLD (pink colonies with or without black centers) and BGA (colourless or pink or opaque-white colonies often surrounded by pink or red zone) 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 h. 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 percent glycerol broth at -20°C for further characterization.

**Antibiotic susceptibility testing**

The antibiotic susceptibility tests were performed as per method described by (Bauer *et al.*, 1966) to find out the antibiotic resistance pattern of all *Salmonella* isolates. Briefly, biochemically confirmed *Salmonella* isolates were grown in Muller-Hinton broth for 6 hr and Muller-Hinton agar plates were seeded with the cultures. Different antibiotic disks like amikacin, ampicillin, cefotaxime, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline and trimethoprim were placed on the inoculated medium. The antibiotic sensitivity plates were incubated at 37°C for 24 h. The standard strain of *Salmonella* (*S. Typhimurium* No.VP81) used in the study were obtained from the National Institute of Cholera and Enteric Disease, Kolkata. The diameter of inhibition zones were measured and compared with the interpretative chart provided by the manufacturer and zones were graded as sensitive, intermediate and resistant.

**Serotyping of *Salmonella* isolates**

Cultures identified as *Salmonella* were serotyped at the 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 the bacterial lysis method. Approximately loopful of culture was taken in microcentrifuge in 100 µl of sterilized DNAse and RNAse-freemilliQwater. The samples were vortexed and then were heated at 95°C for 10 minutes. Cell debris were removed by centrifugation and 3µl of the supernatant was used as a DNA template in the 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*, *stn*, *fimA*, *spvR* and *spvC* following the methodology as described by Kumar *et al.*, 2008; Makino *et al.*, 1999; Naravaneni and Jamil, 2005; Pasmans *et al.*, 2003 and (Oliviera *et al.*, 2003, respectively, with suitable modifications. Standardization of PCR was done by using standard strain of *S. Typhimurium*. PCR was performed with four sets of primer pairs specific for *invA*, *stn*, *fimA*, *spvR* and *spvC* gene as per the details given in Table 1.

The reaction was standardized in thin walled PCR tubes in 25 µl reaction volumes with different concentrations of reactants under different annealing temperatures and cycling conditions. Finally, the reaction mixture was optimized to contain 12.5 µl 2X PCR master mixes, 10 pmol of each forward and reverse primer, 7.5 µl nuclease free distilled water and 3 µl of DNA template.
The reaction was performed in the thermal cycler with pre-heated lid (Lid temp. 105°C). Reaction conditions employed were: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 58°C for 1.5 minutes, and 72°C for 1.5 minutes. A final extension of 7 minutes at 72°C was employed. The reaction conditions were the same as earlier except for the annealing temperature for respective primer used. 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 marker, visualized under UV light and results were noted.

RESULT AND DISCUSSION

Salmonellosis is endemic in nature and responsible for heavy economic losses in India every year. Epidemiological data are needed to inform public health authorities about the nature and magnitude of the problem and to monitor trends over time.

Out of 150 buffalo meat samples, 16 (10.66%) samples were found positive for *Salmonella* spp. All the isolates revealed characteristic features of *Salmonella* producing pink colonies with or without black centers from XLD and colourless or pink or opaque-white colonies often surrounded by pink or red zone from BG agar. On preliminary biochemical characterization they revealed characteristic IMViC pattern (- + - +). Organ wise 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 sample of lung and liver was found positive for *Salmonella* spp. Earlier works indicated variable prevalence ranging from 0 percent (Venkateswaran *et al.*, 1988) to 28.3% (Akoachere *et al.*, 2009) in

<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>
</table>
| invA                | F: GTG AAA TTA TCG CCA CGT TCG GGCAA  
                      | R: TCA TCG CAC CGT CAA AGG AAC C | 64°C | 284bp | Kumar *et al.*, 2008 |
| stn                 | F: CTT TGG TCG TAA AAT AAG GCG  
                      | R: TGC CCA AAG CAG AGA GAT TC | 55°C | 260 bp | Makino *et al.*, 1999 |
| fimA                | F: CCT TTC TCC ATC GTC CTG AA  
                      | R: TGG TGT TAT CTG CCT GAC CA | 56°C | 85 bp | Naravaneni and Jamil, (2005) |
| spvR                | F: CAG GTT CCT TCA GTA TCG CA  
                      | R: TTT GGC CGG AAA TGG TCA GT | 57°C | 310 bp | Pasmans *et al.*, 2003 |
| spvC                | F: ACT CCT TGC ACA ACC AAA TGC GGA  
                      | R: TGT CTT CTG CAT TTC GCC ACC ATC A | 63°C | 571 bp | Oliviera *et al.*, 2003 |

*The forward primer (F) listed first followed by the reverse primer (R).
*aAnnealing temperature in °C.  
bLength of amplification product in base pairs
beef. In addition Alemayehu et al., 2003; Molla et al., 2003; Ejeta et al., 2004; Bosilevac et al., 2009; Soltan et al., 2009; Zewdu and Cornelius, 2009 observed prevalence rates of 3.4 percent, 5.8 percent, 12 percent, 14.4 percent, 8.5 percent, 4.2 percent, 16.9 percent and 8.5 percent, respectively, from beef samples.

In the present investigation all the 16 isolates of *Salmonella* isolated from 150 retail market buffalo meat samples were subjected to PCR assays for the detection of virulence-associated genes. All the 16 isolates of *Salmonella* yielded desired amplified products of approximately 284 bp, 260 bp and 84 bp similar to that of reference strain of *Salmonella* using the primer pairs for *invA*, *stn* and *fimA* respectively. The virulence profile of *Salmonella* isolates is shown in Table 2.

The findings in this study are in agreement with work carried out on detection of these genes in *Salmonella enteritidis* by Galen et al. (1992) and Swamy et al. (1996); Murugkar et al. (2003); Nayak et al. (2004); Skwark et al. (2004); Alphons and Jaap (2005); Soto et al. (2006); Bhatta et al. (2007), Kumar et al. (2008); Madadgar et al. (2008); Minami et al. (2010).

Moreover, among the 16 isolates of *Salmonella*, 14 (87.5%) were positive for the *spvR* gene and eight (50%) were positive for the *spvC* gene. In contrast to our results, higher prevalences of the *spvR* gene and the *spvC* gene were reported by Abouzeed et al. (2000); Oliveira et al. (2003); Geimba et al. (2004); Bacci et al. (2005) and Soto et al. (2006) while Bhatta et al. (2007) and Maria Araque (2009) reported lower prevalences of these genes.

All the sixteen isolates belonged to serovars Typhimurium. Different authors reporting *S. Typhimurium* from beef samples are Basu et al. (1975); Alemayehu et al. (2003); Molla et al. (2003); Ejeta et al. (2004); Bosilevac et al. (2009), Zewdu and Cornelius (2009) and Singh et al. (2010).

The antibiotic susceptibility pattern of positive *Salmonella* isolates from buffalo meat samples is shown in Table 3. The pattern clearly indicated that the overall high percent of *Salmonella* isolates were resistant to ampicillin followed by tetracycline while moderately high percentages of isolates were resistant to Nalidixic acid and Trimethoprim. The findings of the present study similar to the pattern was recorded by Badawy et al. (2004); Miko et al. (2005); Valdezate et al. (2007); Salika et al. (2008); Kumar et al. (2009); Soltan et al. (2009) and Smith et al. (2010). Antimicrobial resistance in the food borne *Salmonella* spp. has been becoming a problem worldwide in recent years. Studies have shown that serotypes of *Salmonella* isolated from humans or from food production or processing facilities are resistant to one or more antibiotics.

In conclusion, our study highlights the adoption of guidelines for the prudent use of antibiotics in food animals and for a reduction in the number of pathogens present on farms and in slaughterhouses. The microbiological hazard of *Salmonella* contamination of raw beef products during storage and improper handling or cooking of beef can lead to human food-borne illness. Hence, there is need to monitor the contamination levels of *Salmonella* as well as other zoonotic pathogens throughout the year to safeguard public health.

ACKNOWLEDGEMENTS

Authors are thankful to Dr. T. Ramamurthy, Deputy Director, Division of Microbiology, National Institute of Cholera and Enteric Diseases
Table 2. The Organwise prevalence and virulence profile of *Salmonella* isolates.

<table>
<thead>
<tr>
<th>Source of Sample</th>
<th>No. of sample analyzed</th>
<th>Total No. of positive samples</th>
<th>invA</th>
<th>stn</th>
<th>fimA</th>
<th>spvR</th>
<th>spvC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Beef</td>
<td>30</td>
<td>8 (26.66%)</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Muscle</td>
<td>30</td>
<td>6 (20%)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Intestine</td>
<td>30</td>
<td>2 (6.66%)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Liver</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>16 (10.66%)</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>14</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3. Antibiotic susceptibility pattern of *Salmonella* isolates from buffalo meat samples.

<table>
<thead>
<tr>
<th>Sr.no</th>
<th>Antibiotic tested (µg)</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amikacin(AK)</td>
<td>14 (87.5%)</td>
<td>2 (12.5%)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Ampicillin (A)</td>
<td>1 (6.25%)</td>
<td>5 (31.25%)</td>
<td>10 (62.5%)</td>
</tr>
<tr>
<td>3</td>
<td>Cefotaxime (CE)</td>
<td>13 (81.25%)</td>
<td>3 (18.75%)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Ceftriaxone (Cl)</td>
<td>16 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Chloramphenicol (C)</td>
<td>16 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Ciprofloxacin (CF)</td>
<td>16 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Gentamicin (G)</td>
<td>15 (93.75%)</td>
<td>1 (6.25%)</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Kanamycin (K)</td>
<td>14 (87.5%)</td>
<td>2 (12.5%)</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Nalidixic acid (NA)</td>
<td>9 (56.25%)</td>
<td>2 (12.5%)</td>
<td>5 (31.25%)</td>
</tr>
<tr>
<td>10</td>
<td>Streptomycin (S)</td>
<td>10 (62.5%)</td>
<td>2 (12.5%)</td>
<td>4 (25%)</td>
</tr>
<tr>
<td>11</td>
<td>Tetracycline (T)</td>
<td>4 (25%)</td>
<td>5 (31.25%)</td>
<td>7 (43.75%)</td>
</tr>
<tr>
<td>12</td>
<td>Trimethoprim (TR)</td>
<td>8 (50%)</td>
<td>3 (18.75%)</td>
<td>5 (31.25%)</td>
</tr>
</tbody>
</table>

S, Sensitive; R, Resistant; I, Intermediate  Buffalo meat samples (n = 150).
Figure 1. Agarose gel showing amplification product of enterotoxin (stn) gene (Approx. 260 bp)
Lane 1-5: Positive sample,
    N: Negative sample,
    S: Standard strain and L: 100 bp DNA ladder.

Figure 2. Agarose gel showing amplification product of invasion (invA) gene (Approx. 284 bp)
Lane 1-4: Positive sample,
    S: Standard strain and L: 100 bp DNA ladder.
Figure 3. Agarose gel showing amplification product of \textit{fimA} (Approx. 85 bp)
Lane 1-5: Positive sample, N: Negative sample,
S: Standard strain and L: 100 bp DNA ladder.

Figure 4. Agarose gel showing amplification product of \textit{spvR} (Approx. 310 bp)
Lane 1-5: Positive sample, N: Negative sample,
S: Standard strain and L: 100 bp DNA ladder.

Figure 5. Agarose gel showing amplification product of \textit{spvC} (Approx. 571 bp)
Lane 1-4 and 8-10: Positive sample, N: Negative sample,
S: Standard strain and L: 100 bp DNA ladder.
(NICED) in Kolkata for assistance in procuring the reference strains and to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India, for serogrouping the *Salmonella* isolates.

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