

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*, *stn* and *fimA* 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.

Isolation and Identification of *Salmonella*

Culture media and antibiotic supplements used in the study were procured from Hi-Media Laboratories, Mumbai. The standard protocol described in *Bacteriological Analytical Manual* (BAM), U.S. Food and Drug Administration (USFDA) method was adopted for the isolation of *Salmonella* spp. from buffalo meat and offals (10). 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 in Rappaport-Vassiliadis Soybean Meal (RVSM) broth and Tetrathionate Broth (TTB), respectively, followed by 24h of incubation at 42°C and 37°C, respectively. The enrichments were streaked on Brilliant Green agar (BGA) and Xylose Lysine Deoxycholate (XLD) agar and incubated for 24 hour 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 Bismuth Sulphite Agar (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.

Table 1: Primer pairs used for virulence characterization of *Salmonella* isolates

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

* 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

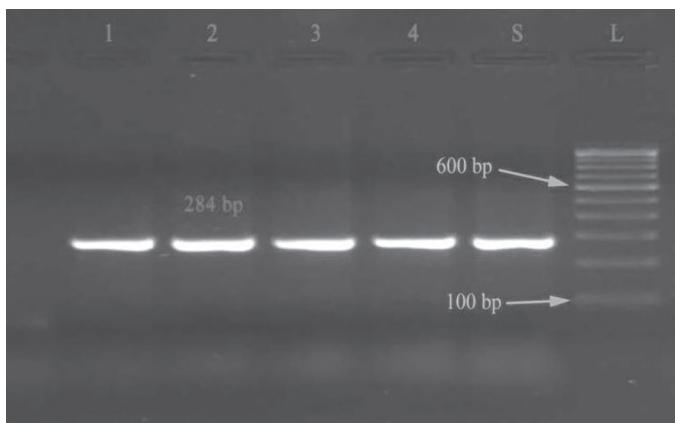


Figure 1: Agarose gel showing PCR Amplified product (284 bp) for *invA* gene in *Salmonella* isolates

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 100 µl 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 3 µl 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*, *stn*, *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 12.5 µl 2X PCR master mixes (MBI Fermentas), 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

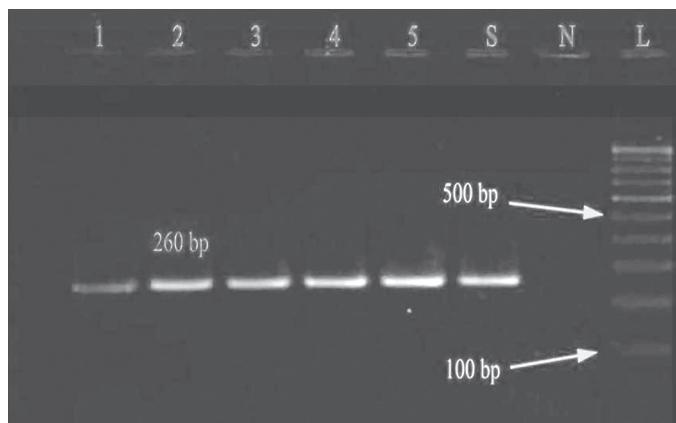


Figure 2: Agarose gel showing PCR Amplified product (260 bp) for *Stn* gene in *Salmonella* isolates

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 marker and 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%,

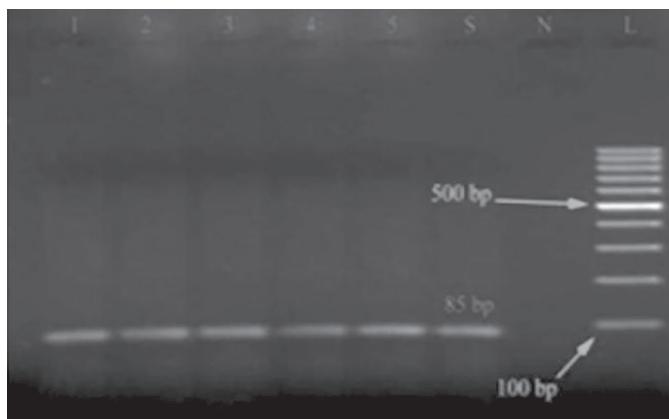


Figure 3: Agarose gel showing PCR Amplified product (85 bp) for *fimbA* gene in *Salmonella* isolates

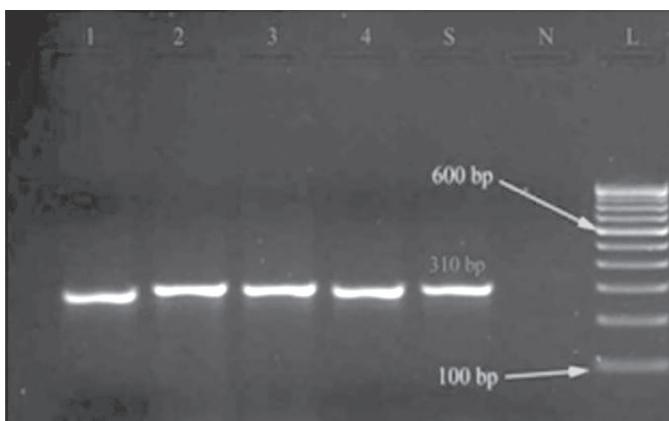


Figure 4: Agarose gel showing PCR Amplified product (310 bp) for *spvR* gene in *Salmonella* isolates

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

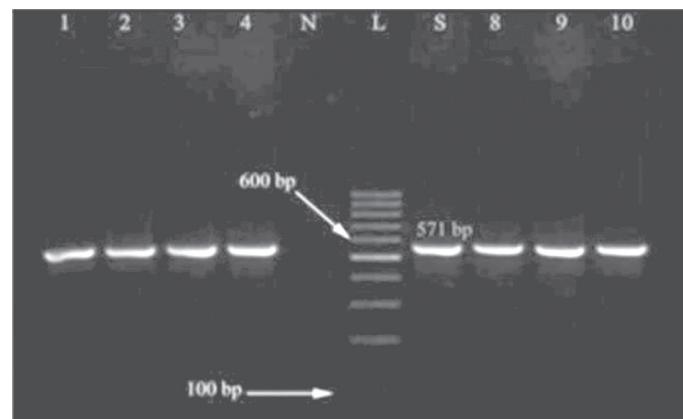


Figure 5: Agarose gel showing PCR Amplified product (571 bp) for *spvC* gene in *Salmonella* isolates

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 2: Specific identification of *Salmonella* isolates-

Test	Typical <i>Salmonella</i> reaction
Catalase	+
Oxidase	-
Indole test	-
Methyl red (MR) test	+
Voges Proskauer (VP)	-
Citrate test	+
Urease test	-
Lysine decarboxylation	+
Triple Sugar Iron agar slant	Red/Yellow/H ₂ S+

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

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

presence of *S. Typhimurium* from buffalo meat samples. In conclusion, our study highlights the microbiological hazard of *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.

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