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Phenotypic and genotypic characterization of *Salmonella* isolated from beef in northern India

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Abstract
Livestock industries are growing tremendously along with the human population. India has rapidly increased its beef exports. The maintenance of quality of food products derived from these animals is on high consideration. In this investigation the prevalence of *Salmonella* species was performed through phenotypic and genotypic characterization in beef samples (n=100) collected from butcher shops locally in Bareilly. A total of 8 isolates (4.0%, n = 8) were identified via. phenotypic assay (H_2S production on TSI, Lysine decarboxylase assay, acid production from lactose and sucrose, IMViC assay). The genotypic characterization was performed using Polymerase Chain Reaction (PCR) assay targeting *inv*A gene (389 bp). Furthermore, antibacterial resistance profile of all *Salmonella* isolates (n=8) was determined against seven antibiotics (ampicillin, cephalexin, ciprofloxacin, nalidixic acid, gentamicin, tetracycline and streptomycin. The antibiotic susceptibility profiles confirmed drug resistance among 50% of *Salmonella* isolates. It was observed that 37.5% of *Salmonella* isolates were resistant to the ampicillin and ciprofloxacin whereas a slight lower resistance of 25.0% against ciprofloxacin and tetracycline was observed. No resistance was observed against nalidixic acid, gentamycin and streptomycin. Overall resistance to multiple drugs (≥ 3) was recorded in 25.0% isolated strains.

Keywords: Beef, *Salmonella* species, Phenotypic and Genotypic characterization

Introduction
Livestock industries are growing tremendously along with the human population (Manyi Loh *et al.*, 2016) [12]. India has rapidly increased its beef exports specifically water buffalo meat, also known as cara beef and narrowly overtaking Brazil as the world's largest beef exporter in 2014. Recent ERS research shows that India's beef exports grew from an average of about 12 percent annually. The rapid expansion in India's beef exports has been driven by three main factors. First, global demand for India's relatively low-cost water buffalo meat is strong, particularly among low- and middle-income countries in Asia and the Middle East. The United States and most other developed-country beef exporters primarily supply higher cost beef products that target higher income markets and consumers. The relatively low price of Indian beef reflects perceived quality differences: it is buffalo rather than cattle meat, it is produced primarily from culled dairy animals, and it cannot meet the stricter sanitary and phytosanitary standards common in more advanced markets (Landes *et al.*, 2016) [11].
The cattle beef has been identified contaminated with variety of life threatening bacterial pathogens including *Salmonella* spp. (Manyi Loh *et al.*, 2016) [12]. *Salmonella* spp are Gram-negative, rod-shaped and non-spore-forming facultative anaerobes. The *salmonella* associated infection is known as Salmonelosis. These are second major cause of foodborne diseases in the world. In the past over one million and twenty-seven thousand cases of foodborne illness are caused by nontyphoidal *Salmonella* spp which resulted in 19,336 hospitalizations and 378 deaths in United State of America (USA) alone. Presently more than 2579 serotypes of *Salmonella* enterica. In India, the most frequently associated serotype among non typhoidal *Salmonella* in chicken meat is *S. typhimurium* (87.8%) (Kiran *et al.*, 2016) [10]. *Salmonella* infections are caused by ingestion of contaminated food or water. The principal clinical syndromes associated with *Salmonella* infection include enteric (typhoid) fever and

gastroenteritis. The characteristic clinical symptoms may include fever, abdominal pain, transient diarrhoea or constipation, and occasionally maculo–papular rashes. The mortality can be 10%–15% in absence of treatment. In contrast, many non–typhoidal *Salmonella* strains, such as *S. enteritidis* and *S. typhimurium*, infect wide range of animal hosts, including cattle. The widespread occurrence of *Salmonella* in natural environment and the intensive husbandry practices used in the meat industries have been a significant problem in public health (Khan *et al*., 2013) [9]. The information on prevalence of *Salmonella* in India is very scattered and no systematic literature is available. Therefore, the present investigation was carried out to study the prevalence through phenotypic and genotypic characterization of *Salmonella* in beef.

Material and Methods

Sample collection

Beef samples (n=100) were collected from local retail shops in Bareilly city, India, as described by Andrews and Hammack, (2003) [1]. Raw beef (100 g each sample) were collected in screw-cap jar containing 100 mL of buffered peptone water (0.1 %) (Hi-media, Pvt. Ltd. Mumbai) aseptically. All samples were transferred to the laboratory on ice away from direct sunlight. Microbiological examinations began at within the 72h from day of sampling.

Phenotypic Characterization of *Salmonella*

Isolation and identification of *Salmonella* by cultural methods

Isolation of *Salmonella* spp. from beef samples was performed according to USFDA Bacteriological Analytical Manual method described by Andrews and Hammack, (1998) [1] with few modifications. Briefly, beef (25 g) sample was homogenized for 2 min in 225 mL of Lactose broth and incubated at room temperature for 1 hour. The pH was adjusted to 6.8 ± 0.2 and incubated mixture at 35ºC for 24 h. one ml mixture was transferred to the 10 ml Tetrathionate (TT) broth and incubated at 35ºC for 48 h. After 48 h of enrichment in Tetrathionate broth, inoculum was streaked onto Hektoen enteric (HE) agar and incubated at 35ºC for 48 h. Plates were checked for growth of typical colonies after 48h.

The confirmation of presumptive *Salmonella* colonies growing on Hektoen enteric (HE) agar was performed by examination of the morphological features of typical *Salmonella* growth on HE agar that appears as black colonies surrounded by a narrow green or green-blue margin or halo with varying radii, with the edge of the colony either entire or irregular. Further confirmations of presumptive *Salmonella* isolates, was carried out by means of biochemical tests determined by H_2S production on TSI, Lysine decarboxylase assay, acid production from lactose and sucrose, IMViC assay as described by Andrews and Hammack, (1998) [1].

Determination of antibiotic resistance

Antibiotic resistance profiles of *Salmonella* isolates were determined by disc diffusion method as described by Giacomelli *et al.* (2014) [7]. Seven commercial antibiotic sensitivity discs (Hi Media) were used: Ampicillin (10 µg), Cephalexin (100 µg), Ciprofloxacin (10 µg), Nalidixic acid (30 µg), Gentamicin (10 µg), Tetracycline (25 µg) and Streptomycin (10 µg). Briefly, 0.1 mL of test bacterial suspension (10^8cfu mL^{-1}) was prepared in Brain Heart Infusion (BHI) broth (Pronadisa, Spain) and was spread onto Muller Hinton (MH) agar (Hi-Media) plates. The plates were incubated at 37 °C for 18 h. Inhibition bacterial growth diameter around the antibiotic disc was measured.

Genotypic Characterization of *Salmonella*

Detection of *Salmonella*

For PCR based detection of *Salmonella spp.* DNA was extracted directly from 1.0mL aliquots taken from *Salmonella* enrichment cultures. The *inv*A gene based detection for the identification of *Salmonella spp.* by PCR was performed as described by Cocolin *et al.* (1997) [4] with some modifications. The DNA extraction method adopted as described by Sambrook and Russel (2001) [15], using DNA extraction kit (Genei, Banglore, India). The primers 5'-GCTGCGCGCGAACGGCGAAG-3'and 5'-TCCCGGCAGAGTTCCCATT-3' used in this study were synthesized by Agile Life Sciences, Mumbai, India. The PCR reaction for amplification of *inv*A gene (389 bp) was optimized as follows: 5 µl of 10X PCR buffer (20 mM Tris HCl, pH 8.0 at 25ºC, 100 mM KCl, 0.1 M EDTA, 1 mM DTT, 50% glycerol, 0.5% tween 20 and 0.5% Nonidet-P40), 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 1 µl of each primer (10 pmol), 1 U (unit) of Taq DNA polymerase (Biogene, USA), 5 µl of DNA as template and final volume made upto 50 µl using nuclease free water. The cycling conditions were including initial denaturation step at 95ºC for 5 min followed by 35 subsequent cycles of heat denaturation of 95ºC for 1 min, annealing at 54ºC for 1 min, and extension at 72ºC for 1 min. A final extension was performed at 72ºC for 5 min to complete the synthesis of all strands. PCR products were separated in 1.5% agarose gel electrophoresis, stained by ethidium bromide (Sigma-Aldrich, USA), and visualized under UV light. The specificity of PCR assay was determined using reference/standard cultures of *Salmonella typhimurium* (MTCC 98), *Salmonella enteritidis* (E 2094) and *E. coli* (MTCC 443), as positive and negative controls.

Results and Discussion

The Phenotypic characters assessed in this study for the identification of the *Salmonella* are depicted in Table 1. Using cultural methods, the phenotypic characterization of *Salmonella spp* was performed and *Salmonella* colonies were detected in 8 beef samples. Therefore a total of 8 isolates (4.0%, n = 8) were identified. All these isolates were further detected by PCR targeting the *inv*A gene (389 bp) for genotypic characterization. After selective enrichment these isolates were found positive in PCR assay. The detail of detection of *Salmonella isolated from* beef samples using phenotypic and genotypic method is listed in Table 2. Our investigation can be supported by other observation available. Kalambhe *et al.* (2016) [8] reported total of 04 isolates of *Salmonella* spp. from cattle and buffalo meat with a prevalence of 4.0%. Arslan and Eyi, (2010) [3] reported a slight higher prevalence of 16.0% in ground beef in Western Turkey. Shafini *et al.* (2017) [16] reported 15.4% beef samples contaminated with *Salmonella* spp. The prevalence of *Salmonella spp.* in beef sample collected in our investigation is presented in Table 3.

The antibacterial resistance profiles of all the 8 isolates of *Salmonella spp* were determined against seven antibiotics (Table 4). The antibiotic susceptibility profiles confirmed drug resistance among 50% of *Salmonella* isolates. It was observed that 37.5% of *Salmonella* isolates were resistant to the ampicillin and ciprofloxacin whereas a slight lower

resistance of 25.0% against ciprofloxacin and tetracycline was observed. No resistance was observed against nalidixic acid, gentamycin and streptomycin. Overall resistance to multiple drugs (≥ 3) was recorded in 25.0% isolated strains. The antimicrobial resistance was observed in our study was in close agreement of other studies (Dallal *et al*., 2010; Dallal *et al.*, 2014; Naik *et al.*, 2015) [5,6,13].

Conclusion

Livestock animals including beef are recognized as source of high-quality foods. The causes of the increased demand for animal products include not only population growth but also increased prosperity, especially in the developing world (Reynolds *et al.*, 2015) [14]. Therefore the maintenance of quality of food products derived from these animals is on high consideration. In the present investigation *Salmonella* in beef samples was found low but a considerable prevalence that can pose a major threat for spread of Salmonellosis in humans. The special concern requires on beef processing because of poor hygienic conditions prevailing in the areas of sampling which ultimately favours contamination of *Salmonella*. The isolates also demonstrated the varied spectrum of antimicrobial resistance, including several multiple drug resistance phenotypes in *Salmonella* isolates from beef. This highlights the need for continued surveillance of zoonotic foodborne pathogens including antimicrobial-resistant variants throughout the beef retail shops.

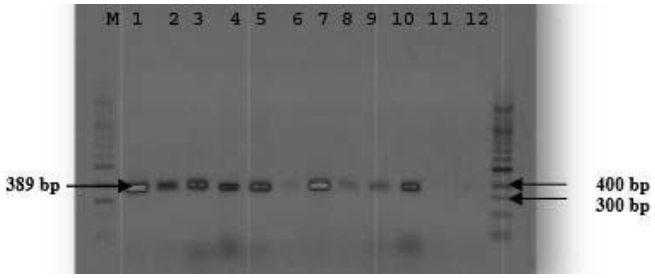

Fig 1: *inv*A gene (389 bp) based identification of *Salmonella* isolates by PCR

Lane M- DNA ladder (100 bp), Lane 1-8- isolates positive for *Salmonella*, Lane 9 - *Salmonella typhimurium* (MTCC 98), *Lane 10- Salmonella enteritidis* (E 2094) Lane 11- *E. coli* (MTCC 443) (negative control), Lane 12- BS- (negative control).

Table 1: Phenotypic Characterization of Salmonella spp. isolated from beef

Isolate number	Biochemical assay								
	Morphology (gram Stain)	Citrate utilization	H2S production on TSI	Urease production	Indole production	Methyl red	Voges Proskauer	Lactose fermentation	Sucrose fermentation
BS-7	rods (-)	+	+	-	-	+	-	-	-
BS-24	rods (-)	+	+	-	-	+	-	-	-
RSB-24	rods (-)	+	+	-	-	+	-	-	-
RSB-25	rods (-)	+	+	-	-	+	-	-	-
RSB-27	rods (-)	+	+	-	-	+	-	-	-
RSB-44	rods (-)	+	+	-	-	+	-	-	-
RSB-45	rods (-)	+	+	-	-	+	-	-	-
RSB-63	rods (-)	+	+	-	-	+	-	-	-

Table 2: Detail of detection of Salmonella spp by cultural and PCR methods in various beef samples.

Isolate number	Detection by cultural methods	Detection by PCR methods
BS-7	+	+
BS-24	+	+
RSB-24	+	+
RSB-25	+	+
RSB-27	+	+
RSB-44	+	+
RSB-45	+	+
RSB-63	+	+

Table 3: Prevalence of Salmonella spp. in beef samples investigated in this study.

Number of beef samples	Prevalence by cultural methods (%)	Prevalence by PCR methods (%)
100	08 (8.0%)	08 (8.0%)

Table 4: Antibiotic Resistance Profile of *Salmonella spp.* Isolated from beef.

Isolate number	Antibiotic resistance Profile	Number of antibiotics against isolate found resistant
BS-7, BS-24, RSB-44, RSB-45	-*	-
RSB-24	Am, Cp, T	3
RSB-25	Am, Cp	2
RSB-27	Cp, T	2
RSB-63	Cf, Cf, Am	3

-* either sensitive/intermediate to antibiotics used.
Am- Ampicillin, Cp- Cephalexin, Cf- Ciprofloxacin, T- Tetracycline

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