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Isolation, Characterization, and Antimicrobial Resistance Profiling of *Salmonella* from Humans, Animals, and Poultry in Pantnagar Region

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ABSTRACT: Salmonellosis is an important disease of public health concern, causing substantial morbidity and mortality in man and is one of the leading cause of food borne disease. Food of animal origin is considered as the important source of salmonellosis. The present study was carried out to isolate and characterize *Salmonella* from humans, animals, and poultry. A total of 722 fecal samples originating from humans (232), cattle (140), poultry (304), pigs (38), and goats (8) were collected and screened by standard culturing methods. Out of which 13 (1.8%) samples revealed the presence of characteristic *Salmonella* colonies, 11 (3.6%) from poultry, one from human (0.43%), and one from cattle (0.71%). The isolates were confirmed by biochemical and serological tests and by serotyping. In a multiplex polymerase chain reaction-based screening of *invA* and *stn* virulence genes, all the isolates revealed their presence. Antimicrobial resistance profiling revealed a high level of resistance to multiple antibiotics, including erythromycin and cephalexin.

Keywords: *Salmonella*, virulence gene, *invA*, *stn*, antimicrobial sensitivity.

INTRODUCTION

Salmonellosis caused by *Salmonella* is an important disease of public health concern, causing substantial morbidity and mortality in man and animals worldwide (Mir *et al.*, 2010; Pui *et al.*, 2011; Das *et al.*, 2012; Kumar *et al.*, 2019). *Salmonella* are Gram-negative bacilli belonging to the family *Enterobacteriaceae*, capable of infecting a wide range of warm and cold-blooded animals. Animals act as important reservoirs of the organism except for *Salmonella* Typhi and *Salmonella* Paratyphi A. Animals contaminate food and water, thereby transmitting the infection to humans (Pui *et al.*, 2011). Approximately 95% of *Salmonella* infections are foodborne, particularly from poultry and its products (Westrell *et al.*, 2009; EFSA, 2010; Kumar *et al.*, 2019). In humans, infection with *Salmonella* results in enteric fever, gastroenteritis, bacteremia, and other complications. It was reported to cause 93.8 million cases of gastroenteritis worldwide annually, with 155,000 deaths (WHO, 2019). In addition, there is also an increasing trend in the incidence of antimicrobial resistance and the emergence of multidrug-resistant *Salmonella* isolates worldwide, further complicating the disease condition (Mir *et al.*, 2010; Parvathi *et al.*, 2011; Putturu *et al.*, 2013; Patra *et al.*, 2021). The virulence of *Salmonella* like colonization, invasiveness, enterotoxin production, intracellular survivability, tissue damage, and others are encoded by virulence genes like *invA, stn, sef, pef, sop,* and *pip* (Mir *et al.*, 2010; Das *et al.*, 2012; Zou *et al.*, 2012). Understanding the virulence gene profile of *Salmonella* isolates helps in better understanding of its nature, ability to cause disease, and public health significance. Numerous studies have been carried out in India to study the prevalence of *Salmonella* in different parts of the country, and the disease is endemic in nature (Kownhar *et al.*, 2007; Mir *et al.*, 2010; Das *et al.*, 2012). The objective of the present study was to isolate and characterize *Salmonella* from fecal samples of humans, animals, and poultry and to study their antimicrobial sensitivity pattern.

MATERIALS AND METHODS

Sample collection. A total of 722 fecal samples were collected from humans (232), cattle (140), poultry (304), pigs (38), and goats (8) from different locations in the Pantnagar region, Uttarakhand, India. The human samples were obtained from apparently healthy

residents staying within Pantnagar. The collected samples were transported to the laboratory with minimum delay, maintaining the cold chain, and processed for *Salmonella* isolation on the same day of collection.

Isolation of *Salmonella.* The fecal samples were processed for isolation of *Salmonella* as per the guidelines of ISO 6579:2002 and published protocols (CDC and WHO, 2003). Human samples were processed for both typhoidal and non-typhoidal *Salmonella* isolation, whereas animal samples were subjected to non-typhoidal *Salmonella* isolation only. For isolation of non-typhoidal *Salmonella*, fecal samples from humans and animals were selectively enriched in Rappaport-Vassiliadis (RV) broth, and selective plating was done on Brilliant Green Agar (BGA) and Xylose Lysine Deoxycholate Agar (XLD). For typhoidal *Salmonella* isolation from human stool samples, Selenite F broth was used for selective enrichment, and Bismuth Sulphite Agar (BSA) and Hektoen Enteric Agar (HEA) were used for selective plating. MacConkey Lactose Agar (MLA) was used for purification of suspected colonies and then transferred to nutrient agar slants. The isolates were then subjected to Gram staining and biochemical tests like triple sugar iron, lysine iron agar, urease, motility, methyl red, Voges-Proskauer, indole, citrate utilization, and carbohydrate fermentation tests (*viz*., dextrose, sucrose, lactose, salicin, mannitol, and dulcitol) for confirmation of the isolate as described by Ewing (1986).

Serological characterization. The biochemically confirmed isolates were tested for auto-agglutination by mixing a loop full of bacterial growth with a drop of sterile normal saline on a clean slide, rotating for one min, and observing for agglutination. Non-agglutinating smooth *Salmonella* were then subjected to a slide agglutination test against poly-O (A-I and Vi) antiserum (Difco) by mixing a loop full of bacterial culture with a drop of antiserum placed on a clean slide and observed for visible agglutination. The isolates were also subjected to a latex agglutination test with the commercial Salmonella-Hi Latex identification kit (HiMedia) as per the recommendations of the manufacturer.

Serotyping of *Salmonella.* The *Salmonella* isolates recovered in the present study were serotyped at the National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh, India.

Genomic DNA extraction. The genomic DNA was extracted using the Hi-pura DNA purification kit (HiMedia) as per the manufacturer's protocol and stored at -20°C until used.

Detection of Virulence genes. A multiplex PCR-based approach for simultaneous screening of both *invA* and *stn* genes among the *Salmonella* isolates recovered in the present study was employed. The oligonucleotide primers previously described by Rahn *et al.* (1992); Murugkar *et al.* (2003) were used in this study (Table 1). The assay was performed in a 25 µl reaction volume containing 10X DreamTaq buffer, dNTP mixture (2 mM each), primers (10 pmol/µl), DreamTaq DNA polymerase (5 U/µl), and genomic DNA. The amplification was carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems) as follows: initial denaturation (94°C for 5 min), followed by 32 cycles of denaturation (94°C for 1 min), annealing (57°C for 1 min), and extension (72°C for 1 min), and a final extension step (72°C for 10 min). The amplicons obtained were analyzed by 2% agarose gel electrophoresis and documented.

Antibacterial sensitivity assay. Antimicrobial sensitivity was determined using the disc diffusion method on Mueller-Hinton agar, as per the guidelines from Baeur and Kirby (1966); NCCLS (2010). The antimicrobial discs (HiMedia) used were ampicillin (10 mcg), cefotaxime (30 mcg), ciprofloxacin (5 mcg), gentamicin (10 mcg), nalidixic acid (30 mcg), oxytetracycline (30 mcg), erythromycin (15 mcg), and cephalexin (30 mcg). The results were noted after 24 h of incubation at 37°C by measuring the diameter of the zone of inhibition, and interpretation as sensitive, intermediate, or resistant was made as per the manufacturer's recommendations.

RESULTS AND DISCUSSION

In recent years, new serological and molecular methods for rapid detection of *Salmonella* have become available, but the cultural method still remains as the 'gold standard' test and lays the foundation of epidemiological studies of *Salmonella* (Singer *et al.*, 2006). The samples collected were screened for *Salmonella* by culture-based methods. Out of 722 samples analyzed, 13 (1.8%) fecal samples revealed the presence of characteristic *Salmonella* colonies, 11 (3.6%) from poultry and one each from human (0.43%) and cattle samples (0.71%). Samples collected from pigs and goats were negative for *Salmonella*. Human samples were also negative for typhoidal *Salmonella*. All 13 isolates were non-autoagglutinating and produced a visible agglutination reaction in the slide agglutination test with poly O (A-I and Vi) antisera and in the latex agglutination test. The overall prevalence rate obtained in this study (1.8%) was low compared to previous studies (Murugkar *et al.*, 2005; Mir *et al.*, 2010; Putturu *et al.*, 2013; Sharma *et al.*, 2019; Pragi *et al.*, 2023), which could be because of the fact that the samples screened here originated from non-clinical, apparently healthy humans, animals, and poultry.

The serotyping results showed that all these 13 isolates were of non-typhoidal serovars consisting of *S. enteritidis*, *S. typhimurium*, and *S. infantis*. From poultry, all three serovars were recovered: *S. enteritidis* (5), *S. typhimurium* (3), and *S. infantis* (3), while the isolate obtained from both human and cattle belonged to the *S. typhimurium* serovar (Table 2). Serotyping indicates that in the case of poultry, *S. enteritidis* was the most common serovar, followed by *S. infantis* and *S. typhimurium*. The serotyping results align with global patterns where these serovars are commonly associated with foodborne infections (EFSA, 2010). Isolation of non-host-specific *Salmonella serovars*, *S. enteritidis* and *S. typhimurium*, from poultry could pose serious threats to public health (Murgarkar *et al.*, 2005; Mir *et al.*, 2010; Putturu *et al.*, 2013). The non-typhoidal serovars, *S. typhimurium* and *S. enteritidis*,

were reported to be the most common cause of Salmonella-induced food poisoning in humans, especially in neonates and young children (Murugkar *et al.,* 2005; Putturu *et al.,* 2013). In a recent report from the National *Salmonella* and *Escherichia* Centre, among all the poultry *Salmonella* isolates received between 2011 and 2016, *S. typhimurium, S. gallinarum,* and *S. enteritidis* were reported as the most common serotypes, accounting for 96.2 percent, and *S. infantis* accounted for 2.7% only (Kumar *et al.,* 2019).

The isolate obtained from humans and cattle in the present study belonged to the *S. typhimurium* serovar, which was reported as the most common serovar isolated from both humans (Murugkar *et al.,* 2005; Zaidi *et al.,* 2006) and cattle (Sato *et al.,* 2001; Murugkar *et al.,* 2005; EFSA, 2010). In this study we could not detect any typhoidal *Salmonella* from human stool samples, which could be due to the fact that blood samples are mainly used for isolation of *Salmonella* Typhi rather than stool samples (Tankhiwale *et al.,* 2003; Saha *et al.,* 2003).

Polymerase chain reaction-based screening of field isolates for the presence of virulence genes was described as an important tool in the molecular characterization of the isolates (Barman *et al.,* 2013). In this study a multiplex PCR assay was standardized for simultaneous screening of two virulence genes, wherein all 13 isolates of *Salmonella* were found to carry both *invA* and *stn* virulence genes as evidenced by the production of 284 bp and 617 bp amplicons, respectively (Fig 1). Similar findings were also reported by Mir *et al*. (2010); Murugkar *et al.* (2005). The *invA* gene has been reported to be found in all known serovars of *Salmonella* (Galan *et al.,* 1992; Rahn *et al.,* 1992; Chiu and Ou 1996; Swamy *et al.,* 1996), and in several studies this gene is being targeted for identification of the *Salmonella* in different samples (Lampel *et al.,* 2000; Putturu *et al.,* 2013). *Salmonella* enterotoxin determinant *stn* gene was also reported to be present in all *Salmonella* enterica serovars (Prager *et al.,* 1995; Ziemer and Steadham 2003; Murugkar *et al.,* 2005).

The emergence and spread of multi-drug resistance in *Salmonella* species have reinforced the need for epidemiological studies describing the prevalence and the patterns of resistance among these strains. In this study, the antibiotic sensitivity pattern revealed that all five *S. enteritidis* isolates of poultry were sensitive to cefotaxime and ciprofloxacin and 100% resistant to erythromycin and cephalexin. The *S. infantis* isolated from poultry was found to be sensitive to ciprofloxacin and resistant to erythromycin, cephalexin, and nalidixic acid. While the *S. typhimurium* isolates of poultry were found to be 100% sensitive to cefotaxime and ciprofloxacin and 100% resistant to erythromycin. Similar results were also reported by Sharma *et al.* (2019).

The *S. typhimurium* isolate of human origin was found to be resistant to all antibiotics tested. The *S. typhimurium* isolate from cattle was also found to be resistant to all antibiotics except gentamicin (Table 3). Among all the *Salmonella* isolates, 30.7% of isolates were resistant while 15.3% were sensitive to ampicillin. The majority of the isolates were sensitive to ciprofloxacin (84.6%) and cefotaxime (77%) and resistant to cephalexin (92.3%) and nalidixic acid (77%). All the isolates of *Salmonella* were found to be resistant to erythromycin. About 38.4% of all the isolates were sensitive, and 15.3% of isolates were resistant to gentamicin. About 53.8% of isolates were resistant, and 30.7% were sensitive to oxytetracycline. The resistance patterns observed, including the resistance of *S. typhimurium* from both human and cattle samples to multiple antibiotics, underscore the issue of antimicrobial resistance in *Salmonella* (Putturu *et al.,* 2013; Sharma *et al.,* 2019). This emphasizes the need for stricter regulations on the use of antibiotics in both human and animal health sectors to control the spread of resistant strains.

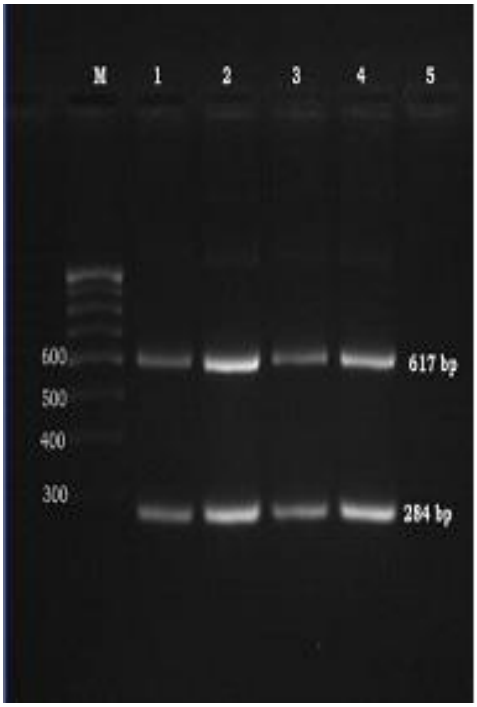

Fig. 1. Agarose gel electrophoresis of multiplex PCR amplified *stn* and *invA* virulence genes of *Salmonella* Lane M: 100 bp DNA ladder: Lane 1-5, *Salmonella* isolates showing amplicons.

Table 1: Details of the primers used.

Target gene		Sequence of primers (5'-3')	Amplicon size(bp)	Reference
*inv*A	Forward	GTG AAA TTA TCG CCA CGT TCG GGC AA	284	Rahn *et al.* (1992)
	Reverse	TCA TCG CAC CGT CAA AGG AAC C		
stn	Forward	TTG TGT CGC TAT CAC TGG CAA CC	617	Murugkar *et al.* (2003)
	Reverse	ATT CGT AAC CCG CTC TCG TCC		

Table 2: Details of *Salmonella* isolates obtained from different samples.

Sr. No.	Sample Type	No. of sample Processed	No. of positive sample (%)	Serovars identified	No. of Isolate
1.	Poultry droppings	304	11(3.6)	*S. enteritidis* *S. infantis* *S. typhimurium*	5 3 3
2.	Human stools	232	1(0.43)	*S. typhimurium*	1
3.	Cattle dung	140	1(0.71)	*S. typhimurium*	1
4.	Pig faeces	38	0(0)	—	—
5.	Goat faeces	8	0(0)	—	—

Table 3: Antibiotic sensitivity pattern of *Salmonella* serovars.

Serovar	Sample source	No.	Ampicillin	Gentamicin	Cefotaxime	Cephalexin	Ciprofloxacin	Nalidixic acid	Erythromycin	Oxytetracyline
			R I S	R I S	R I S	R I S	R I S	R I S	R I S	R I S
S. Enteritidis	P	5	0 5 0	1 4 0	0 0 5	5 0 0	0 0 5	3 0 2	5 0 0	2 1 2
S. Infantis	P	3	1 1 1	0 1 2	1 0 2	3 0 0	0 0 3	3 0 0	3 0 0	2 0 1
S. Typhimurium	P	3	1 1 1	0 1 2	0 0 3	2 0 1	0 0 3	2 0 1	3 0 0	1 1 1
	H	1	1 0 0	1 0 0	1 0 0	1 0 0	0 1 0	1 0 0	1 0 0	1 0 0
	C	1	1 0 0	0 0 1	1 0 0	1 0 0	1 0 0	1 0 0	1 0 0	1 0 0
Total		13	4 7 2	2 6 5	3 0 10	12 0 1	1 1 11	10 0 3	13 0 0	7 2 4

R- Resistant, I - Intermediate, S – Sensitive, P – Poultry, H – Human, C - Cattle

CONCLUSIONS

In conclusion, this study highlights the low prevalence of *Salmonella* in the Pantnagar region but underscores the importance of poultry as a major source of *Salmonella* infection, with significant public health implications. The identification of multi-drug-resistant strains, particularly from *S. typhimurium*, signifies the need to regulate antimicrobial use.

FUTURE SCOPE

This study must be expanded to a wider region across the state to better understand the prevalence of *Salmonella* and its potential threats to food safety and public health.

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Conflict of interest. None.

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