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Characterization of non-typhoidal *Salmonella* from poultry in Punjab, India

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ABSTRACT

Present study was done to determine the prevalence, virulence and antimicrobial spectrum of identified serotypes. A total of 693 samples, comprising 585 poultry fecal samples (420 from layer, 120 from broiler and 45 from backyard poultry), 54 each poultry feed and farm water were collected from 31 poultry farms of three districts of Punjab. A total of 6 *Salmonella* isolates were obtained from these samples. Out of 585 fecal samples, 6 (1.02%) were positive for *Salmonella*. Out of 31 farms, 2 (6.45%) farms were positive for *Salmonella*. Out of 6 *Salmonella* isolates, four isolates from one farm were serotype *Salmonella* IIIa, 35: z24: z23 and two *Salmonella* isolates from another farm were untypable and also carried *spvC* gene. Isolates showed resistance to gentamicin (2/6, 33.3%), co-trimoxazole (1/6, 16.7%). Antibiotics ampicillin, tetracycline, ciprofloxacin, chloramphenicol and enrofloxacin were effective against all the isolates. On PFGE analysis, four isolates from one farm were clustered in two clusters and two isolates from other farm were similar and clustered together. Based on the results we can say that drug resistant *Salmonella* is present in poultry flock in Punjab.

Keywords: Antimicrobial susceptibility, PCR, PFGE, Poultry, *Salmonella*

Salmonellosis is an important food-borne disease in the world. In India also it is a major public health problem (Mir *et al.* 2015). According to CDC, out of 9.4 million food-borne illnesses occurring each year in the United States, salmonellosis accounts for 1,000,000 illness, 19,000 hospitalization and 380 deaths annually (Scallan *et al.* 2011). *Salmonella* had been reported from different parts of India such as poultry cloacal samples from Kolkata (Sudhanthirakodi *et al.* 2016), backyard poultry in Odisha (Das *et al.* 2017), chicken and slaughter house environment in Thanjavur, Tamil Nadu (Balakrishnan *et al.* 2018). Poultry and meat products, eggs and egg containing food products are important sources of *Salmonella* infection in human. Being a facultative intracellular pathogen, the virulence and pathogenicity of *Salmonella* depends upon multiple factors encoded by genes that lead to their colonization, invasiveness, intracellular survival and damage to host tissues. The genus *Salmonella* is subdivided into 2,541 serovars and non-typhoidal *Salmonella* spp. affect both human and animals. In recent years, antibiotic resistance in *Salmonella* has raised concern worldwide due to the practice of using antimicrobial agents in livestock and poultry industries to prevent and treat diseases and use them as growth promoters. Antimicrobial resistant and even multi drug resistant *Salmonella* presence in India is documented (Bhuvaneswari *et al.* 2015). Punjab is a progressive state with several farmers involved in poultry farming as well. The present study was conducted to determine the prevalence, serotype, virulence status and antimicrobial resistance profile of *Salmonella* spp. isolated from poultry environment of Punjab, India.

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MATERIALS AND METHODS

In this study, a total of 693 samples comprising 585 poultry fecal samples (420 from layers, 120 from broilers and 45 from backyard poultry), 54 poultry feed samples (2/farm), 54 poultry water samples (2/farm) were collected from 27 commercial poultry (20 samples/farm) and 4 backyard farms (10–15 sample/farm) located in three districts (Ludhiana, Sangrur, Hoshiarpur) of Punjab, India. Samples were collected from September 2015 to April 2016 (Table 1). Sterile cotton swab was used for sampling. Before sampling, swab was moistened with sterile buffer peptone water (BPW). Freshly passed fecal sample on the farm was picked with the moistened swab taking care not to touch the cage or floor. The person collecting sample wore gloves at the time of sampling. Samples were maintained and transported on ice until processed.

*Isolation of Salmonella***:** For isolation of *Salmonella* spp., 1 g feed and 1 ml water were processed separately in 9 ml BPW each, fecal swabs in 10 ml of BPW and incubated at 37°C for 18–24 h. Thereafter, 1 ml of the inoculum from BPW was transferred to 9 ml of Rappaport-Vassiliadis (RV) enrichment broth (Himedia, Mumbai) and incubated at 42°C for 24–48 h. A loopful of inoculum from RV broth was streaked onto Xylose-Lysine-Tergitol-4 (XLT_4) agar (Himedia, Mumbai) and incubated at 37°C for 18–24 h. Presumptive *Salmonella* colonies (translucent with black

Table 1. Samples collected from poultry farms

District	No of poultry farm	Type of farm	No of bird	Type and no of samples examined			Total
				Faecal sample	Water sample	Feed sample	
Ludhiana	14	Layer	2,85,000	280	28	28	336
	4	Backyard	62	45	0	0	45
Sangrur	7	Layer	1,61,000	140	14	14	168
Hoshiarpur	6	Broiler	7,700	120	12	12	144
Total	31		4,53,762	585	54	54	693

center) were subjected to urease test, those which were urease negative were further streaked on Triple sugar iron test (TSI), whereas urease positive colonies were discarded. Colonies showing typical reaction on TSI were subjected to biochemical test using kit (HiMedia Labs, Mumbai). (Bacteriological Analytical Manual, FDA method)

Confirmation of Salmonellaby PCR: S. Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076 were used as positive control in PCR targeting genus specific gene invasion A (*inv*A) and virulence gene, *Salmonella* Plasmid Virulence (*spvC*), respectively. All the *Salmonella* isolates identified through biochemical tests were further confirmed and Genomic DNA of the isolate was obtained by snap chill method. Freshly grown colonies were inoculated in 1.5 ml nuclease free water (NFW) and kept for boiling at 100°C for 10 min. Immediately after boiling, cold shock was given by keeping it in ice for 10–15 min, followed by centrifugation at 10,000 rpm for 10 min. The supernatant containing DNA was aliquoted in a sterile tube and stored at –20°C until further use.

The primers used for the reaction were *inv*A-F **'**TTGTTACGGCTA TTT TGA CCA', *inv*A-R **'**CTG ACT GCT ACC TTG CTG ATG' yielding 521 bp product and *spv*C-F 'ACT CCT TGC ACA ACC AAA TGC GGA', *spv*C-R **'**TGT CTT CTG CAT TTC GCC ACC ATC A' yielding 571 bp (Mir *et al*. 2010). The PCR reaction conditions consisted of an initial denaturation of DNA at 94°C for 2 min followed by 35 cycles each of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, followed by a final extension at 72°C of 5 min and hold at 4°C. Nuclease free water (NFW) was used as negative control. The amplified PCR product was analyzed in 1% agarose gel electrophoresis with ethidium bromide. Gel documentation system (Syngene, USA) was used for the visualization of the bands.

Serotyping of Salmonella: Isolates confirmed biochemically and by PCR were sent for serotyping to Indian Veterinary Research Institute (IVRI), Bareilly UP, India.

Antimicrobial sensitivity testing (*AST*): Antibiotic sensitivity of confirmed *Salmonella* isolates to ampicillin (10 µg/unit), gentamicin (10 µg/unit), tetracycline (30 µg/unit), enrofloxacin (5 µg/unit), trimethoprim (µg/unit), ciprofloxacin (5 µg/unit), chloramphenicol (30 µg/unit), cotrimoxazole (25 µg/unit) was performed by Kirby-Bauer disc diffusion method as per Clinical and Laboratory Standards Institute (CLSI) Guidelines 2013. The antimicrobial discs from HiMedia, Laboratory, India were used.

Pulse field gel electrophoresis of Salmonella isolates: Genetic relatedness of six *Salmonella* isolates in present study was determined by Pulse field gel electrophoresis (PFGE) following protocol for *Salmonell*a described in PulseNet methods of PulseNet, Centre for Disease Control, United States (CDC, USA). Restriction enzyme *Xba*I (New England Biolabs, USA) was used for digestion of genomic DNA in plugs. Plugs were then loaded in the wells of the 1% Pulse field certified agarose gel (Bio-Rad) and sealed with the single drop of same agarose on the top of the well. Genomic DNA in plugs was separated for 19 h by electrophoresis on CHEF MAPPER (Bio-Rad) at 200 volts (6v/cm) with the following conditions: temperature 14°C, initial switch time of 2.16 sec, final switch time of 63.8 sec and angle 120°. A lambda ladder molecular weight marker (Bio-Rad laboratories, USA) was included in three lanes in gel as molecular standard. After the run, gel was stained with ethidium bromide (0.5 mg/l) for 20 min and then de-stained for 1 h in distilled water. The control strain *Salmonella* Braenderup H9812 could not be used in the experiment as it was not available. The gel was visualized using Gel Documentation system (Syngene, USA). The gel image was subjected to analysis using Gel Compare 2 (Applied Maths, College Station, TX). Band molecular weights were determined by sample lanes to a standard molecular weight ladder (Lambda Ladder – Bio Rad). Banding patterns were converted into binary data based on the presence or absence of bands with each molecular weight and fingerprints were then compared using the Dice algorithm. The dendrogram was drawn using unweighted pair group method with arithmetic mean (UPGMA). Fingerprints were analyzed using the Dice algorithm. *Salmonella* Typhimurium ATCC 14028 and *Salmonella* Enteritidis ATCC 13076 were used as control for comparison between our isolates.

RESULTS AND DISCUSSION

Isolation, identification and serotyping of Salmonella: In the present study, out of 585 fecal samples from 27 organized and four backyard farms, six fecal samples collected from organized commercial farm were positive for *Salmonella*, resulting in six *Salmonella* isolates giving overall isolation rate and fecal prevalence of 1% (Table 2). Out of 27 organized commercial farms, 2 layer farms were positive for *Salmonella* spp. giving overall prevalence of

Table 2. Prevalence of *Salmonella* in poultry farms of Punjab

District	No of poultry farms	Type of farms	Type of rearing	No of faecal sample examined	No of positive samples (%)	No. of positive farm (%)	No of feed sample examined	No of positive samples (%)	No of water sample examined	No of positive samples (%)
Ludhiana	14	Layers	Cage	280	6 (2.14%)	2 (14.0%)	28	0	28	0
	4		Free ranging	45	0	0	0	0	0	0
Sangrur	7	Layer	Cage	140	0	0	14	0	14	0
Hoshiarpur	6	Broiler	Cage	120	0	0	12	0	12	0
Total	31	–	–	585	6 (1.02%)	2 (6.45%)	54	0	54	0

Table 3. *Salmonella* serotypes and virulence plasmid from the isolates

Farm No.	Types of farm	Isolate No.	Antigen structure	Serotypes	Virulence plasmid (*spvC*)
1	Layer	Isolate 16	35: z24: z23: -	*Salmonella* IIIa, 35: z24: z23	–
		Isolate 18	35: z24: z23: -	*Salmonella* IIIa, 35: z24: z23	–
		Isolate 21	35: z24: z23: -	*Salmonella* IIIa, 35: z24: z23	–
		Isolate 35S	35: z24: z23: -	*Salmonella* IIIa, 35: z24: z23	–
9	Layer	Isolate 35L	–	*Salmonella* Untypable	+
		Isolate 46	–	*Salmonella* Untypable	+

6.45%. Whereas, none of the broiler and back yard poultry farms were positive for *Salmonella* (Table 2). None of the feed and water samples collected from the farms in the study were positive for *Salmonella* spp. Four isolates from one farm were serotyped as *Salmonella* enterica subspecies IIIa or subspecies Arizona with antigenic formula as 35: z24: z23, whereas other two isolates from second farm were untypable (Table 3).

Poultry accounts for one of the most common sources of *Salmonella* serotypes responsible for human disease through contaminated meat and egg. However, in the present study, there was 1% incidence of *Salmonella* from fecal samples. *Salmonella* has also been detected by Suresh *et al.* (2010) who reported 1.4% *Salmonella* isolation from cloacal samples of poultry in Coimbatore. Similarly, Tiwari *et al.* (2014) also reported 0.75% prevalence of *Salmonella* spp. Comparatively higher prevalence of 6.31% was recorded by Mir *et al.* (2015) from different species of poultry in Rajasthan. Li *et al.* (2018) found varying levels of *Salmonella* isolates ranging from 3.7–33.3% at layer farms. In contrast, Orji *et al.* (2004), found high incidence of 38.3% *Salmonella* from fecal samples in Awka, Nigeria. Similarly, Akhtar *et al.* (2009) found 55% of the poultry fecal samples in their study positive for *Salmonella* in Faisalabad, Pakistan. Difference in prevalence may be due to geo-graphical variations, difference in management (Al-Abadi and Mayah 2012). *Salmonella* exhibit intermittent shedding in feces which can also add to the differences in prevalence level (Fanelli *et al.* 1991). Poultry farms operating in organized sector for commercial gains are also resorting to use of antibiotics in poultry feed for early growth and better return, leading to lower detection of *Salmonella* serotypes from poultry production environment (Jarolman *et al.* 1976). Site of sample collection for *Salmonella* isolation also influence its detection rate. Al-Abadi and Mayah (2012), reported higher (19%) *Salmonella* detection from cloacal swabs compared to fecal samples in Basrah province, Iraq.

Based on the findings of serotyping, *Salmonella* serotype IIIa isolated from layer farm no. 1 could be zoonotic in nature (CDC 1999). *Salmonella enterica* or subspecies I, serovar enterica is responsible for the majority of infections in mammals and birds. While subspecies other than I causes sporadic diseases in human and birds and primarily colonize cold blooded vertebrates (Weiss *et al.* 1986, Waterman *et al*. 1990). *Salmonella enterica* subspecies IIIa has also been found to colonize the human intestinal tract and has been isolated from fecal culture of infected individuals (Hall and Rowe 1992, Schroter *et al.* 2004). It could also cause severe systemic infection in young children and immuno compromised individuals (CDC 1999). The other two serotypes from layer farm no. 9 could not be typed with available antisera, but carried virulence determinants.

Virulence genes: All the isolates carried *inv*A gene and only two out of six *Salmonella* isolates, from one farm also carried *Salmonella* virulence *spvC* plasmid indicating their virulence potential (Table 3). Large numbers of genes are involved in virulence of *Salmonella* serotypes. Based on the results of the present study we can say that the two untypable *Salmonella* serotypes are potentially pathogenic as they were positive for both invasion gene and virulence plasmid. Both these genes are believed to be responsible for invasion and persistence of *Salmonella* serotypes, in host organs (Finlay and Falkow 1989, Gulig 1990). This *spv* operon has been found to be conserved in different virulence plasmids of various *Salmonella* seroptypes responsible for systemic infection (Guiney *et al.* 1994). At the same time it is difficult to designate other four isolates that carried only *inv*A gene but were negative for virulence plasmid as non-pathogenic as numbers of other genes are

Table 4. Phenotypes of antibiotic resistance *Salmonella* isolates

Farm	Isolate	Antibiotic (% resistance)								
		AMP	GEN	ERY	TET	ENRO	AMI	CIP	CHL	COT
Farm 1	Isolate 16	S	R	S	S	S	S	S	S	R
Farm 1	Isolate 18	S	S	S	S	S	R	S	S	S
Farm 1	Isolate 21	S	S	R	S	S	R	S	S	S
Farm 1	Isolate 35S	S	S	R	S	S	S	S	S	S
Farm 9	Isolate 35L	S	R	R	S	S	S	S	S	S
Farm 9	Isolate 46	S	S	R	S	S	R	S	S	S
Total		(0%)	(33.3%)	(66.7%)	(0%)	(0%)	(50%)	(0%)	(0%)	(16.7%)

R, Resistance; S, Sensitive; AMP, Ampicillin; GEN, Gentamicin; ERY, Erythromycin; TET, Tetracycline; ENRO, Enrofloxacin; AMI, Amikacin; CIP, Ciprofloxacin; CHL, Chloramphenicol; COT, Cotrimoxazole.

also responsible for *Salmonella* serotype virulence whose detection was not attempted in the present study. Some studies have provided evidence that the virulence plasmid plays a significant role in human disease and poses a serious threat to public health (Guiney *et al* 1994, Chu and Chiu 2006). In this study, presence of *spv*C virulent gene in *Salmonella* isolates indicated the risk of zoonotic transfer of this pathogen to human indirectly through contaminated food and water or directly through contact thereby posing a threat to public health.

Antimicrobial resistance of Salmonella: All the *Salmonella* serotypes (100%) were sensitive to antibiotics ampicillin, tetracycline, ciprofloxacin, and chloramphenicol (Table 4). Whereas the isolates showed resistance to gentamicin (2/6, 33.33%) and co-trimoxazole (1/6, 16.66%).

The use of antimicrobials is important for the control and treatment of Salmonellosis. However their unregulated use in poultry has resulted in emergence and spread of antibiotic resistance strains. Antimicrobials such as penicillin, doxycycline, cephalosporins, fluoroquinolones, tetracyclines, sulphonamides and aminoglycosides are used in poultry industries. (Marshall and Levy 2011, Silbergeld *et al*. 2008). Poultry can be one another source of antimicrobial resistant microorganisms, especially *Salmonella* spp. In the present study, all or majority of the isolates were sensitive to ampicillin, chloramphenicol and cotrimoxazole which are considered first line of treatment for salmonellosis cases in human beings, which has also been documented by El-Tayeba *et al.* (2017). All the isolates were also sensitive to tetracycline, enrofloxacin and ciprofloxacin. On the contrary, Mir *et al.* (2010) and Mir *et al*. (2015) found *Salmonella* isolates from poultry resistant to ampicillin and contrimoxazole. Mir *et al.* (2010) found 33 *Salmonella* isolates from poultry fecal samples in Kashmir resistant to gentamicin, cotrimoxazole, and ampicillin. In another study by Mir *et al.* (2015) they found *Salmonella* enterica isolates from different species of poultry resistant to gentamicin, tetracycline, ciprofloxacin, and cotrimoxazole. Akhtar *et al*. (2009) found 55 *Salmonella* isolates from 100 poultry droppings in Faisalabad, Pakistan resistant to gentamicin, and trimethoprim. Differences in the resistance pattern occur mainly due to chemotherapeutic management as poultry feed has antimicrobials as growth promoters.

*Pulse field gel electrophoresis of Salmonella isolates***:** PFGE analysis of six *Salmonella* isolates revealed that the four isolates namely 16, 18, 21 and 35S from a layer farm (LF1) in Ludhiana district with same serotype and antigenic structure were clustered in three clusters indicating genetic variability among them. The other two untypable serotypes from the backyard poultry farm in Ludhiana were similar to each other (90–95% similarity) and clustered together (Fig. 1). Based on the results we can say that there was variation among the *Salmonella* strains present in one farm while strains of other farms were similar to each other. As only fecal samples in the study were positive for *Samonella* that too from two farms only and none of the water and feed samples were positive, therefore not many conclusions could be drawn on *Salmonella* serotypes presence in different sources and region.

PFGE is considered the gold-standard method for subtyping food-borne pathogens (Whittam and Bergholz

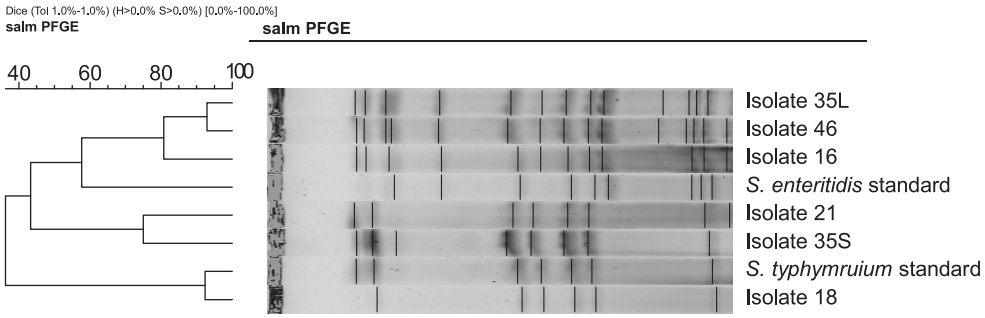

Fig. 1. Dendrogram showing the relatedness of *Salmonella* isolates from fecal samples.

2007). PFGE is widely used molecular method and has been shown to be very effective for both epidemiological surveillance and outbreak investigations in several serotypes of *Salmonella*. The four isolates although of same serotype showed some diversity among them. Whereas, the other two untypable strains were similar. Boonmar *et al.* (1998) analysed 53 isolates of *S*. Enteritidis, where in 45 showed an indistinguishable pattern and the other 8 isolates showed similar pattern that differed by only a few bands which indicated the spread of a genetically identical clone of *S.* Enteritidis in humans and poultry in Thailand. Mir *et al.* (2010) analyzed 33 isolates of *Salmonella* and observed that the majority of these isolates were related but a few belonged to different clones that indicated a potential threat not only to poultry but also to human health in Kashmir.
No association between different factors included in the study and *Salmonella* could be established as only two farms of layers flock turned positive and rest of the other samples negative. Feed and water being used at the selected farms were not contaminated which could be important source of infection at farm.

Based on the results, it can be concluded that there is a 1% incidence of *Salmonella* in poultry farms of three districts in Punjab. The serotypes reported were of *Salmonella* enterica subsp. Arizonae (IIIa) and some were untypable serotypes. The isolates carried *inv A* gene and virulence plasmid *spv.* The isolates were resistant to majority of the antibiotics tested with little resistance to gentamicin and co-trimoxazole. *Salmonella* isolates at one farm were similar, while at other farms showed some genetic variations. Therefore, there is need for continued surveillance to identify emerging serotypes, changing pattern of resistance for effective control of salmonellosis.

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