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CONTENTS

Assessment of faecal shedding of salmonellae in poultry farms of Uttarakhand

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ABSTRACT: Salmonellosis is one of the leading causes of food borne illnesses around the world and has been known to cause diseases in a number of hosts. Meat products especially poultry products have been known to serve as a source of disease transmission in humans since the major route of transmission is faeco-oral. In the present study, isolation of *Salmonella* was attempted from faecal samples of poultry and the prevalence was determined to ascertain the likelihood of a public health threat in the area.

Key words: Poultry faeces, prevalence, *Salmonella*

The bacteria belonging to genus *Salmonella* are one of the most widely known causes of food borne infections around the world. Estimates suggest that around 95 percent of the *Salmonella* infections are food borne (Frenzen *et al.,* 1999). A wide array of foods is linked to human infections but the most important food are poultry products, both meat and eggs(Kiran *et al.*, 2016). Apart from causing human infections, *Salmonella* also causes infections in a wide range of other animals- mammals, birds, reptiles etc. The animals mostly become infected via feed (faecal-oral route), water (contaminated water) or the general environment they reside in. In many economically backward countries *Salmonella* infections are endemic in nature. *Salmonella* organisms can adapt to a wide variety of ecological niches with ease and that is what makes them versatile and ubiquitous. The reservoir potential of the bacteria is enormous because of its wide host range, the large number of convalescent and chronic healthy carriers and environmental sources in the communities and is also responsible for the enhancement in its endemicity, especially in areas with low environmental hygiene. Animal droppings have been shown to be a potential reservoir for many enteric organisms.

There have been multiple reports of *Salmonella* in intensive animal husbandry practises like poultry production (Ashfaw Ali *et al.,*2020). The major routes of transmission to the birds on such farms are either vertical or horizontal (Cosby *et al.,* 2015), the former occurring when the laying hen is infected with the bacteria (i.e., the reproductive organs are infected) and the infection is then transmitted to the eggs being laid (Jajere, 2019). During horizontal transmission, poultry are exposed to contaminated litter, faeces, feed, water, equipment, other chickens, rodents, other animals, and farm personnel colonised/infected with *Salmonella*.These bacteria belong to *Enterobacteriaceae* family and are gram negative rods with peritrichous flagella except S. Pullorum and *S. Gallinarum*. The genus consists of only two species- *S. Enterica* and *S. Bongori* (Popoff, 2001).

The present study aims to isolate *Salmonella* from faecal samples collected from poultry farms situated in and around areas of Pantnagar,Udham Singh Nagar district.

MATERIALS AND METHODS

Sample collection

A total of 965 samples were collected from poultry farms in Ramnagar (n=46),Kiccha (n=49), Jawaharnagar (n=51), Shantipuri (47), Pantnagar (698) and Haldwani (74) of Uttarakhand state, India in sterile 100 ml Whirl-Pak bags. Thereafter, the samples were transported to the laboratory and

processed further under sterile conditions.

Isolation

The isolation of non –Typhoidal *Salmonella* organisms was done as per Keelara *et al.*(2013).All the samples were inoculated into buffered peptone water in the ratio of 1:9, mixed well and incubated at 37ºC for 24 hours. Henceforth, they were enriched in 9.9 ml Rappaport Vassiliadis (RV) broth (Difco, United States) by taking 100 µl of the sample from BPW inoculum. They were then incubated for 18-24 hours at 42ºC. After that selective plating was done by streaking the samples from RV broth onto XLT4 agar (Hi-Media, India) and the plates were kept at 37ºC for 12 hours.

Morphological Identification

The analysis of the samples was done on the basis of characteristic colony morphology on selective plating media i.e., XLT4. The colonies which appeared small, moist and black with pink periphery were suspected to be *Salmonella* and further tested.

Biochemical Identification

The biochemical tests performed were Urease Test and Triple Sugar Iron (TSI) test. The suspected colonies from the XLT4 agar plates were streaked onto and stabbed into the TSI slants and kept at 37ºC for 24h. The positive colony gives red alkaline slants and yellow acidic butts, some black discoloration is also observed in case the colonies are H_2S producing.For the Urease test, the suspected colonies are picked from the XLT4 agar plates and streaked onto the Christensen's Urea agar slants and incubated at 37ºC overnight. Negative reaction (no colour change) is characteristic of *Salmonella*.

Polymerase Chain Reaction (PCR)for genus(*invA* gene) and virulence genes (*sip*Aand*stn*) detection

For PCR reaction, DNA of the presumptive positive isolates (on the basis of morphological and biochemical identification)was extracted by using Hi-pura DNA purification kit (Himedia) as per the manufacturer's instructions supplied with the kit. The kit works by lysing the bacterial cells by the various buffers, RNAase removes the RNA and proteinase K removes the proteins from the target cells. After extraction, the DNA is stored at -20ºC for further use.

The genes targeted for confirmation include *inv*A gene (Rahn *et al.,* 1992) with primer sequence- 5' GTGAAATTATCGCCACGTTCG GGCAA-3' (forward) and 5'- TCATCGCACCGTC AAAGGAACC-3' (reverse) which is responsible for invasion of the bacteria into the cell and gives a band at 284 bp.

Virulence genes *stn*with primer sequence 5'-TTGTGTCGCTATCACTGGCAACC-3' (F) and 5'-ATTCGTAACCCGCTCTCGTCC-3'(R) (Murugkar *et al.,* 2003) and *sip*A, primer sequence 5'-TTCGACTAACAGCAGCA-3' (F) and 5'-CCGTCGTACCGGCTTTATTA-3' (R) (Wang *et al.,* 2009) were also used, which give characteristic bands at 617 bp and 449 bp respectively. The separate reactions involving the target genes were carried out in 25 µl mixture, the composition of the mixtures is given in tables 1,2,3 and 4.The PCR tubes were spun to properly mix the contents andput in the thermal cycler. The conditions set for the cycler are given in Tables 1b, 2b and 3b.

Electrophoresis of PCR products

The amplified products were then run in a horizontal gel electrophoresis assembly using 2% agarose gel. Along with the samples, a 100 bp ladder was also

Table 1: PCR for *inv*A gene

Contents	Quantity (µl)
10X PCR assay buffer	2.5
dNTP mix	0.5
Primers (Forward and Reverse) (100 pmol/ul)	0.5 each
Taq polymerase (5 U/ul)	0.2
Genomic DNA	1
Sterile Triple Distilled water	To make quantity upto 25

Table 1b: PCR conditions for *inv*A gene

Stage	Time	Temperature (ºC)
Initial Denaturation	5 min	94
Denaturation (30 cycles)	1 min	94
Annealing	1 min	55
Extension	1 min	72
Final Extension	5 min	72

Table 2: PCR for *stn* gene

Contents	Quantity (µl)
10X PCR assay buffer	2.5 µl
dNTP mix	1
Primers (Forward and Reverse) (100 pmol/ul)	0.5 each
Taq polymerase (5 U/ul)	0.2
Genomic DNA	1
Sterile Triple Distilled water	To make quantity upto 25

Table 2b: PCR conditions for *stn* gene

Stage	Time	Temperature (ºC)
Initial Denaturation	5 min	94
Denaturation (30 cycles)	1 min	94
Annealing	1 min	59
Extension	1 min	72
Final Extension	10 min	72

Table 3: PCR for *sip*A gene

Contents	Quantity (µl)
10X PCR assay buffer	2.5 ul
dNTP mix	0.5
Primers (Forward and Reverse) (100 pmol/ul)	0.5 each
Taq polymerase (5 U/ul)	0.25
Genomic DNA	1
Sterile Triple Distilled water	To make quantity upto 25

Table 3b: PCR conditions for *sip*A gene

Stage	Time	Temperature (ºC)
Initial Denaturation	5 min	95
Denaturation (30 cycles)	30 sec	95
Annealing	30 sec	55
Extension	2 min	72
Final Extension	5 min	72

run and the gel was then visualized in gel documentation system.

Serotyping

The isolates showing desired biochemical and molecular confirmation were then transferred to a semi solid media and sent for serotyping to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh.

RESULTS AND DISCUSSION

Genus Identification

Of the total 965 samples screened, 41 (4.249%) isolates amplified *invA* gene (284bp) confirming them to be belonging to genus *Salmonella*.For this study *inv*A gene which is responsible for the invasion of the bacteria into the epithelial cells of the host was taken for confirmation of the genus. The PCR with *inv*A gene is now recognized as an international standard for detecting *Salmonella* genus as it is rapid, sensitive and specific (Shanmugasamy *et al.,* 2011).

Prevalence

An overall prevalence of *Salmonella*was found to be 4.24% from the faecal samples of poultry which is much lower than 12.50% found by Tabo and co-workers (2013). Studies conducted by Tabo*et al.*(2013) and Suresh *et al.*(2006) found that the prevalence of *Salmonella* was much higher in the litter and water samples compared to the poultry droppings. Though when compared with previous studies conducted around the area of study, the prevalence was found to be in agreement with Sharma and Thapliyal(1995) who found a prevalence of 6.49%. The reason for the low prevalence might be because the disease is not endemic to the area. Another reason for the low prevalence might be because shedding of *Salmonella* in faeces is intermittent (VanImmerseel *et al.,* 2004). The shedding of *Salmonella* is highest around 2 weeks of rearing which can be attributed to an immature immune system of the birds and hence the time of sampling might have also affected the prevalence rate in the flocks.

Location wise prevalence

Out of the total 965 samples collected, the highest prevalence which was 23.40% (11/47) was recorded from Shantipuri. It was followed by Haldwani with a prevalence of 21.62% (16/74) and then Pantnagar 2.01% (14/698), which is shown in figure 1. The bacteria could not be isolated from samples collected from Ramnagar, Kiccha and Jawaharnagar. Variety of risk factors have been linked to elevated environmental *Salmonella* prevalence, including larger flock size, greater flock age, and housing in older facilities (Gast *et al.*, 2015).Since the samples were taken from organized farms, it can also be said that the good hygienic and rearing practices can help

curtail the transmission to other birds.

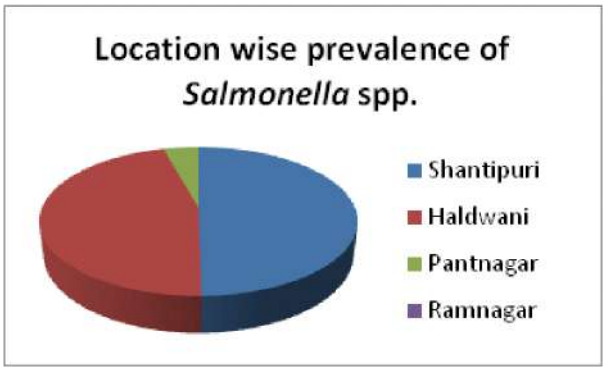

Fig 1: Location wise distribution of the *Salmonella* spp.

Serotypes

The different *Salmonella* serotypes and their rate of isolation from faecal samples of poultry are shown in Table 4 and in figure 2. Many of the *Salmonella* serotypes isolated from the samples are known to be pathogenic to man. *Salmonella* Kentucky was isolated from 43.9% of the poultry droppings, *S.* Typhimurium from 17.07%, *S.* Virchow from 7.31%, *S.* Enteritidis and *S.* Infantis from 12.19% of the isolates respectively, while 5 isolates were found non-typable. *Salmonella* Pullorum and *Salmonella* Gallinarum were not isolated from the sampled poultry droppings. This shows that the serotypes of *Salmonella* can vary from region to region and from time to time. Also, the isolation percentage as well as the serotypes found will depend on the location, time of sampling, method of sampling, endemicity of the bacteria and many other factors.

The serotype found prominently in the area was *S.* Kentucky with a prevalence of 43.9%, followed by *S.* Typhimurium (17.07%). Surprisingly the 'poultry isolates' viz., *S.* Gallinarum and *S.* Pullorum were not found in any of the samples. This is different

Table 4: Serotypes of *Salmonella*

Isolate Serotype	No. of positives
S. Kentucky	18
S. Virchow	3
S. Typhimurium	7
S. Enteritidis	5
S. Infantis	3
Non-Typable	5
Total	41

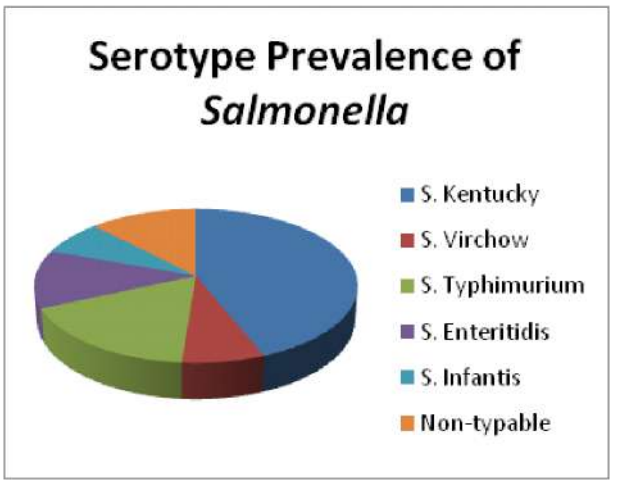

Fig 2: Serotype wise distribution of *Salmonella* spp.

from the prevalence that was found by Marin and Lainez (2009) where they found *S.* Enteritidis to be the most prevalent serotype. In another study conducted by Ibrahim *et al.*(2013), *S.* Kentucky was found predominantly from chickens. *S.* Kentucky was also found to be the major serotype in poultry in the works of Byrd *et al.*(1997). In another work done by Caffrey *et al.* (2021) in Canada, *S.* Kentucky was found to the prevalent serotype in the faecal samples of broiler chickens with 36% presence, followed by *S.* Enteritidis (20%) and *S.* Heidelberg (8%).

Virulence genes

The prevalence of virulence gene *sip*A, mainly responsible for invasion in the study was found to be 95.12% (39/41) and of *stn* was 56.09% (23/41). The prevalence of virulence genes *sip*A and *stn* in our study was 95.122% and 56.09% respectively. The former is in agreement with the previous study conducted by Sharma *et al.*(2019) in the same area while *stn* gene, an enterotoxin coding gene, was found in only 34.29% of the samples. A study conducted by Fardsanei *et al.* (2017) revealed the prevalence of *sip*A to be 100% (24/24) and of *stn* to be 91.67% (22/24). Fardsanei *et al.* (2021), in Iran found the prevalence of *sip*A to be 100% while *stn* was present in 92.6% isolates.

CONCLUSION

Salmonella spp. is one of the most important

pathogen in poultry because it causes a severe economic loss in poultry industry as well as the food industry related to poultry and poultry products. Eating of raw or undercooked poultry meat or meat which has not been handled properly has been reported to cause salmonellosis in humans.Faecal shedding of salmonellae is a product of their ability to adhere to cells of the avian intestinal tract. Persistent shedding of *Salmonella* organisms by even a small proportion of hens in a flock could enhance the opportunities of horizontal transmission to other birds and consequently can lead to production of contaminated eggs.

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