

Occurrence and antimicrobial resistance of non-typhoidal *Salmonella* isolates belonging to layer breeds and the farm environment

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ABSTRACT

This study assessed the occurrence and antimicrobial resistance of NTS organisms isolated from faecal, caecal and environmental samples of five-layer breeds [Uttara fowl, Kadaknath, Rhode Island Red (RIR), White Leghorn and Australorp] raised in an organized farm. A total of 470 samples were examined using the culture method, followed by biochemical and molecular confirmation of the isolates. All *Salmonella* strains were tested for their susceptibility to the antibiotics using the disk diffusion method. The overall occurrence of NTS was 4.89% (23/470). RIR showed a highest occurrence (17.58%), followed by Uttara fowl (2.73%) and Kadaknath (1.82%). Water samples had the highest occurrence (10.77%), followed by poultry faeces (6.67%) and caeca (6.0%). On serotyping, a single serovar viz. *Salmonella* Typhimurium (91.3%) was found to be circulated on the farm. The highest resistance was observed for erythromycin (100%), followed by ampicillin (65.22%) and nalidixic acid (56.52%). About 78.26% of isolates were multidrug-resistant (MDR). The multiple antibiotic resistance (MAR) index ranged from 0.15-0.69. The study confirmed the presence of MDR *S. Typhimurium* in the breeds and the farm environment, warranting strict control and surveillance.

Keywords: Antimicrobial susceptibility, Layers, Non-typhoidal *Salmonella*, Occurrence, Serotyping

Food safety, a major concern for developing countries, is still in its infancy. Safe food is a mandatory requirement for everyone to prevent many foodborne illnesses, which today seem to be growing at an alarming rate. Non-typhoidal *Salmonella* (NTS) is responsible for the majority of foodborne illnesses worldwide, infecting the gastrointestinal tract and resulting in cramps, nausea, and diarrhoea in humans (Jung *et al.* 2022). Humans can contract NTS infections mostly via poultry and poultry-derived products, including meat and eggs (Diaz *et al.* 2022). Epidemiological investigation has also revealed the occurrence of NTS in poultry to be a common cause of human salmonellosis (Shaji *et al.* 2023). Apart from the poultry itself, the contaminated poultry environment has also been identified as the potential source responsible for its transmission (O'Bryan *et al.* 2022).

Salmonella infection is a major problem for the poultry industry due to the illness and possible mortality risk it poses to the birds as well as the losses and decreased productivity brought as by clinical or subclinical infection (Ruvalcaba-Gómez *et al.* 2022). NTS linked to poultry causes substantial health expenses and financial difficulties for society, with an estimated 2.79 billion in costs per year (Scharff 2020).

Moreover, the NTS organisms are also burdened with a wide range of antimicrobial resistance in both human and

animal hosts, thus appearing as a major public health threat (Borah *et al.* 2022). Of all the zoonotic *Salmonella* serovars, *Salmonella* Typhimurium is found to be the most prevalent (Ferrari *et al.* 2019), as shown in a meta-analysis done on animal-based foods. Also, *S. Typhimurium* serves as an excellent model to address antibiotic-resistant bacterial persistence and transmittance through the food chain (Wang *et al.* 2019). Therefore, a farm harbouring both indigenous and exotic poultry breeds and rearing an indigenous poultry breed, Uttara fowl, which is the first recognized breed of Uttarakhand State of India (National Bureau of Animal Genetic Resources, Karnal), was selected to assess the status of NTS and its resistance to antimicrobials.

MATERIALS AND METHODS

Ethical statement: This research study was approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary and Animal Sciences. This committee functions based on the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Study design and sample collection: A cross-sectional study using simple random sampling was employed. The sample size was calculated using online software named 'Sampsize'. The calculation assumed of 5% prevalence in a farm (Samanta *et al.* 2014), 2% precision and a 95% confidence level. The average population size of layer birds in the selected farm was 4000. Samples (n=470) were collected from an organized poultry farm in Pantnagar

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Table 1. Distribution of samples collected from different breeds of layers

Sample	Uttara fowl	Kadakhnath	RIR	White leghorn	Australorp	Total
Poultry faeces	41	14	15	10	10	90
Litter	32	09	16	09	09	75
Feed	31	08	15	08	08	70
Water	26	09	15	08	07	65
Eggs	Surface	05	10	05	05	60
	Content	05	10	05	05	60
Caecal content	20	05	10	10	05	50
Total	220	55	91	55	49	470

town of Uttarakhand State from July 2018–June 2019. Details of the samples collected from different breeds are given in Table 1. Poultry faeces and litter samples were collected randomly from different spots within the flock, while feed and water samples were collected from the feeder and waterer, respectively. Eggs (unwashed) were picked from the flock, while the caeca of the dead birds, belonging to the farm and brought to the post-mortem unit of the college, were aseptically removed. All these samples were taken aseptically in an individual sterile container/bag and immediately brought to the laboratory in an ice-cooled box for further processing.

Isolation and confirmation of *Salmonella* organisms: The isolation of NTS was carried out with modifications as previously described (Keelara *et al.* 2013). Egg and caecal samples were processed for isolation as described by Pande *et al.* (2016) and Abed and Ali (2018), respectively. Further, the biochemically confirmed isolates were subjected to DNA isolation using the thermal lysis method as described previously with minor modifications (Reischl *et al.* 2000). Briefly, a loopful of culture from triple sugar iron slant was mixed into 0.1 ml nuclease-free water (Hi Media, India) in sterile 1.5 ml Eppendorf tubes to obtain a turbid suspension of bacteria. The bacterial suspension was kept in a boiling water bath for 10 min, immediately cooled at -20°C for 15 min and centrifuged at 12000 g for 5 min. The supernatant containing genomic DNA was collected in a new tube and used as a DNA template for PCR reactions. Conventional PCR targeting the *ompC* gene (204 bp) specific for the confirmation of the *Salmonella* genus was detected using forward (5'-ATCGCTGACTTATGCAATCG-3') and reverse primers (5'-CGGGTTGCGTTATAGGTCTG-3') (Alvarez *et al.* 2004). Primers were standardized using *S. Typhimurium* DNA as a positive control obtained from the department. PCR reagent concentrations and thermal cycler conditions were used as described earlier in the respective reference. Further, the amplified PCR product was resolved on agarose gel electrophoresis and visualized over a gel documentation system. All the PCR-confirmed isolates were stored as 20% glycerol stock in a -80°C deep freezer. These isolates were then sent for serotyping using the Kauffmann-White scheme (Grimont and Weill 2007) at the National Salmonella Center, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly (U.P.).

Antimicrobial susceptibility testing: The antimicrobial

resistance profile of all the isolates was determined using the standard Kirby-Bauer disk diffusion method (Bauer *et al.* 1966). A panel of 13 antimicrobials representing 7 different classes were selected as per the recommendations of CLSI (CLSI 2018) including ampicillin (AMP) 10 µg, streptomycin (S) 10 µg, nalidixic acid (NA) 30 µg, ciprofloxacin (CIP) 5 µg, levofloxacin (LE) 5 µg, gatifloxacin (GAT) 5 µg, enrofloxacin (EX) 10 µg, cefotaxime (CTX) 30 µg, ceftiofur (CX) 30 µg, cefazolin (CZ) 30 µg, sulfisoxazole (SF) 300 µg, tetracycline (TE) 30 µg and erythromycin (E) 15 µg. Diameter of zone of inhibition was measured, and breakpoints were interpreted based on the recommendations of CLSI for the disk-diffusion assay (CLSI 2018). ATCC 25922 *Escherichia coli* culture was used as a reference strain. The isolates showing resistance to three or more classes of antimicrobials were termed multidrug-resistant (Magiorakos *et al.* 2012). The MAR index of an isolate was also detected and calculated as a/b (Jayaraman *et al.* 2012), where 'a' represents the number of antibiotics to which an isolate was resistant, and 'b' represents the number of antibiotics to which an isolate was subjected to the disk diffusion assay.

RESULTS AND DISCUSSION

Occurrence and serovar distribution: An overall occurrence of *Salmonella* was found to be 4.89% (23/470) in the poultry breeds of the farm. In previous studies, a prevalence of 6.1% (Samanta *et al.* 2014) and 4.17% (Kumar *et al.* 2015) was reported from poultry flocks. Thus, it was observed that infective birds harbour and disseminate the organisms through the environment to other birds, and the extent of prevalence also depends upon the management of the flock. Amongst the different layer breeds, the highest occurrence was recorded from RIR (17.58%, 16/91), followed by Uttara fowl (2.73%, 6/220) and Kadakhnath (1.82%, 1/55). At the same time, none of the samples from White Leghorn and Australorp showed the presence. The occurrence of 0, 26.67%, 46.67% and 10% was reported from feed, faeces, water and egg samples, respectively. Samanta *et al.* (2014) observed a prevalence of 6.1% from RIR, with 10%, 15%, 20% and 10% in feed, cloacal swabs, water, and egg samples, respectively. A low presence was found in the Uttara fowl flock. As it is an indigenous breed, low prevalence and higher resistance against infection are well documented (Girmay *et al.*

2015). Moreover, in the absence of any NTS prevalence data of this breed, no evidence in support/contradiction is available. Likewise, Kadaknath, also an indigenous breed, had a low occurrence (1.82%) of *Salmonella*, explaining either the resistance or the good management of the flock. The presence of *Salmonella* was also not detected in the flocks of White Leghorn and Australorp. Hutt and Crawford (1960) reported that White Leghorn seem to be more resistant than heavy breeds such as RIR, New Hampshire or their crosses.

Among the different samples screened, the highest occurrence was found in water (7/65, 10.77%), followed by poultry faeces (6/90, 6.67%), caecal content (3/50, 6.0%), litter (4/75, 5.33%), feed (2/70, 2.86%) and egg (1/120, 0.83%). Only one sample of egg surface rinse was positive (1/60; 1.67%). Higher prevalence of 13% (Nahashon *et al.* 2008) and 20% (Samanta *et al.* 2014) was reported previously from water samples. Contamination of water might have resulted from dust particles, faecal dropping or infected birds; also, improper washing and irregular sanitization of the waterer could have further increased the contamination levels. Faecal prevalence (6.67%) in this study is similar (7.01%) to a previous study (Sharma *et al.* 2019). However, reports of high faecal prevalence (41.8%) in layers have also been observed (Im *et al.* 2015). It was expected, as *Salmonella* is a common inhabitant of poultry intestines, though better management conditions can reduce faecal excretion and prevalence. The 6% of caecal prevalence observed in this study was concurrent (6.66%) with the previous study (Tiwari *et al.* 2014). The recovery of *Salmonella* from poultry caeca is higher than from any other organs, tissues, or other parts of the gastrointestinal tract (Khan *et al.* 2015a). For egg shell samples, Jamshidi *et al.* (2010) had reported a prevalence of 1.6%, almost similar as ours (1.67%), to be positive for *Salmonella*. They also could not isolate *Salmonella* from egg content samples. On the contrary, Im *et al.* (2015) detected *Salmonella* in 17.2% and 5.2% of egg shells and egg contents, respectively. The presence of *Salmonella* in environmental samples, viz. feed and litter, is well documented in previous studies (Tabo *et al.* 2013, Hussain 2019). Singh *et al.* (2013) also reported a 2.5% prevalence from feed samples in poultry farms. *Salmonella*, being an enteric pathogen that is shed predominantly in faeces, can result in contamination of environmental samples. Serotyping revealed *Salmonella* Typhimurium as the predominant serovar (91.3%; 21/23) found, while two isolates were untypable. Akeem *et al.* (2017) also reported *S. Typhimurium* as the most frequent serovar from poultry. NARMS (2012) highlighted Typhimurium as the most associated serotype (87.8%) among NTS in chicken meat. Earlier reports also state *S. Typhimurium* as being the most common serovar isolated from poultry and animals in Pantnagar (Shekhar and Singh 2014, Tiwari *et al.* 2014).

Phenotypic antimicrobial susceptibility: Out of 23 isolates, 18 (78.26%) were MDR. Of these 23 isolates, 21 were *S. Typhimurium* and two were untypable. Since data for untypable serovars couldn't be interpreted, thereby resistance

findings were analysed for *S. Typhimurium* (n=21). The highest resistance was observed for erythromycin (21/21; 100%), which was also reported previously (Sharma *et al.* 2019). Amongst quinolones, maximum resistance for was detected for nalidixic acid (12/21; 57.14%) and ciprofloxacin (11/21; 52.38%), while low resistance was seen for enrofloxacin (5/21; 23.81%), gatifloxacin (4/21; 19.05%) and levofloxacin (2/21; 9.52%). Sharma *et al.* (2019) reported higher resistance for nalidixic acid (100%), ciprofloxacin (80%) and gatifloxacin (80%), while low resistance for levofloxacin (20%) is in harmony with this study. Nagappa *et al.* (2007) found resistance to enrofloxacin in 50% of isolates. High resistance was also seen towards ampicillin (14/21; 66.67%). Zhao *et al.* (2017) had earlier reported complete resistance against ampicillin (100%). Low resistance was expressed towards three generations of cephalosporins, viz., cefazolin (7/21; 33.33%), cefoxitin (3/21; 14.29%) and cefotaxime (6/21; 28.57%), which are also considered as clinically important antibiotics. On the contrary, Sharma *et al.* (2019) observed high resistance for all 3 generations (cefazolin and cefotaxime: 60% each and cefoxitin: 40%). In agreement with this study, Zhao *et al.* (2017) reported 30% resistance against cefotaxime. Sulfisoxazole resistance was seen in 28.57% (6/21) isolates, similar to Kumar (2016). Low resistance was also observed for streptomycin (2/21; 9.52%) and tetracycline (4/21; 19.05%). This finding is in harmony with reports of Shekhar (2012), who found 9.52% and 14.29% resistance against streptomycin and tetracycline, respectively.

MAR analysis has been used to differentiate bacteria from different sources using antibiotics that are frequently used for human treatment. Compared to other methods of source tracing of bacteria, such as genotypic characterization, MAR indexing is rapid, cost-effective and easy to perform (Khan *et al.* 2015b). In this study, the MAR index of *Salmonella* isolates was found in a range of 0.15-0.69. Compared to the previous study, Shekhar (2012) reported the MAR index in a range of 0.07-0.67. Khan *et al.* (2015b) observed the MAR index in a range of 0.06-0.56, with 0.37 being the common MAR index found in 8 isolates. MAR index values greater than 0.2 indicate a high-risk source of contamination where antibiotics are regularly used (Khan *et al.* 2015b). Details of the resistance pattern and MAR index for the isolates are given in Table 2.

The overall occurrence of NTS recorded from the layer flocks of an organized farm in the study area was low, which may be a reflection of good management procedures on the farm compared to unorganized (backyard) farming. The presence of antimicrobial resistance, particularly multidrug resistance among the isolates, represents a potential public health risk. The resistance to fluoroquinolones by NTS is of particular concern, as it is the drug of choice for treating invasive salmonellosis in adults. Therefore, WHO specifically ranked fluoroquinolone-resistant *Salmonella* as a high-priority pathogen for research and development of new antibiotics. Appropriate measures must be taken to combat increased resistance by creating awareness among

Table 2. Details of the antimicrobial resistance profile of different *Salmonella* isolates

Isolate Id	Breed	Sample	Resistance pattern (a)	MAR Index (a/b)
S-1	RIR	Egg surface	NA CIP CZ E	0.31
S-2	Uttara fowl	Feed	CTX E TE SF	0.31
S-3	Uttara fowl	Feed	E TE SF	0.23
S-4	Uttara fowl	Faeces	CZ CTX E AMP	0.31
S-5	Uttara fowl	Faeces	CZ CTX E AMP	0.31
S-6	Kadaknath	Caecal content	NA CIP LE GAT EX CZ CTX E SF	0.69
S-7	Uttara fowl	Caecal content	NA CIP LE GAT EX E AMP SF	0.62
S-8	Uttara fowl	Caecal content	NA CIP LE GAT EX E AMP SF	0.62
S-9	RIR	Water	S E	0.15
S-10	RIR	Water	NA E AMP	0.23
S-11	RIR	Water	S E	0.15
S-12	RIR	Water	NA E	0.15
S-13	RIR	Water	CIP GAT EX E AMP	0.38
S-14	RIR	Water	NA CIP CTX E AMP TE SF	0.54
S-15	RIR	Water	NA CIP CTX E AMP TE SF	0.54
S-16	RIR	Litter	CIP GAT EX E	0.31
S-17	RIR	Litter	E AMP	0.15
S-18	RIR	Litter	NA CIP CZ CX CTX E AMP	0.54
S-19	RIR	Litter	E AMP	0.15
S-20	RIR	Faeces	NA EX CZ E AMP	0.38
S-21	RIR	Faeces	NA CIP CZ CX CTX E AMP	0.54
S-22	RIR	Faeces	NA CIP CZ CX E AMP	0.46
S-23	RIR	Faeces	NA CIP CZ CX E AMP	0.46

S-5 & S-6: untypable strains

a: number of antibiotics to which an isolate was resistant; b: number of antibiotics to which an isolate was subjected, i.e., 13

high-risk groups, seeking better alternatives to resistant antimicrobials, implementing policies and laws on national and international levels, and most importantly, prohibiting the use of higher-generation antimicrobials in poultry that are supposed to be the last resort for treating *Salmonella* infections. More extensive studies on layer breeds are required, particularly Uttara fowl (a newly registered local breed of Uttarakhand), to assess the status of NTS in this breed and to suggest different ways to curb its infection.

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