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Isolation and characterisation of multi-drug resistant non-typhoidal Salmonella spp. from cloacal samples of broilers in the Wayanad district

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Abstract

The purpose of this study was to investigate the occurrence of multi-drug resistant non-typhoidal Salmonella spp. (MDR-NTS) in Wayanad district, Kerala. Initially, the cloacal swabs (n = 31) collected from different broiler farms in the Wayanad district were subjected to isolation and identification of Salmonella spp. and confirmation by polymerase chain reaction (PCR). A total of 24 (77.42 %) Salmonella spp. isolates were recovered by isolation and identification in selective media and confirmed by PCR assay. Out of the 24 confirmed isolates, 20 (83.33 %) were confirmed to be NTS strains, of which 2 (8.33 %) belonged to S. Typhimurium and 18 (75 %) were S. Enteritidis. Later, the antibiotic susceptibility testing was carried out by a disc diffusion assay using 8 different classes of antibiotics. Of the 24 isolates tested, 16 (66.67 %) were resistant to more than 4 classes of antibiotics, 10 (41.67 %) were resistant to more than or equal to 6 classes of antibiotics, and 2 (8.33 %) were found to produce extended-spectrum beta-lactamase. Out of the 24 Salmonella spp. isolated, 21 (87.5 %) were confirmed to be MDR in nature, while 18 NTS strains exhibited MDR pattern. The occurrence of MDR-NTS strains in broilers necessitates strict biosecurity measures to be adopted to reduce disease incidence and avoid indiscriminate use of antibiotics in broiler farms to safeguard public health.

Keywords: *Antibiotic, broiler chicken, multi-drug resistance, non-typhoidal Salmonella*

Poultry meat represents the fastest-growing segment of worldwide meat production and export. The broiler industry plays a crucial role in meeting human nutritional requirements, particularly the demand for protein. According to BAHS (2023), about 51.14% of the total meat production in the country is contributed by the poultry sector which has shown consistent growth in 2022-23 with a 4.52% increase when compared to the previous year. One of the primary challenges in broiler production is the occurrence of clinical or subclinical bacterial infections. The chicken gut typically hosts organisms such as *Salmonella* spp., *Escherichia coli*, *Staphylococcus* spp. and *Streptococcus* spp. which can lead to pathological burdens resulting in reduced feed efficiency and production losses.

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Poultry and poultry products constitute the main sources of *Salmonella* spp. compared to other animal products. *Salmonella* spp. of poultry origin passes through the food chain and is finally transmitted to humans creating huge public health problems worldwide (CDC, 2019; Wang *et al*., 2020). Among *Salmonella* spp., *S. enterica* sub spp. *enterica* comprises the zoonotic *Salmonella* spp. of which *S.* Typhimurium and *S*. Enteritidis are the major pathogens associated with food-borne illnesses. The outbreaks of salmonellosis were reported internationally as well as nationally (Kumar *et al*., 2010; Scallan *et al*., 2011; Rajagopal and Mini, 2013; Stanaway *et al*., 2019) resulting in a significant increase in in-house mortality and huge economic loss to the farmers.

The increasing demand for poultry products, coupled with the rising incidence of bacterial infections in flocks, has led to the utilisation of antibiotic growth promoters. These promoters, known for their cost-effectiveness and simplicity, have been employed for several decades to enhance poultry farm performance (Anand *et al*., 2018). Apart from the economic benefit, food safety issues also arise due to the potential misuse of antibiotics as growth promoters in food animals. There are restrictions on the sub-therapeutic use of antibiotics in poultry as it can lead to the development of antibiotic resistance (Apata, 2009). In both animal and human populations, *Salmonella* spp. has been consistently developing resistance to commonly used antibiotics (Molla *et al*., 2003). Considering these facts, the present study was conducted to investigate the prevalence of multi-drug resistant non-typhoidal *Salmonella* spp. (MDR-NTS) in the selected broiler farms of Wayanad district, Kerala.

Materials and methods

Isolation of Salmonella spp.

The cloacal swabs (n = 31) collected aseptically from five organised poultry farms of the Wayanad district of Kerala were brought to the laboratory under chilling conditions. The samples were subjected to pre-enrichment in sterile buffered peptone water (BPW; 1:9 proportion) at 37 °C for 18-24 h. Later, enrichment was carried out in Rappaport-Vassiliadis (RV) broth at 42 °C for 18-24 h. After enrichment, a loopful of culture was streaked on Xylose Lysine Deoxycholate (XLD) agar and incubated at 37 °C for 18-24 h to observe characteristic red colonies, with black centres, which were identified presumptively as *Salmonella* spp.

Confirmation of Salmonella spp.

The presumptive colonies were then subjected to a polymerase chain reaction (PCR) assay employing *invA* gene for genus-level identification. The confirmed *Salmonella* spp. was then subjected to PCR assays employing *spy* and *sdf1* genes for the confirmation of non-typhoidal *Salmonella* (NTS) strains *i.e.*, *S.* Typhimurium and *S*. Enteritidis, respectively (Nair *et al.,* 2015). The PCR was carried out using the bacterial DNA extracted by the hot-cold lysis method and performed in an automated thermal cycler (Bio-Rad, USA). The amplified genomic DNA was separated on 1.40 % agarose gel prepared in 1X Tris-acetate- EDTA buffer containing ethidium bromide. The obtained bands were visualized and documented under a gel documentation system (Bio-Rad, USA).

Antibiotic susceptibility testing

The confirmed isolates obtained in the present study were subjected to antibiotic susceptibility testing employing disc diffusion assay (Bauer *et al.*, 1966) as per the guidelines provided by the Clinical Laboratory Standards Institute (2019). The commercial antibiotic discs used in this study included doxycycline hydrochloride (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ampicillin (2 µg), azithromycin (15 µg) and meropenem (10 µg). Further, the antibiotics ceftazidime (30 µg), ceftazidime- clavulanic acid (30/10 µg), cefotaxime (30 µg) and cefotaxime - clavulanic acid (30/10 µg) were used to determine the extended-spectrum beta-lactamase (ESBL) producing ability of the confirmed isolates.

Results and discussion

Out of the 31 samples collected from the broiler farms in the Wayanad district, 24 (77.42 %) were identified to be *Salmonella* spp. on selective culturing in XLD agar. Further, all 24 (100 %) recovered *Salmonella* spp. were confirmed by PCR employing *invA* gene and 20 (83.33 %) were identified as NTS. In PCR studies employing *spy* and *sdf1* genes, 18 (75 %) of recovered NTS were identified as *S.* Enteritidis and 2 (8.34 %) samples were *S*. Typhimurium

Fig. 1. PCR- based confirmation of *S*. Typhimurium (1-24)
L- DNA Marker ladder (100 bp), N- Negative Control

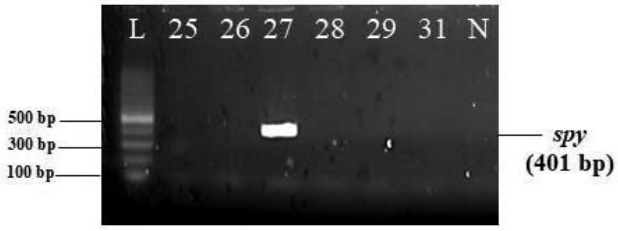

Fig. 2. PCR- based confirmation of *S*. Typhimurium (25- 31)
L- DNA Marker ladder (100 bp), N- Negative Control

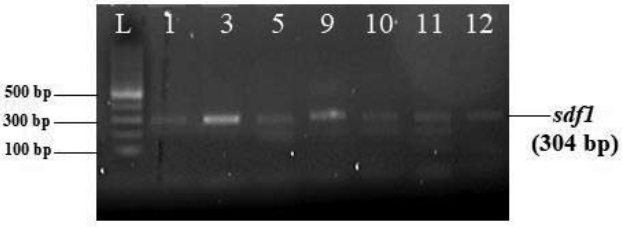

Fig. 3. PCR- based confirmation of *S*. Enteritidis (1- 12)
L- DNA Marker ladder (100 bp), N- Negative Control

(Fig 1- 4). Kerr *et al.* (2013) reported that 22.3 per cent of cloacal swabs collected from poultry exhibited the presence of *Salmonella* spp. Elkenany *et al.* (2019) also reported that *S.* Enteritidis (30 %) and *S.* Typhimurium (28.5 %) were the most common *Salmonella* spp. isolated from poultry farms through the cloacal swab method. In the present study also, molecular characterisation confirmed the presence of 83.33 % NTS in the samples collected *i.e*., *S.* Enteritidis (75 %) and *S.* Typhimurium (8.34 %).

All the confirmed isolates were evaluated by antibiotic susceptibility testing using a disc diffusion assay. Out of the 24 *Salmonella* spp. isolates, 21 (87.5 %) were observed to be MDR- *Salmonella* spp. and among the NTS isolates, 18 (75 %) were MDR-NTS. Interestingly, two of the isolates were found to exhibit ESBL-producing ability. Out of 24 isolates, 41.60 % (10/24) of the *Salmonella* isolates were showing resistance against 6 classes of antibiotics. Among NTS isolates, 45% (9/20) were showing resistance to more than 6 classes of antibiotics. Earlier studies by Salehi *et al*. (2005), Alcaine *et al.* (2007), McDermott *et al.* (2016), Liljebjelke *et al*. (2017), Sharma *et al*. (2019), Gayathridevi *et al*. (2021) and Jayaweera *et al*. (2020) also stated that the isolated NTS strains from broilers, carcass and poultry farm environment exhibited MDR pattern.

The *Salmonella* isolates recovered in our study were resistant to ciprofloxacin (87.5 %), azithromycin (87.5 %), doxycycline (70.83 %), ampicillin (79.17 %), cefotaxime (62.5 %), ceftazidime (58.33 %), gentamicin (50 %), ceftazidime and clavulanic acid (37.50 %), chloramphenicol (29.17 %), meropenem (29.17 %), cefotaxime and clavulanic acid (8.33 %), and intermediate to meropenem (45.83 %), gentamicin (25 %), chloramphenicol (20.83 %), doxycycline hydrochloride (12.5 %), ciprofloxacin (8.33 %), azithromycin (8.33 %). Earlier reports by Gayathridevi *et al*. (2021) and Liljebjelke *et al*. (2017) also stated that the isolated *Salmonella* spp. were resistant to ciprofloxacin (53.00 %), gentamicin (80 %), erythromycin (100 %), ampicillin (100 %), tetracycline (87 %), chloramphenicol (87 %), streptomycin (80 %), gentamicin (12.6 %), trimethoprim-sulfamethoxazole (8.6 %), sulfadimethoxine (20.9 %), streptomycin (30.9 %), and tetracycline (13.9 %). The alarming drug resistance observed among *Salmonella* spp. could be due to the presence of drug-resistant *Salmonella* residing in the intestine and liver of broiler chickens in most of the cases (Salehi *et al*., 2005) and attributed to the plasmid-encoded gene transfer (Molla *et al*., 2003). Antibiotic resistance, intermediate resistance and susceptibility patterns of isolated *Salmonella* spp. are presented in Table 1 and Fig 5.

The alarming rise of antibiotic resistance in NTS strains has critical public health implications, as it not only compromises treatment options but also risks transmission of resistance to humans, highlighting the urgent need for stringent biosecurity measures and the development of safer antibiotic alternatives to mitigate this threat.

Conclusion

The present study investigated the occurrence of MDR-NTS strains recovered from five organised poultry farms in the Wayanad district of Kerala. In the present study, an alarming drug resistance pattern was observed among the recovered NTS strains isolated from the poultry farms. This study warrants stringent biosecurity measures to reduce the incidence of disease and a vigil

Fig. 4. PCR- based confirmation of *S*. Enteritidis (13- 31)
L- DNA Marker ladder (100 bp), N- Negative Control

Table 1. Antibiotic susceptibility pattern of *Salmonella* spp. isolates

ISOLATES	DO (30 µg)	C (30 µg)	GEN (10 µg)	CIP (5 µg)	AMP (2 µg)	AZM (15 µg)	MRP (10 µg)	ESBL			
								CAZ (30 µg)	CAC (30/10 µg)	CTX (30 µg)	CEC (30/10 µg)
1	R	R	R	R	R	R	R	R	R	R	S
3	R	S	R	R	R	I	I	S	S	I	S
5	R	S	R	R	S	R	I	R	S	S	S
9	I	S	R	R	R	R	R	R	R	R	S
10	R	I	I	R	R	R	S	R	S	I	S
11	R	R	R	R	S	R	R	S	S	R	S
12	R	S	I	R	S	R	I	R	R	R	S
13	R	I	S	R	R	R	S	S	S	S	S
15	S	S	S	I	R	I	S	R	R	R	S
16	R	I	I	R	R	R	I	R	R	R	R
17	R	R	R	R	R	R	I	R	R	R	S
18	S	S	S	I	S	S	S	R	S	R	S
19	S	S	S	S	R	R	S	S	S	S	S
20	I	S	R	R	R	R	R	I	S	R	S
21	R	R	R	R	R	R	I	S	S	I	S
22	I	S	R	R	S	R	R	S	S	R	S
23	R	R	R	R	R	R	R	R	R	R	S
24	R	S	I	R	R	R	I	S	S	I	S
25	R	S	I	R	R	R	R	R	S	R	S
26	S	S	S	R	R	R	S	S	S	S	S
27	R	R	R	R	R	R	I	R	R	R	S
28	R	I	R	R	R	R	I	R	R	R	R
29	R	I	I	R	R	R	I	R	S	R	S
31	R	R	S	R	R	R	I	S	S	S	S
R (%)	70.83	29.17	50.00	87.50	79.17	87.50	29.17	58.33	37.50	62.50	8.33
I (%)	12.50	20.83	25.00	8.33	0.00	8.33	45.83	4.17	0.00	16.67	0.00
S (%)	16.67	50.00	25.00	4.17	20.83	4.17	25.00	37.50	62.50	20.83	91.67

(R- Resistance, I- Intermediate Resistance, S- Sensitivity)

(DO- Doxycycline, C- Chloramphenicol, GEN- Gentamicin, CIP- Ciprofloxacin, AMP- Ampicillin, AZM- Azithromycin, MRP Meropenem, CAZ- Ceftazidime, CAC- Ceftazidime/ clavulanic acid, CTX- Cefotaxime, CEC- Cefotaxime/ clavulanic acid).

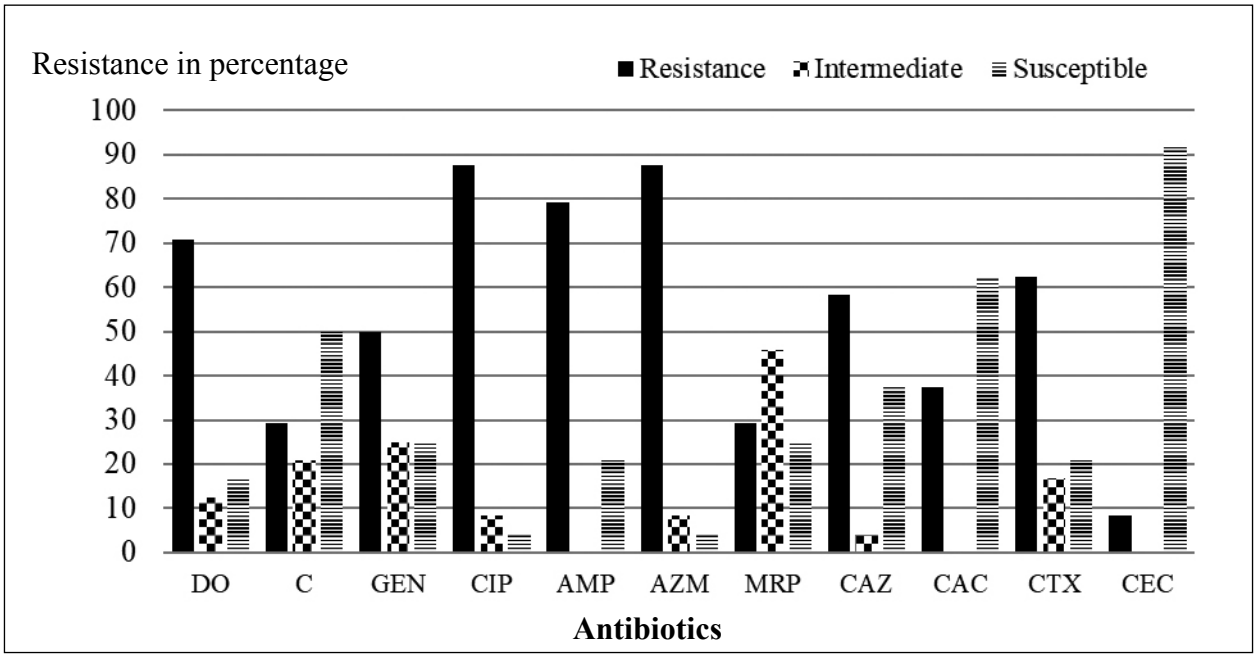

Fig. 5: Antibiotic resistance pattern of *Salmonella* spp.

on the indiscriminate use of antibiotics in broiler farms to safeguard public health.

Conflict of interest

Authors have no conflicts of interest to disclose.

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