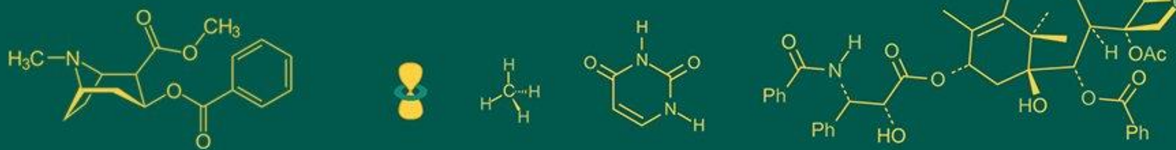

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Antibiotic resistance genes characterization of non-typhoidal *Salmonella* serovars of layers

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Abstract
This study aimed to detect the presence of antibiotic resistance genes (ARGs) in 23 non-typhoidal *Salmonella* (NTS) isolated from layer breeds of an organized poultry farm. Phenotypically resistant isolates (including intermediate) for different antibiotics were subjected to PCR for detection of ARGs. Among quinolone-resistant isolates, *gyrA, parC* and *qnrS* genes were found in 100%, 95.65% and 4.35%, respectively. Other genes such as β-lactams resistant bla_{TEM}, streptomycin-resistant *aadA1* and sulfisoxazole-resistant *sul1* were found in 21.74%, 50% and 42.86%, respectively. None of the isolates was found positive for tetracycline-resistant genes screened. Overall, this study detected a few genetic determinants of antibiotic resistance in the isolates that can give rise to the possibility of transmission to the environment or other bacteria. Regular monitoring and surveillance of NTS for various antibiotic resistance genes need to be emphasized to address public health threats.

Keywords: Antibiotic resistance genes, non-typhoidal *Salmonella*, layer breeds, surveillance, public health

Introduction

Non-typhoidal *Salmonella* (NTS) is one of the leading causes of food poisoning in the world with an approximate yearly incidence of 1.3 billion cases and 3 million fatalities (Pragasam *et al*., 2019) [28]. The burden caused by NTS can be more complicated due to infections by multidrug-resistant (MDR) bacteria (Prestinaci *et al*., 2015) [29]. Food-producing animals, particularly poultry and their products have emerged as significant reservoirs for drug-resistant bacteria due to the widespread use or misuse of common antimicrobial agents in veterinary and human medicine for treating and preventing infections, aside from their application for growth-promoting purposes (Jajere, 2019) [16]. Furthermore, through the food chain, drug-resistant bacteria like *Salmonella enterica* serovars can spread from these animals to humans, reducing the number of antimicrobial treatments available for severe salmonellosis (Ghazaey and Mirmomeni, 2012) [14]. As *Salmonella* infection is becoming more prevalent, concern over its resistance to last-line treatments is growing (Borowiak *et al*., 2020) [7].

Antimicrobial resistance (AMR) has been identified as a significant threat to public health worldwide (Hernando-Amado *et al*., 2019) [15]. AMR was directly responsible for 1.27 million deaths in 2019, according to a global study published in The Lancet (Murray *et al*., 2022) [38]. According to a report commissioned by the UK government, AMR may kill 10 million people annually by 2050 (O'Neill, 2016) [25]. AMR in bacteria can be linked to mutations in endogenous genes or acquisitions of antibiotic resistance genes (ARGs) through plasmids, transposons, and integron-associated resistance gene cassettes. Antibiotic resistance genes, which bacteria can acquire to develop antibiotic resistance, are primarily responsible for the rising risk of AMR. ARGs in the environment can be transferred both horizontally and vertically among the microbial population, which promotes the spread of AMR (Larsson and Flach, 2022) [19]. ARGs induce resistance against many antibiotics, such as beta-lactams, quinolones, sulfonamide, tetracycline, aminoglycoside, macrolide, chloramphenicol, glycopeptide, trimethoprim, etc. (Ezzariai *et al*., 2018; Singh *et al*., 2019) [12, 35]. The integron-associated resistance gene cassettes provide flexibility to the bacterial host and facilitate the dissemination of these genes (ARGs) across diverse bacterial

populations (Blair *et al*., 2015) [5]. Class 1 integrons are predominant in the *Enterobacteriaceae* family and encompass different gene cassettes encoding different resistance genes (Lamas *et al*., 2016) [18]. Taking into account the importance of these resistance genes in accelerating the spread of AMR, the present study was undertaken to detect different antibiotic resistance genes in non-typhoidal *Salmonella* serovars obtained from layer breeds of an organized poultry farm.

Materials and Methods

Salmonella isolates

The study was conducted on 23 non-typhoidal *Salmonella* isolates detected from different layer breeds of an organized poultry farm in Pantnagar town of Uttarakhand State (Nagpal, 2019) [22]. The isolates consisted of *Salmonella* Typhimurium (n=21) and untypable strains (n=2) obtained from different samples *viz.* water (n=7), poultry faeces (n=6), caecal content (n=3), litter (n=4), feed (n=2) and egg surface (n=1) of layer breeds *viz.* Rhode Island Red (n=16), Uttara fowl (n=6), and Kadaknath (n=1). All the isolates were revived in nutrient broth (HiMedia, India) from the respective 20% glycerol stock kept at −80 °C. After 24 hours of incubation at 37 ºC, cultures were streaked on XLT-4 agar (BD Difco, USA) and confirmed again for *Salmonella*.

Detection of antibiotic resistance genes (ARGs)

All resistant and intermediate-resistant *Salmonella* isolates (n=23) obtained upon disk diffusion test were subjected to PCR for detecting antibiotic resistance genes (ARGs) against the respective antibiotics. The presence of 16 ARGs was detected *viz.* β-lactams (ampicillin, cefazolin, cefoxitin, and cefotaxime) resistance coding bla_{TEM} (Randall *et al.,* 2004) [30], bla_{PSE-1} (Carlson *et al.,* 1999) [8], and bla_{CMY-2} (Zhao *et al.,* 2001) [37], streptomycin resistance coding *aadA1, aadA2, strA* and *strB* (Madsen *et al.,* 2000) [20], sulphonamide (sulfisoxazole) resistance coding *sul1* (Randall *et al.,* 2004) [30] and *sul2* (Aarestrup *et al.,* 2003) [1], tetracycline resistance coding *tetA*, *tetB,* and *tetG* (Ng *et al.,* 1999) [24] and quinolones (nalidixic acid, ciprofloxacin, levofloxacin, gatifloxacin and enrofloxacin) resistance coding *gyrA* (Chau *et al*., 2007) [9], *parC* (Chau *et al*., 2007) [9], *qnrA* (Minh Vien *et al*., 2009) [21] and *qnrS* (Minh Vien *et al*., 2009) [21]. Primer sequences and respective amplicon sizes were used as described earlier in respective references. DNA isolation was done using the thermal lysis method (Reischl *et al.,* 2000) with some modifications. Briefly, a loopful of culture from XLT-4 agar was mixed into 0·1 ml nuclease-free water (HiMedia, 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 12000g for 5 min. The supernatant containing genomic DNA was collected in a new tube and used as a DNA template for PCR reactions. The reaction mixture of 25 µl was set up for each gene containing 2.5 µl of 10X Taq buffer (Tris HCl with 15 mM $MgCl_2$) (GeNei, India), 1 µl of each dNTP (2.5 mM) (GeNei, India), 1 µl of each primer (10 pmol) (IDT, India), 1U Taq DNA polymerase (GeNei, India) and 2 µl of DNA template (50-100 ng per µl). Nuclease-free water was added to make the final volume. Cycling conditions for PCR including annealing temperature for different genes were used as described earlier in respective references. Amplified PCR products were electrophoresed on 1.5% agarose gel visualized over a gel documentation system.

Results and Discussion

Presence of antibiotic resistance genes (ARGs)

Salmonella isolates showing resistance (including intermediate) to β-lactams, streptomycin, sulfisoxazole, tetracycline and quinolones were further subjected to PCR for the detection of corresponding resistance genes (bla_{TEM}, bla_{PSE-1}, and bla_{CMY-2}; *aadA1, aadA2, strA* and *strB*; *sul1* and *sul2; tetA*, *tetB* and *tetG; gyrA*, *parC*, *qnrA* and *qnrS*). Of the 23 isolates showing resistance to β-lactams, 5 isolates (21.74%) carried bla_{TEM} while none of them showed the presence of bla_{PSE-1} and bla_{CMY-2} genes. The gene bla_{TEM} was found in 5 *S.* Typhimurium serovar. Of the 4 isolates showing resistance to streptomycin, 2 isolates (50%) carried *aadA1,* while none of them were found positive for *aadA2, strA,* and *strB* genes. The gene *aadA1* was found in 2 *S.* Typhimurium serovar. Of the 7 isolates showing resistance to sulfisoxazole, 3 isolates (42.86%) carried *sul1*, while none showed the presence of *sul2* gene. The gene *sul1* was found in 2 *S.* Typhimurium and 1 untypable serovar. Of the 4 isolates showing resistance to tetracycline, none of them was found to carry any of the studied genes (*tetA, tetB,* and *tetG*). Of the 23 isolates showing resistance to quinolones, all the isolates (21 *S.* Typhimurium and 2 untypable) carried *gyrA*, 22 isolates (20 *S.* Typhimurium and 2 untypable) carried *parC* (95.65%), 1 isolate (*S.* Typhimurium) carried *qnrS* (4.35%) while the *qnrA* gene was not found in any isolate (Table 1). The details of the antibiotic resistance genes profile of *Salmonella* isolates are shown in Table 2.

Table 1: Distribution of ARGs in the resistant *Salmonella* serovar

Antibiotics	Phenotypical resistant isolates (R+I)	Genes detected upon PCR	No. of isolates (%)	*S.* Typhimurium (%)	Untypable (%)
β-lactams	23	bla_{TEM}	5 (21.74)	5 (23.81)	0
		bla_{PSE-1}	0	0	0
		bla_{CMY-2}	0	0	0
Streptomycin	4	*aadA1*	2 (50)	2 (50)	0
		aadA2	0	0	0
		strA	0	0	0
		strB	0	0	0
Sulfisoxazole	7	*sul1*	3 (42.86)	2 (33.33)	1 (100)
		sul2	0	0	0
Tetracycline	4	*tetA*	0	0	0
		tetB	0	0	0
		tetG	0	0	0
Quinolones	23	*gyrA*	23 (100)	21 (100)	2 (100)
		parC	22 (95.65)	20 (95.24)	2 (100)
		qnrA	0	0	0
		qnrS	1 (4.35)	1 (4.76)	0

*R- Resistant; I- Intermediate

Table 2: Antibiotic resistance genes (ARGs) profile of *Salmonella* isolates (n=23)

Isolate Id.	Layer Breed	Sample type	Serovar	Phenotypic Resistance Pattern	Resistance genes
S-1	Rhode Island Red	Egg surface	Typhimurium	NA CIP CZ E	*gyrA*
S-2	Uttara fowl	Feed	Typhimurium	CTX E TE SF	*aadA1, gyrA, parC*
S-3	Uttara fowl	Feed	Typhimurium	E TE SF	*aadA1, gyrA, parC*
S-4	Uttara fowl	Faeces	Typhimurium	CZ CTX E AMP	*gyrA, parC*
S-5	Uttara fowl	Faeces	Untypable	CZ CTX E AMP	*gyrA, parC*
S-6	Kadaknath	Caeca	Untypable	NA CIP LE GAT EX CZ CTX E SF	*sul1, gyrA, parC*
S-7	Uttara fowl	Caeca	Typhimurium	NA CIP LE GAT EX E AMP SF	*sul1, gyrA, parC*
S-8	Uttara fowl	Caeca	Typhimurium	NA CIP LE GAT EX E AMP SF	*sul1, gyrA, parC*
S-9	Rhode Island Red	Water	Typhimurium	S E	*gyrA, parC*
S-10	Rhode Island Red	Water	Typhimurium	NA E AMP	*gyrA, parC*
S-11	Rhode Island Red	Water	Typhimurium	S E	bla_{TEM}, *gyrA, parC*
S-12	Rhode Island Red	Water	Typhimurium	NA E	bla_{TEM}, *gyrA, parC*
S-13	Rhode Island Red	Water	Typhimurium	CIP GAT EX E AMP	bla_{TEM}, *gyrA, parC*
S-14	Rhode Island Red	Water	Typhimurium	NA CIP CTX E AMP TE SF	bla_{TEM}, *gyrA, parC, qnrS*
S-15	Rhode Island Red	Water	Typhimurium	NA CIP CTX E AMP TE SF	bla_{TEM}, *gyrA, parC*
S-16	Rhode Island Red	Litter	Typhimurium	CIP GAT EX E	*gyrA, parC*
S-17	Rhode Island Red	Litter	Typhimurium	E AMP	*gyrA, parC*
S-18	Rhode Island Red	Litter	Typhimurium	NA CIP CZ CX CTX E AMP	*gyrA, parC*
S-19	Rhode Island Red	Litter	Typhimurium	E AMP	*gyrA, parC*
S-20	Rhode Island Red	Faeces	Typhimurium	NA EX CZ E AMP	*gyrA, parC*
S-21	Rhode Island Red	Faeces	Typhimurium	NA CIP CZ CX CTX E AMP	*gyrA, parC*
S-22	Rhode Island Red	Faeces	Typhimurium	NA CIP CZ CX E AMP	*gyrA, parC*
S-23	Rhode Island Red	Faeces	Typhimurium	NA CIP CZ CX E AMP	*gyrA, parC*

Among the β-lactams resistance genes screened (bla_{TEM}, bla_{PSE-1}, and bla_{CMY-2}), bla_{TEM} (21.74%) was the single gene found in β-lactam resistant *S.* Typhimurium (23.81%). In agreement with this finding, Siddiky *et al.* (2022) [34] detected only the bla_{TEM} gene out of all β-lactam resistance genes tested for *S.* Typhimurium in poultry processing environmental samples. This gene has been also reported in *Salmonella* isolated from poultry and humans (Djeffal *et al.,* 2017) [11] and pigs (Trongjit *et al.,* 2017) [36]. Due to the acquisition of resistance genes encoding extended-spectrum β-lactamase (ESBL), the attainment of resistance to β-lactams among NTS isolates has been increasing gradually and is a major public health concern. Among streptomycin resistance genes screened (*aadA1, aadA2, strA, and strB*), *aadA1* (50%) was the only gene found in streptomycin-resistant *S.* Typhimurium (50%). A high prevalence of the *aadA1* gene among all streptomycin resistance genes tested (*aadA1*, *aadA2, aacC2*, *Kn*, *aph(3)-IIa* and *aac(3)-Iva*) was also reported previously (Chen *et al*., 2004) [10]. On the contrary, Pezzella *et al*. (2004) [27] detected *strA and strB* genes in 84% of the streptomycin-resistant isolates.

Sulfisoxazole-resistant isolates carried *sul1* (42.86%), while none showed the presence of *sul2* gene. The prevalence of *sul1* over the other genes (*sul2* and *sul3*) was also observed by Siddiky *et al*. (2022) [34]. Ahmed *et al.* (2016) [3] identified the *sul1* gene in 96.7% of sulfonamide-resistant *S.* Typhimurium from chicken and humans. On the contrary, Keelara *et al.* (2014) [17] reported a predominance of *sul2* gene in MDR *S.* Typhimurium. None of the tetracycline-resistant isolates in the study carried *tetA, tetB,* or *tetG* genes. On the contrary, the occurrence of the *tetA* gene among the other genes screened in all tetracycline-resistant *S.* Typhimurium isolates was reported earlier (Sharma *et al*., 2019; Siddiky *et al*., 2022) [33, 34]. Adesiji *et al.* (2014) [2] identified the presence of four tetracycline-resistant genes (*tetA*, *tetB*, *tetC,* and *tetG)* in MDR *Salmonella* isolates from retail meat, poultry feces, and clams. The absence of the respective genes in the studied isolates suggests the involvement of some other resistance determinants in those isolates that conferred resistance to tetracycline (Borah *et al*., 2022) [6].

Predominance of *gyrA* (100%) and *parC* (95.65%) genes were found in quinolone-resistant isolates. Nahar and Rashid (2018) [23] reported the presence of *gyrA* and *parC* in 95.56% and 68.89% of the isolates, respectively. One isolate (4.35%) in the study carried *qnrS* and none of the isolates showed the presence of *qnrA* gene. Ata *et al*. (2014) [4] detected *qnrS* in 2% of the isolates while, all the isolates were found negative for *qnrA, qnrB,* and *aac(6')-Ib-cr* genes. The fluoroquinolone resistance can be linked to multiple mutations in quinolone resistance-determining regions (QRDRs) of the genes that encode for DNA gyrase (i.e., *gyrA* and *gyrB*) and DNA topoisomerase IV (i.e., *parC* and *parE*), which are the targets for fluoroquinolones in bacterial cells or acquisition of plasmid-mediated quinolones resistance (PMQR) genes such as *qnrA, qnrS,* etc. The presence of genes (*gyrA* and *parC*) in almost all the isolates was confirmed using PCR but further investigation in accessing the mutation in these genes can be carried out through sequencing to confirm their role in exhibiting resistance to fluoroquinolones.

Conclusion

The findings of this study show the presence of few antibiotic resistance genes in the non-typhoidal *Salmonella* serovars of layers with the potential to spread to other bacteria or the environment, further aggravating the risks to public health. Thus, assessment of the distribution of resistance genes in NTS denotes a more comprehensive and possibly an additional beneficial tool for improving our understanding of antibiotic resistance epidemiology. More extensive studies on the investigation of other genetic determinants in the isolates responsible for resistance can be carried out.

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