Antibiotic Resistance Profile of Rarely Isolated Salmonella Serotypes *from Poultry in Turkey*

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Veterinaria Italiana 2023, **59** (4), xx-xx doi: 10.12834/VetIt.3004.20698.2
Accepted: 08.03.2023 | Available on line: 31.12.2023

Keywords
Salmonella, Antibiotic Resistance, Poultry

Summary
This study investigated five strains of each serotype of *Salmonella* Agona, *Salmonella* Heidelberg, *Salmonella* Hindmarsh, *Salmonella* Kouka, *Salmonella* Muenchen, *Salmonella* Ottmarchen, *Salmonella* Saintpaul and *Salmonella* II, isolated between 2014-2017. Disc diffusion was used to identify the phenotypic profiles of antibiotic resistance to 12 antimicrobials while the presence of antibiotic resistance genes (ARGs) was detected by PCR. The most sensitive serotype was *S.* Kouka while the most resistant serotypes were *S.* Agona and *S.* Heidelberg. MDR was detected most frequently in *S.* Agona strains, followed by *S.* Saintpaul, *S.* Hindmarsch, and *S.* Ottmarchen. The samples were most susceptible to chloramphenicol and ceftazidime and most resistant to sulfonamide. The resistance genes were detected in phenotypically resistant strains. Among the tetracycline-resistant strains, *tet (A)* was the most prevalent gene. The results of this study highlight the importance of monitoring antibiotic resistance profiles and related genes, which can spread to form MDR bacteria. *Salmonella* spp., which significantly contribute to ARG dissemination, should be monitored constantly to protect the closely related health of humans, animals, and the environment. The level of antibiotic resistance observed in this study, even in rarely isolated *Salmonella* serotypes, also indicates the need for careful and selective use of antibiotics.

Introduction

Salmonellosis is a major foodborne infection caused by non-typhoidal *Salmonella* (NTS) serotypes. NTS has a significant global impact on human health, with approximately 93.8 million infections and 155,000 deaths each year (Majowicz *et al*. 2010). *Salmonellae* agents are transmitted to humans both directly and indirectly. Contaminated poultry and its products are major source of *Salmonella* in human salmonellosis (Shang *et al*. 2021). According to the EFSA zoonotic agents report in 2021 the most common serotypes in humans and poultry are *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Infantis. Other *Salmonella* serotypes have been reported with prevalence of less than 0.01% (EFSA, 2021). In recent years, the prevalence of *Salmonella* serotypes (*S.* Derby, *S.* London, *S.* Rissen, *S.* Anatum) other than *S.* Enteritidis, *S.* Typhimurium, and *S.* Infantis has increased in the poultry industry (Chen *et al*. 2019, Chang *et al*. 2020) suggesting that previously non-prevalent *Salmonella* serotypes may become dominant.

Antimicrobial therapy is the most common method for treating human and animal salmonellosis infections. However, this is hindered by both misuse and overuse, leading to treatment failures, higher costs, and increased mortality. One of the most important negative consequences is the development of resistant bacterial strains since *Salmonella* is one of the critical resistant pathogens in foodborne outbreaks worldwide (Hashempour-Baltork *et al*. 2019). In recent years, the emergence and spread of antimicrobial-resistance (AMR) in *Salmonella* spp. isolates have been reported worldwide (EFSA, 2020). In particular, the increase in multi-drug resistant (MDR) *Salmonella* strains

Please refer to the forthcoming article as: Müştak *et al*. 2023. Antibiotic Resistance Profile of Rarely Isolated *Salmonella Serotypes* from Poultry in Turkey. Vet Ital. doi: 10.12834/VetIt.3004.20698.2

has caused global concern (Shang *et al*. 2021). Accordingly, the World Health Organization (WHO) includes *Salmonella* in its list of critical pathogens (WHO, 2014).

According to the WHO's global antimicrobial surveillance report, the emergence of multidrug-resistant non-typhoidal *Salmonella* in many countries is an important public health problem (WHO, 2022). The only way to protect human health is the proper use of antibiotics, and monitoring of antibiotic resistance profiles and related genes. *Salmonella* spp. can provide important reservoirs of antibiotic resistance genes that contribute to the emergence multidrug-resistant bacteria (Bakkeren *et al*. 2019). AMR is now recognized as a zoonosis causing problems in hospitals, the environment, and animals (Polianciuc *et al*. 2020).

In Turkey, *Salmonella* spp. strains have high resistance profiles to nalidixic acid, sulphonamides, tetracycline, trimethoprim, streptomycin, and ampicillin (Sahan *et al*. 2016, Kızıl, 2020). This study aims to contribute to the analysis of these antibiotic resistance profiles and investigate related resistance genes in *Salmonella* serotypes, which are rarely isolated from chickens. It is known that serotypes such as *S.* Infantis and *S.* Newport, which were rarely seen in other countries before 1997, became a dominant serotype (Zhao *et al*. 2003, Asai *et al*. 2007). Given the potential of *Salmonella* serotypes included in the study to become predominant serotype in Turkey, it is crucial to monitor the antibiotic resistance profiles of these *Salmonella* serotypes.

Material and Methods

Bacterial strains

Five strains of each serotype of *S.* Agona, *S.* Heidelberg, *S.* Hindmarsh, *S.* Kouka, *S.* Muenchen, *S.* Ottmarchen, *S.* Saintpaul, and *S.* II (9,12: g, m ,t:-) were used. These were isolated in the Faculty of Veterinary Medicine Department of Microbiology, Ankara University, Turkey between 2014 and 2017. Table I shows the origins and serotypes of the *Salmonella* strains.

Table I. *Source and serotypes of* Salmonella *strains*

Serotype	Origin	Number of Strains
S. Agona	Cloacal swab/ Broilers Environmental sample/ Broilers	3 2
S. Heidelberg	Litter/ Broilers	5
S. Hindmarsch	Pellet feed/ Broilers Environmental sample/ Broilers	2 3
S. Kouka	Environmental sample/ Broilers	5
S. Muenchen	Cloacal swab/ Broilers	5
S. Ottmarchen	Litter/ Broilers Environmental sample/ Broilers	4 1
S. Saintpaul	Cloacal swab/ Broilers Litter/ Broilers	4 1
S. II	Litter/ Broilers	5

Antimicrobial Susceptibility by Disc Diffusion

Each strain's antimicrobial susceptibility was detected by disc diffusion (Kirby- Bauer), following the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2021). The discs were produced by Oxoid (Thermo Fisher Scientific, USA). The antimicrobials and concentration used were: ampicillin (AMP: 10 µg); cefotaxime (CTX: 30µg); chloramphenicol (C: 30 µg); ciprofloxacin (CIP: 5 µg); gentamicin (GM: 10 µg); kanamycin (K: 30 µg); nalidixic acid (NA: 30 µg); streptomycin (S: 10 µg); tetracycline (TE: 30 µg); trimethoprim-sulfamethoxazole (SXT: 5 µg); sulfonamide (S3: 250 µg); ceftazidime (CAZ: 30 µg). *Escherichia coli* ATCC 25922 was used as the positive control in all tests.

A bacterial suspension (0.5 McFarland) was prepared from each strain, spread on Mueller-Hinton agar, and incubated at 37°C for 24h. After incubation the isolates were determined as resistant (R), susceptible (S), or intermediate (I) according to the diameters of the inhibition zones.

The results was evaluated according to CLSI guidelines (CLSI, 2021).

DNA Extraction

Nucleic acid extraction was performed using the boiling method (Millemann *et al*. 2000). All samples were then kept in -20°C until the PCR assay was performed.

PCR Amplification of Antimicrobial Resistance Genes

Detection of resistance genes was performed by PCR amplification with specific primers (Table II). For this purpose, *sul1, aphA1, floR, tet(A), tet(B),* and *tet(G)* genes were amplified according to Guerra and colleagues (Guerra *et al*. 2004). The resistant isolates were screened as follows using PCR for the presence of resistance genes corresponding to their resistance phenotypes: trimethoprim-sulfamethoxazole resistant isolates were screened for the presence of *sul1*; kanamycin-resistant isolates for *aphA1*; chloramphenicol-resistant isolates for *floR*; Tetracycline-resistant isolates for *tet(A), tet(B,)* and *tet(G)*.

Table II. *Genes and PCR primers used*

Gene	Sequence 5'-3'
sul1	CTT CGA TGA GAG CCG GCG GC GCA AGG CGG AAA CCC GCG CC
aphA1	AAA CGT CTT GCT CGA GGC CAA ACC GTT ATT CAT TCG TGA
floR	CAC GTT GAG CCT CTA TAT ATG CAG AAG TAG AAC GCG
tet(A)	GCT ACA TCC TGC TTG CCT CAT AGA TCG CCG TGA AGA
tet(G)	GCT CGG TGG TAT CTC TGC AGC AAC AGA ATC GGG AAC
tet(B)	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG

PCR amplification was performed with 0.2 µM of each primer for the target gene, 0.2 mM dNTPs (10 mM dNTP mix; Thermo Fisher Scientific, USA), 3 mM of $MgCl_2$ (Thermo Fisher Scientific, USA), 2.5 µl PCR reaction buffer, 2U of Taq DNA polymerase (Thermo Fisher Scientific; EP0402), and nuclease-free water to a final volume of 25 µl. In the reaction, 2 µL of DNA was used as the template.

The amplification was performed as follows: strand separation at 94°C for 3 min; 30 cycles of 94°C for 1 min; appropriate annealing temperature for each primer for 1 min; 72°C for 1 min; 7 min at 72°C for further strand extension.

The amplified PCR products were then analyzed by electrophoresis.

Results

Antimicrobial Susceptibility

The disc diffusion results indicated that the most sensitive serotypes were *S*. Kouka while the most resistant serotypes were *S*. Agona and *S*. Heidelberg (Table III). In all strains, the serotypes were most susceptible to chloramphenicol and ceftazidime and most resistant to sulfonamide (Table IV). We defined MDR in line with the CLSI as resistance to at least one antimicrobial in three or more drug classes. Multiple antibiotic resistance was most frequent in *S*. Agona strains, followed by *S*. Saintpaul, *S*. Hindmarsch, and *S*. Ottmarchen strains. The most frequently observed resistance pattern was NA, K, S, TE, SXT, S3 in 25% of isolates, followed by Amp, K, S, S3 in 16% of isolates. Table V presents the other MDR patterns.

Detection of Antimicrobial Resistance Genes

The strains were investigated for the presence of resistance genes for trimethoprim-sulfamethoxazole (*sul1),* kanamycin (*aphA1),* chloramphenicol (*floR)*, and tetracycline *tet(A), tet(B),* and *tet(G)*. The analysis showed compatibility between the presence of resistance genes and phenotypic resistance to the corresponding antimicrobials.

The most prevalent gene among tetracycline-resistant strains was *tet (A)*, present in 10/12 strains. The second most prevalent was *tet (B)*, found in 2/12 strains. In contrast, *tet (G)* was not found in any strains.

Table III. *Results of antimicrobial susceptibility by the Kirby-Bauer method*

ID Strain	Serotype	AMP	C	CAZ	CIP	CTX	GM	K	NA	S	SXT	S3	TE
S1	S. Agona	S	S	S	S	S	S	R	S	S	S	R	S
S4	S. Agona	S	S	S	S	S	S	R	R	R	R	R	R
S5	S. Agona	R	S	S	R	S	R	S	R	R	S	R	R
S6	S. Agona	S	S	S	S	S	S	R	R	R	R	R	R
S10	S. Agona	S	S	S	S	S	S	S	S	S	S	R	S
S16	S. Heidelberg	S	S	S	S	S	S	S	S	S	S	S	R
S17	S. Heidelberg	S	S	S	S	S	S	S	S	S	S	S	R
S18	S. Heidelberg	S	S	S	S	S	S	S	S	S	S	S	R
S21	S. Heidelberg	S	S	S	S	S	S	S	S	S	S	R	S
S22	S. Heidelberg	S	S	S	S	S	S	S	S	S	S	R	S
S23	S. Hindmarsh	R	S	S	S	S	S	S	R	R	S	R	R
S24	S. Hindmarsh	S	S	S	S	S	S	S	R	S	S	S	S
S25	S. Hindmarsh	S	S	S	S	R	S	R	R	R	R	R	R
S26	S. Hindmarsh	S	S	S	S	S	S	S	S	S	S	S	S
S27	S. Hindmarsh	S	S	S	S	S	S	S	R	R	S	R	R
S31	S. Kouka	S	S	S	S	S	S	S	S	S	S	S	S
S32	S. Kouka	S	S	S	S	S	S	S	S	S	S	S	S
S33	S. Kouka	S	S	S	S	S	S	S	S	S	S	S	S
S34	S. Kouka	S	S	S	S	S	S	S	S	S	S	S	S
S35	S. Kouka	S	S	S	S	S	S	S	S	S	S	S	S
S42	S. Muenchen	R	S	S	S	S	S	S	S	S	S	S	S
S43	S. Muenchen	R	S	S	S	S	S	S	S	S	S	S	S
S44	S. Muenchen	S	S	S	S	S	S	S	S	S	S	S	S
S45	S. Muenchen	S	S	S	S	S	S	S	S	S	S	S	S
S46	S. Muenchen	S	S	S	S	S	S	S	R	S	S	S	S
S47	S. Ottmarchen	R	S	S	S	S	S	R	S	R	S	R	S
S48	S. Ottmarchen	S	S	S	S	S	S	S	S	S	S	S	S
S49	S. Ottmarchen	S	S	S	S	S	S	S	S	S	S	S	S
S50	S. Ottmarchen	S	S	S	S	S	S	R	R	R	R	R	R
S51	S. Ottmarchen	S	S	S	S	S	S	S	S	S	S	S	S
S53	S. Saintpaul	R	S	S	S	S	R	R	S	R	S	R	S
S54	S. Saintpaul	R	S	S	S	S	I	R	S	R	S	R	S
S56	S. Saintpaul	S	S	S	S	S	S	S	S	S	S	S	S
S59	S. Saintpaul	R	S	S	S	S	I	R	R	R	R	vvR	R
S60	S. Saintpaul	S	S	S	S	S	S	S	S	S	S	S	S
S73	S. II	R	S	S	S	S	S	R	I	S	S	S	S
S74	S. II	S	S	S	S	S	S	S	S	S	S	S	S
S75	S. II	S	S	S	S	S	S	S	S	S	S	S	S
S76	S. II	S	S	S	I	S	S	S	R	S	R	S	R
S77	S. II	S	S	S	S	S	S	S	S	S	S	S	S

S.II refers to 9,12:g, m, t:- formula

Table IV. *The antimicrobial resistance rates of* Salmonella *strains*

Antimicrobials	Number of isolates	Resistance Rates (%)
AMP	9	22.5
CTX	1	2.5
CAZ	0	0
S	11	27.5
GM	2	5
K	10	2.5
NA	11	27.5
CIP	1	2.5
C	0	0
TE	12	30
SXT	6	15
S3	15	37.5

Ampicillin (AMP), Cefotaxime (CTX), Ceftazidime (CAZ), Streptomycin (S), Gentamicin (GM),Kanamycin (K), Nalidixic acid (NA), Ciprofloxacin (CIP), Chloramphenicol (C), Tetracycline (TE), Trimethoprim-Sulfamethoxazole (SXT), Sulfonamides (S3)

Table V. *Antimicrobial resistance patterns MDR* Salmonella *strains*

Multiple Resistance Patterns	Number of Isolates
AMP, NA, CIP, GN, S, TE, S3	1
AMP, NA, K, S, TE, SXT, S3	1
CTX, NA, K, S, TE, SXT, S3	1
NA, K, S, TE, SXT, S3	3
AMP, NA, S, TE, S3	1
AMP, GN, K, S, S3	1
NA, S, TE, SE	1
AMP, K, S, S3	2
NA, TE, SXT	1

Discussion

This study identified the antimicrobial resistance profiles of 40 *Salmonella* strains and their associated resistance genes for four antibiotics, namely trimethoprim-sulfamethoxazole, kanamycin, chloramphenicol, and tetracycline. The strains originated from different poultry matrices (cloacal swab, litter, environmental sample, and pellet feed). Resistance to at least one antibiotic among the 16 tested was found in 24 (60%) strains. Resistance was most frequently found to sulfonamides (37.5%) and tetracycline (30%), which are widely used in poultry (Chopra *et al*. 2001, Castro-Vargas *et al*. 2020). Resistance to cephalosporins was very low and there was no resistance at all to chloramphenicol and ceftazidime. In contrast, resistance was high to nalidixic acid, kanamycin, streptomycin, tetracycline, and sulfonamides. All strains were susceptible to chloramphenicol, which confirms previous research on various *Salmonella* serotypes (Gargano *et al*. 2021). Regarding the cephalosporins family, resistance to ceftazidime (0%) was similar to that reported in Kaya and colleagues (Kaya *et al*. (2017) for *Salmonella* isolated from broiler chickens. Among the 40 *Salmonella* strains, there was frequent resistance to tetracycline (30%), which confirms previous findings for *Salmonella* spp. isolates (Kaya *et al*. 2017, Castro-Vargas *et al*. 2019, Gargano *et al*. 2021).

Resistance genes [*sul1, aphA1, floR, tet(A), tet(B)* and *tet(G)*] were also detected in phenotypically resistant strains. Several *tet* genes responsible for tetracycline resistance in *Salmonella* spp. have been described (Hall, 2010), of which the most frequent are A, B,

C, D, and G classes (Brunelle *et al*. 2013). These genes can be localized within mobile elements of the *Salmonella* genome, such as transposons or plasmids, which can be easily transferred to other bacteria (Hall, 2010). We also detected *tet(A*) and *tet(B)* genes in tetracycline-resistant strains. The most frequently detected gene was *tet(A)*, which encodes a subunit of the tetracycline efflux pump found in many species of Gram-negative bacteria (Aldema *et al.* 1996). This result mirrors that reported by Gargano and colleagues (Gargano *et al*. 2021), who investigated tetracycline resistance genes in *Salmonella* spp. isolated from animals and food. In our study, *tet(A)* and *tet(B)* were detected in those isolates that were resistant to tetracycline. Khoshbakht and colleagues (Khoshbakht *et al*. 2018) reported that six out of 60 *Salmonella* serotypes had *tet(A)* and *tet(C)* genes.

The most common serotypes among *S. enterica* strains are serovar Typhimurium and Enteritidis. Since 2015, *S*. Infantis, which was rarely seen before, was the most frequently isolated serovar from both humans and animals. Accordingly, it is now considered a "zoonotic salmonella" (EFSA, 2018)

Therefore, given that rarely isolated *Salmonella* serotypes have the potential to become predominant, it is crucial to monitor their antibiotic resistance profiles. In addition to their high pathogenic potential, *Salmonella* genus particularly interesting because of their contribution to spreading antibiotic resistance as they are able to accumulate and spread ARGs (McDermott *et al*. 2018). Indeed, given the high level of attention to antimicrobial resistance and the ease with which ARGs spread, it is important to monitor the presence of ARGs, especially in zoonotic bacteria. Our data show that the majority of strains in our Turkish sample harbor antibiotic resistance genes.

Conclusion

In conclusion, our results show that there is a problem of antibiotic resistance among rarely isolated strains. This suggests that more frequent isolation of these strains will be needed because they may affect the spread of antibiotic resistance. To our knowledge, this was the first study to investigate antibiotic resistance profiles in these serotypes.

Acknowledgments

This study was presented as a poster presentation on 2nd International Congress of Veterinary Microbiology, 16-19 October 2018 Sherwood Breezes Resort- Lara/ Antalya.

Conflict of Interest

There is no conflict of interest, including among the letter the financial, personal or other relationships with other people or organizations that could inappropriately influence the work.

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