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Phenotypic and genotypic characterization of antimicrobial resistance in commonly isolated Salmonella serovars from chickens

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Abstract: Salmonellosis caused by Salmonella agents is the second most common zoonotic infection in humans. In recent years, Salmonella's increasing antimicrobial resistance (AMR) has been a concern. The major transmission route of Salmonella is consumption of contaminated poultry products. Therefore, monitoring of antimicrobial resistance in chicken-originated Salmonella is critically important. This study investigated AMR in four commonly isolated Salmonella serovars from chickens, namely Salmonella Enteritidis (S. Enteritidis), Salmonella Infantis (S. Infantis), Salmonella Kentucky (S. Kentucky), and Salmonella Typhimurium (S. Typhimurium). A total of 133 isolates were examined by phenotypic and genotypic AMR characterization. Resistance to 14 different antimicrobials and eight resistance genes were investigated in all isolates. The AMR test indicated that there was no resistant isolate to all antimicrobials while 14.3% were susceptible to all antimicrobials. The highest resistance was to sulfonamides (57.1%), nalidixic acid (48.1%), and tetracycline (39.1%). The highest susceptibilities were to cefotaxime (86.5%), cefoxitin (92.5%), ceftazidime (78.2%), and ceftriaxone (97%). S. Infantis and S. Kentucky isolates had higher resistance to all antimicrobials than S. Enteritidis and S. Typhimurium isolates. Significantly high multidrug-resistance (MDR) was detected in 50.4% of all isolates, although MDR prevalence varied widely between serovars: 78.7% of all S. Infantis isolates were MDR whereas only 18.8% of S. Enteritidis isolates were MDR. The most prevalent resistance genes were tetA (35.2%) and sul1 (31.6%), with 12.5% and 3.1% of S. Enteritidis isolates being positive for tetA and sul1, respectively, whereas 17.4% and 8.7% of S. Typhimurium isolates were positive. These rather low prevalence rates are probably due to effective monitoring of these serovars by control programs in Türkiye. The nondetection of mcr1 and mcr2 can be explained by the rare use of colistin in chicken flocks in Türkiye. The obtained findings emphasize the importance of AMR monitoring for Salmonella and the risks of chicken-originated isolates to humans.

Key words: Antimicrobial resistance, chicken, MDR, resistance gene, Salmonella, S. Infantis

1. Introduction
Salmonella is the second most common zoonotic agent worldwide. According to the European Food Safety Authority (EFSA) zoonoses report, Salmonella agents caused 90,105 human salmonellosis cases in 2019. Salmonella spp. consists of two species, Salmonella enterica and Salmonella bongori, with approximately 2700 serovars. The most prevalent serovars in human salmonellosis are S. Enteritidis (50.3%), S. Typhimurium (11.9%), S. Infantis (2.4%), and S. Kentucky (0.07%). However, control programs generally focus on S. Enteritidis and S. Typhimurium as they cause over 70% of human cases. S. Infantis is the most frequent serovar in broilers while S. Kentucky is another common serovar that spreads to humans via food products [1, 2].

Antimicrobial therapy is commonly used to treat bacterial infections in both humans and animals. However, improper use of antimicrobials has led to treatment failure, increased costs and mortality, and the spread of resistant pathogens [3]. According to the World Health Organization’s (WHO) surveillance report, the spread of AMR and MDR nontyphoidal Salmonella is a global challenge [4]. WHO has included Salmonella in its high-priority pathogen list due to increasing AMR [5]. In recent years, AMR in Salmonella has increased in food-producing animals [6, 7]. Many studies conclude that resistant Salmonella of animal origin poses a risk to humans [8]. Humans are exposed to resistant Salmonella through the consumption of contaminated foods, particularly contaminated poultry products (eggs and chicken meat). Thus, the monitoring of AMR in chicken-originated Salmonella is critical for detecting cases and reducing the risk to public health [9].

Accordingly, this study determined the phenotypic and genotypic AMR in the most common Salmonella serovars isolated from chickens. The findings provide valuable

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information regarding AMR in commonly isolated *Salmonella* serovars, based on evaluation of serovars, antimicrobials, resistance genes, and breeding types.

2. Materials and methods

2.1. *Salmonella* isolation and identification

*Salmonella* isolates were obtained from litter/feces samples of broiler and layer chicken flocks. *Salmonella* isolation was performed by the ISO 6579-1:2017 procedure [10]. Serotyping was performed with specific somatic and flagella antisera (Biorad, France), using the Kauffmann–White–Le Minor scheme [11]. A total of 133 isolates were used, including 32 *S. Enteritidis*, 47 *S. Infantis*, 31 *S. Kentucky*, and 23 *S. Typhimurium* (Table 1). The 20% glycerol stocks were prepared to store bacterial cultures until molecular characterization.

2.2. Antimicrobial resistance test

All isolates were tested for AMR using the Kirby-Bauer Disc Diffusion method on Mueller Hinton agar (Oxoid, UK) as defined in the Clinical and Laboratory Standards Institute (CLSI) manual. The following antimicrobials were used (Oxoid, UK): ampicillin (AMP: 10 μg), cefotaxime (CTX: 30 μg), cefoxitin (FOX: 30 μg), ceftazidime (CAZ: 30 μg), ceftriaxone (CRO: 30 μg), chloramphenicol (C: 30 μg), ciprofloxacin (CIP: 5 μg), gentamycin (CN: 10 μg), meropenem (MEM: 30 μg), nalidixic acid (NA: 2 μg), sulfonamides (S3: 300 μg), trimethoprim (W: 5 μg) and trimethoprim-sulfamethoxazole (SXT: 25 μg). *Escherichia coli* ATCC25922 strain was preferred as the quality control strain. The colony suspensions were adjusted equivalent to a 0.5 McFarland turbidity standard on a densitometer (Biosan, Latvia). After incubation at 36 °C ± 1 °C for 16–18 h, the zone diameters (mm) were evaluated as resistant, intermediate, or susceptible based on CLSI criteria [12]. *Salmonella* isolates resistant to three or more antimicrobial classes were considered to exhibit MDR [9].

2.3. Bacterial DNA extraction

Bacterial DNA was obtained from all isolates using the conventional boiling method. The bacterial suspensions were respectively incubated at 100 °C for 10 min and on ice for 5 min. DNA concentrations and qualities were checked using NanoDrop equipment (Thermo Scientific, USA).

2.4. Molecular characterization of resistance

The antimicrobial resistance genes to ampicillin (*bla*<sub>TEM</sub>), colistin (*mrc1, mrc2*), fluoroquinolones (*qnrB*), sulfonamides (*sul1*), tetracyclines (*tetA, tetB*) and trimethoprim (*dfrA1*) were amplified by polymerase chain reaction (PCR). The PCR analyses were performed using specific primers as previously reported (Table 2). The reactions were conducted in a total of 25 μL of mixture volume containing 0.2 μL of Taq polymerase (2U/μL) (Thermo Scientific, USA), 0.5 μL of 10 mM dNTPs, 1 μL of each 10 mM primer, 2.5 μL of 10X buffer, 3 μL of MgCl₂, 14.8 μL of nuclease-free water, and 2 μL of template DNA. Amplifications were performed as follows: initial denaturation for 5 min at 95 °C, 34 cycles of denaturation for 20 s at 94 °C, annealing for 20 s at a defined temperature, extension for 20 s at 72 °C, and final extension for 5 min at 72 °C. Positive and negative controls were included for each reaction. The amplicons were analyzed with 1.5% agarose gel electrophoresis (Thermo Scientific, USA). The samples were visualized by G: Box Chemi UV transillumination (SynGene, India).

3. Results

The AMR test findings indicated that 14.3% (19/133) of the *Salmonella* isolates were susceptible to all tested antimicrobials. There was no resistant isolate to all antimicrobials while 78.9% (105/133) were resistant to at least one. Resistance to sulfonamides 57.1% (76/133) was the most common while high resistances were also found to nalidixic acid 48.1% (64/133), tetracycline 39.1% (52/133), and ampicillin 37.6% (50/133). The highest intermediate resistance was to ciprofloxacin at 48.1% (64/133). The highest susceptibilities were found to cephalosporin group antimicrobials, cefotaxime 86.5% (115/133), cefoxitin 92.5% (123/133), ceftazidime 78.2% (104/133), and ceftriaxone 97% (129/133) (Figure 1).

The antimicrobial resistance rates based on the serovars are shown in Figure 2. The highest resistances were in *S. Infantis* isolates for sulfonamides (85.1%) and nalidixic acid (78.7%). All *S. Enteritidis* isolates were susceptible to ceftriaxone and ciprofloxacin whereas *S. Typhimurium* isolates were susceptible to cefoxitin, ceftriaxone and meropenem. A significantly high MDR rate of 50.4% (67/133) was detected in all isolates. Moreover, 55.2% of the isolates were resistant to three or more antimicrobial classes.

<table>
<thead>
<tr>
<th>Breeding type</th>
<th><em>S. Enteritidis</em></th>
<th><em>S. Infantis</em></th>
<th><em>S. Kentucky</em></th>
<th><em>S. Typhimurium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler</td>
<td>20</td>
<td>38</td>
<td>27</td>
<td>21</td>
</tr>
<tr>
<td>Layer</td>
<td>12</td>
<td>9</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>47</td>
<td>31</td>
<td>23</td>
</tr>
</tbody>
</table>

Table 1. Distribution of *Salmonella* isolates by serovar and breeding type.
of all MDR isolates were S. Infantis. At 78.7% (37/47), S. Infantis isolates had higher MDR rates than the other serovars.

The antimicrobial resistance rates based on the breeding types were shown on Figure 3. The highest resistance rates were to the same antimicrobials in both breeding types. The broiler isolates showed the highest resistance to sulfonamides (59.4%) and nalidixic acid (53.8%) compared to 48.1% and 25.9%, respectively, of the layer isolates that were resistant. All layer isolates were susceptible to four different agents, namely cefotaxime, ceftriaxone, and chloramphenicol.

The Salmonella isolates for these four serovars were investigated for antimicrobial resistance genes. None of the isolates had mrc1 and mrc2 genes. In all isolates, the most commonly detected genes were in tetA (35.2%), sul1 (31.6%), and blaTEM (15.0%). Prevalences were low for all other genes. Regarding the prevalence of resistance genes in terms of serovars, S. Infantis had the highest prevalence, particularly sul1 (72.3%) and tetA (70.2%) (Figure 4).

Table 2. Primers, sequences, and annealing temperatures for the resistance genes.

<table>
<thead>
<tr>
<th>Antimicrobial class</th>
<th>Resistance genes</th>
<th>Primer sequence (5’-3’)*</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-lactams</td>
<td>bld&lt;sub&gt;TEM&lt;/sub&gt;</td>
<td>F: GCACGAGTGTTACATCGA</td>
<td>310</td>
<td>60</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGTCCTCCGATGTTGTCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colistin</td>
<td>mrc1</td>
<td>F: AGTCGGTTTTGTTCTTGTCGC</td>
<td>320</td>
<td>58</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>mrc2</td>
<td>R: AGATCCCTTGCTCAGGCTTG</td>
<td>715</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F: CAAGTGCTGTGTCGAGTTTTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCTAGCCCCGACAAGCATACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>qnrB</td>
<td>F: GATCGTGAAGCCAGAAAGGG</td>
<td>469</td>
<td>53</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ACATGCCTGGTATGTGCCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>sul1</td>
<td>F: TCGGATCGACGTCTGG</td>
<td>258</td>
<td>60</td>
<td>[29]</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>tetA</td>
<td>F: GGTTCACTCGAGCAGGTCGA</td>
<td>577</td>
<td>55</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>tetB</td>
<td>R: CTGTCCGACAGTTGCGATGA</td>
<td>634</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F: CCGAGCTCTCAGCGCGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCACCTTGCTGATGACTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate pathway inhibitors</td>
<td>dfrA1</td>
<td>F: GAGGTGCCAAAGGTGAACAGC</td>
<td>367</td>
<td>55</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAGGCCGAATTGCTGGGTAAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*F, forward; R, reverse.

Figure 1. Distribution of resistant, intermediate, and susceptible isolates by the antimicrobials.
In addition, phenotypic and genotypic findings were compared for the most prevalent resistance genes. Fifty-two isolates were found tetA- or tetB-positive. Similarly, fifty-two isolates were resistant to tetracycline also by the disc diffusion test. Seventy-six isolates were resistant to sulfonamides, with the presence of sul1 in forty-two isolates while fifty isolates were resistant to ampicillin, with the presence of blaTEM in twenty isolates.

4. Discussion
Salmonellosis is one of the most common foodborne infections worldwide, transmitted to humans through infected animals and consumption of contaminated food. Being very effective vectors, contaminated poultry animals and poultry products are the major source of Salmonella transmission to humans. Due to cross-contamination, humans are exposed to antimicrobial resistant strains
of *Salmonella* [13], which poses a serious risk to public health. Many studies have reported increasing prevalence and spread of antimicrobial resistant *Salmonella*, especially MDR *Salmonella*. Accordingly, WHO has added *Salmonella* to its priority pathogens list for global challenge. Therefore, monitoring of AMR *Salmonella* in chickens is recommended to control AMR [9, 14].

In this study, we detected that the highest resistance was to sulfonamides (57.1%), nalidixic acid (48.1%), tetracycline (39.1%), and ampicillin (37.6%). This is consistent with the resistance rates reported by Thi et al. (2020) to sulfonamides (75.86%), tetracycline (51.72%), and ampicillin (31.03%). These high resistance levels may be due to wide use of sulfonamides and nalidixic acid in chickens. Tetracycline and ampicillin have been used as antimicrobial agents in recent years, although their effectiveness has been decreasing in veterinary implementation. Our findings are thus in line with previous reports detailing increasing resistance of *Salmonella*.

Many studies have reported significantly high resistance to ciprofloxacin in recent years. Utrarachki et al. (2016), Fardsanei et al. (2018), Güran et al. (2020), and Jiang et al. (2021) reported high ciprofloxacin resistance, such as levels of 51.1%, 90.9%, 100%, and 57.6%, respectively [3, 15-17]. In contrast, we found low resistance (14.3%) and high intermediate resistance (48.1%) to ciprofloxacin. Given that ciprofloxacin is a fluoroquinolone antimicrobial recommended as a first choice for treating human salmonellosis [4], ciprofloxacin-resistant *Salmonella* in contaminated food presents a serious risk to humans by hindering effective treatment. We therefore recommend monitoring resistance trends in ciprofloxacin.

The tested serovars were compared in terms of antimicrobial resistance rates. Significant differences were detected in the distribution of resistant isolates. S. Infantis and S. Kentucky isolates had higher resistance to almost all antimicrobials than S. Enteritidis and S. Typhimurium isolates. Compared with other *Salmonella* serovars, S. Infantis isolates had the highest resistance rates to the most of tested antimicrobials, such as ampicillin (42.6%), nalidixic acid (78.7%), sulfonamides (85.1%), and tetracycline (68.1%). The next highest rates were for S. Kentucky, such as ampicillin (54.8%), nalidixic acid (54.8%), sulfonamides (51.6%), and tetracycline (38.7%). Abdel-Maksoud et al. (2015) also found high rates of resistance to ampicillin (97%), and nalidixic acid (94%), sulfonamides (100%), and tetracycline (97%) [18]. However, one S. Kentucky isolate was resistant to at least 12 of the 14 tested antimicrobials and had five of the eight tested resistance genes. This finding is compatible with a previous study that reported particularly high resistance in S. Kentucky [19].

In our study, all isolates showed high susceptibility to cephalosporins. These low rates of cephalosporin resistance are in line with the findings of Abdel-Maksoud et al. (2015), who reported low resistance to cephalosporins among poultry-originated *Salmonella* isolates [18]. None of the layer isolates was resistant to cefotaxime, ceftazidime, ceftriaxone, or chloramphenicol while layer isolates showed only limited resistance to all tested antimicrobials except nalidixic acid and sulfonamides. These findings are compatible with Pande et al. (2015) [20], who reported low antimicrobial resistance in layer-originated most common *Salmonella* serovars.

While we found a high rate of MDR *Salmonella* (50.4%), this is lower than that reported in some previous studies. Wei et al. (2019) and Queslati et al. (2021), for example, reported high MDR rates (respectively 81% and 87.5%) in chickens [21, 22] whereas we detected MDR S. Enteritidis in only 18.8% of S. Enteritidis isolates, which contrasts with some previous studies. For example, Medeiros et al. (2011), Lu et al. (2014), and Asif et al. (2017) reported high rates of MDR S. Enteritidis isolates from chickens, at 63.9%, 92.6%, and 54.8%, respectively [14, 23, 24].

Regarding resistance genes, the highest prevalences were for tetA (35.3%) and sul1 (31.6%), which encode tetracycline and sulfonamide resistance, respectively, in *Salmonella*. These findings are supported by the high positivity for tetA and sul1 reported by Lu et al. (2014) and Thi et al. (2020). Comparing tetA and sul1 rates by serovars, 12.5% and 3.1% of S. Enteritidis isolates were positive for tetA and sul1, respectively, while 17.4% and 8.7% of S. Typhimurium isolates were positive. These rates are significantly lower than those previously reported [24], which may reflect effective monitoring of S. Enteritidis and S. Typhimurium serovars with control programs in Türkiye. These low prevalences indicate the risk that resistance genes may be acquired by horizontal transfer remains low. We did not detect mcr1 and mcr2 in any isolate, which, we believe, is because colistin is rarely used in chicken flocks. In addition, colistin is only used in salmonellosis cases after resistance has been detected to commonly used antimicrobials. The low resistance to colistin can be explained with this approach [21].

Finally, we found no correlation between phenotypic resistance and genotypic resistance among the serovars. Some resistance genes were not detected in AMR-positive isolates, which could be due to silent genes, the presence of other genes, nonintegrated genes, or lack expression of existing genes in *Salmonella* isolates [21, 25].

Our findings provide valuable information about AMR in commonly isolated *Salmonella* serovars from chickens. We investigated AMR in S. Enteritidis, S. Infantis, S. Kentucky, and S. Typhimurium isolates by phenotypic and genotypic characterization. The findings were then evaluated in terms of serovars, antimicrobials, resistance genes and breeding type.
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Conflict of interest
The authors declare no conflict of interests.

References


