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Phenotypic Colistin Resistance and *mcr* Genes Presence in *Salmonella* Serovars Originating from Poultry Farms

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ABSTRACT: The prevalence of antibiotic resistance in bacteria is a matter of concern. Colistin is the last resort for treating infections caused by resistant *Enterobacteriaceae*. The *mcr* genes carried and transferred to the plasmid are responsible for the resistance of the bacteria to colistin. The objective of this study was to determine the resistance of *Salmonella* strains to colistin, isolated from environmental samples taken from poultry farms and serologically identified, and to investigate the presence of *mcr* genes in resistant strains. A total of 300 *Salmonella* strains isolated and identified from poultry farms in Turkey between 2014 and 2018 were subjected to phenotypic colistin resistance testing using the microdilution method. The presence of *mcr* genes was evaluated by multiplex PCR. The antibiotic resistance status of the *Salmonella* isolates with phenotypic resistance to colistin was analyzed using the Kerby Bauer method. A total of 72 out of 300 *Salmonella* isolates were phenotypically resistant to colistin. Additionally, resistance to pefloxacin, ampicillin, sulfamethoxasol/trimethoprim, gentamycin, and cefotaxime antibiotics was observed in 34.7%, 5.6%, 4.2%, 2.8%, and 1.4%, respectively. Furthermore, the *mcr* genes were not detected in the *Salmonella* strains examined in this study. The results of this study indicate that phenotypic colistin resistance in *Salmonella* strains isolated from poultry environmental samples is not related to the *mcr* genes analyzed. The mechanism of resistance may be chromosomal resistance, and the mechanisms should be investigated by whole-genome analysis.

Keyword: Antimicrobial resistance; EUCAST; colistin; *mcr*; *Salmonella*.

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INTRODUCTION

Antibiotic resistance has reached an alarming level owing to the overuse and inappropriate use of antibiotics in humans, animals, and agriculture. Death from antimicrobial-resistant bacteria is expected to increase in the future (Bello & Dingle, 2018). Colistin is a peptide antibiotic effective against gram-negative bacteria, including the polymyxin group (Biswas et al., 2012). Colistin is a frequently used antimicrobial agent in animal production in numerous countries (Rhouma and Letellier, 2017). Non-typhoidal *Salmonella enterica* serotypes are zoonotic pathogens that can be transmitted from animals to humans through the consumption of contaminated food, most commonly from poultry. These pathogens, especially multi-antibiotic-resistant strains, have the potential to cause severe infections (Lima et al., 2019). The mechanism of action of colistin begins with binding to Lipid A found in the outer membrane (OM) of gram-negative bacteria (Velkov et al., 2010). Although colistin is not an antibiotic used for the treatment of *Salmonella* infections in humans, it is becoming increasingly important as one of the last treatment options for human infections caused by multi-resistant *Salmonella*, especially carbapenem-resistant and extended-spectrum beta-lactamase-producing (ESBL) strains (WHO, 2019).

In recent decades, the identification of *mcr* genes carried by mobile genetic elements responsible for colistin resistance worldwide and the possibility that these mobile elements may also carry and transmit resistance genes to quinolones and beta-lactams, along with colistin resistance, has raised global concern (Campos et al., 2016; Lima et al., 2019; Sun et al., 2020). Investigating the presence and origin of *mcr* genes, especially in zoonotic pathogens such as non-typhoidal *S. enterica*, is extremely important. In China (Li et al., 2016), Taiwan (Yi et al., 2017), and Portugal (Campos et al., 2016), *mcr*-1 has been identified in *S. enterica* serotypes of human, poultry, pig, and chicken meat (Figueiredo et al., 2016). A polymyxin-sensitive *Salmonella* strain of animal origin was recently found to contain the *mcr*-9 gene (Braga et al., 2023). It is postulated that the *mcr*-9 harboring strains may be spreading quietly, as the resistance phenotype is not expressed. However, there is limited information on *mcr* genes in non-typhoidal *S. enterica* isolates compared with studies on the carriage of colistin resistance genes in *Escherichia coli* isolates (Luk-In et al., 2021). This study aimed to examine colistin resistance in *S. enterica* serotypes isolated from poultry environmental samples and to investigate the *mcr* genes related to mobile colistin resistance.

MATERIAL AND METHODS

Sampling

A total of 300 non-typhoidal *Salmonella* strains with 37 different serotypes, which were isolated from various environmental samples taken by boot swabs and/or sock swabs from poultry production farms in Turkey, sent to Aviagen Anatolia Poultry Diagnosis and Analysis Laboratory for *Salmonella* isolation between 2014-2018 and serologically identified in the Bacteriological Diagnostic Laboratory of the Ministry of Agriculture and Forestry, Etlik Veterinary Control Central Research Institute Directorate, were studied (Table 1).

Samples used for isolation were collected from various locations in the houses using random sampling methods to represent the flock. Approximately 250 samples from eight different companies were sent weekly to the Aviagen Anadolu AŞ laboratory as part of the *Salmonella* Monitoring Program.

For each house, dust and swab samples were collected to cover all sections of the house. A minimum of two sets of dust samples (collected in two sterile containers of 250 ml each) were collected from at least 10 different points in the house, with a maximum weight of 25 g.

At least six drag swab samples (socks and boot swabs) were collected from each poultry house. The drag swabs were taken from the litter and placed in a sterile 250 ml container, with a maximum of three drag swabs per container. This corresponds to 2 containers per poultry house.

Bacteria were isolated from the samples mentioned above in line with the monitoring program of the farms by inoculation on different selective culture media. The purified colonies were identified by conventional biochemical procedures followed by a rapid biochemical-test kit (API 20E, BioMérieux)

Phenotypic Colistin Resistance Determination

Colistin resistance in *the Salmonella* isolates (n=300) was studied using the broth microdilution method (BMM). BMM was performed and evaluated according to EUCAST criteria (ECAST, 2018). In this study, colistin sulfate (Sigma Aldrich, USA) was reconstituted in two layers in 96-well, U-bottom, polystyrene microplates in CAMHB liquid medium using BMM, and the final bacterial concentration was adjusted to 5×10^5 CFU/mL. Quality control

Table 1. *Salmonella* serotypes names

Serotype Name	Antigenic Formula of the Isolate	Group	Number of Samples (%)
S. Abony	(1,4,[5],12,[27] ; b ;e,n,x)	B	18 (6,00)
S. Anatum	(3,[10],[15],[15,34]; e,h ;1,6)	E	5 (1,67)
S. Bispebjerg	(1,4,[5],12 ; a; e,n,x)	B	2 (0.67)
S. Charity	([1],6,14,[25] ; d ; e,n,x)	H	3 (1,00)
S. Corvallis	(8,20 ; Z4,Z23 ; [Z6])	C	1 (0,33)
S. Enteritidis	(1,9,12; g,m ; -)	D	66 (22,00)
S. Ferruch	(8; e,h ;1,5)	C	1 (0,33)
S. Hadar	(6,8 ; Z10 ;e,n,x)	C	3 (1,00)
S. Havana	(1,13,23 ; f,g,[s] ; -)	G	9 (3,00)
S. Infantis	(6,7,14; r ;1,5)	C	115 (38,33)
S. Kentucky	(8,20 ; i ; Z6)	C	3 (1,00)
S. Kikoma	(16 ; y ;e,n,x)	I	1 (0,33)
S. Kottbus	(6,8 ;e,h ;1,5)	C	7 (2,33)
S. Leeuwarden	(11 ; b ; 1,5)	F	1 (0,33)
S. Lexington	(3,10,15,34; Z10;15;[Z49])	E	1 (0,33)
S. Liverpool	(1,3,19 ; d ; e,n,Z15)	E	4 (1,33)
S. Matopeni	(30 ;y ; 1,2)	N	1 (0,33)
S. Mbandaka	(6,7,14;Z10; e,n,Z15)	C	3 (1,00)
S. Mikawasima	(6,7,14 ; y ;e,n,Z15)	C	3 (1,00)
S. Newport	(6,8,20:e,h:1,2)	C	4 (1,33)
S. Paratyphi B	(1,4,[5],12 ; b ; 1,2)	B	5 (1,67)
S. Poona	(13,22 ; z ;1,6,Z44)	G	2 (0.67)
S Richmond	(6,7 ; y ; 1,2)	C	1 (0,33)
S. Salford	(16 ; 1,v ; e,n,x)	I	4 (1,33)
S. Thompson	(6,7,14 ; k ;1,5)	C	1 (0,33)
S. Tomegbe	(1,42 ; b ; e,n,Z15)	T	9 (3,00)
S. Typhimurium	(1,4,[5],12; i ;1,2)	B	11 (3,67)
S. Vitkin	(28 :1,v :e,n,x)	V	1 (0,33)
Salmonella Grup B	(1,4,12,27 : d : ?)	B	2 (0.67)
Salmonella Grup C1	(6,7 : Z29 : ?)	C	1 (0,33)
Salmonella Grup D1	(1,9.12;g,m:?)	D	3 (1,00)
Salmonella Grup G1	(13,22 : Z29 : ?)	G	2 (0.67)
Salmonella Grup G2	(1,13,23 : g? : ?)	G	2 (0.67)
Salmonella Grup H	(14,25;d;?)	H	1 (0,33)
S. enterica subsp. *salamae* serotip II	(13,22 : Z29 :1,5)	G	2 (0.67)
S. enterica subsp. *salamae* serotip II	(42 : z :1,5)	T	1 (0,33)
S. enterica subsp. *salamae* IIIb	(50 : k : Z35)	Z	1 (0,33)
All samples			300 (100)

of the test was conducted using two strains, *E. coli* ATCC 25922 and colistin-resistant *E. coli* NCTC 13846 (carrying *mcr*-1). Antibiotic-free wells were left in each tested microplate for bacterial growth control, and media sterility control wells were used to control media without bacterial inoculation. The inoculated 96-well microplates were covered with a microfilm and incubated in an incubator at 35˚C for 16-18 hours under aerobic conditions, and then the growth in the wells was evaluated with the naked eye. Limit values were evaluated according to the EUCAST criteria.

Determination of Resistance of Colistin-Resistant Isolates to Other Antibiotics

Resistance of phenotypic-colistin-resistant isolates to other antibiotics was determined by the Disk Diffusion test. Bacteria suspensions prepared from fresh pure cultures at 0.5 McFarland turbidity were rubbed on Mueller-Hinton Agar (MHA) with sterile swab and then including antibiotic discs; gentamycin (30 µg), kanamycin (30 µg), sulphamethoxasol/trimethoprim (1.2-23), ceftiofur (30 µg), ampicillin (10 µg), pefloxacin (5 µg), meropenem (10 µg), and cefotaxime (30 µg), were placed on agar at appropriate intervals. The agar was then incubated at 35˚C for 16-24 hours. Zone diameters were measured using a compass and evaluated according to EUCAST and CLSI criteria for ceftiofur.

Investigation of *mcr* Genes of Colistin Resistant Isolates

The European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR) established a multiplex PCR protocol to determine the presence of the *mcr* gene in *Salmonella* isolates that were phenotypically resistant to colistin. Table 2 provides details of the positive controls and primers used in this study. *Salmonella* isolates were incubated on Columbia Agar with 5% Sheep Blood and Plate Count Agar medium. Following purity control, sterile extracts were obtained and DNA was extracted using the conventional boiling method. The PCR reaction was prepared by adding 0.5 µl of each reconstituted stock F and R primer (10 µl in total) and 12.5 µl of Green PCR buffer (DNA polymerase) to a final reaction volume of 25 µl. Then, 2 µl of the previously prepared DNA samples of each isolate were added to each tube, and the remainder was filled with water without nuclease to arrange the final volume to 25 µl. The tubes were placed in a thermal cycler for amplification.

Amplification conditions: DNA was amplified for 25 cycles of initial denaturation at 94 °C for 15 min, followed by denaturation at 94 °C for 30 s, binding at 58 °C for 90 s and extension at 72 °C for 60 s. The final extension step was performed at 72 °C for 10 min. To visualize the amplified DNA products, 1.5% agarose gel was prepared and electrophoresed at 130 volts for 45 min. Finally, the gel was placed in the imaging system and specific bands were visualized.

RESULTS

Three hundred *Salmonella* isolates for which microdilution tests were conducted to determine colistin resistance were isolated between 2014 and 2018 (Table 1). According to the EUCAST criteria, 72 of 300 isolates (24%) were phenotypically resistant to colistin, while the remaining 228 (76%) were susceptible (EUCAST, 2018) (Table 3). The colistin MIC values of the isolates ranged from 0.122–256 µg/mL. The colistin MIC 50 and MIC 90 for these isolates were 1 µg/mL and 8 µg/mL, respectively.

Of the 72 phenotypic colistin-resistant *Salmonella* serotypes, the most prevalent were *Salmonella* Infantis (n=27) and *S.* Enteritidis (n=19). Other serotypes include *S.* Typhimurium (n=5), *S.* Abony (n=2), *S.* Liverpool (n=2), *S.* Kottbus (n=2), *S.* Hadar (n=2), *S.* Newport (n=2), *S.* Kentucky (n=1), *S.* Kikoma (n=1), *S.* Havana (n=1), *S.* Anatum (n=1), *Salmonella* Group G1 (n=1), *Salmonella* II (n=1), *Salmonella* Group B (n=1), *S.* Mbandaka (n=1), *S.* Paratyphi B (n=1), *S.* Thompson (n=1), and *S.* Lexington (n=1). Seventy-two phenotypically colistin-resistant isolates were tested for antibiotic resistance rates against pefloxacin, ampicillin, sulfa-

Table 2. Primers used in this study and positive strains carrying *mcr* genes (Rebelo et al. 2018).

Genes	Primer 5 '-3'	Size (bp)	Positive control strain
mcr-1	Fw-5'AGTCCGTTTGTTCTTGTGGC-3' Rev-5' AGATCCTTGGTCTCGGCTTG-3'	320	*E. coli* (2012-60-1176-27)
mcr-2	Fw 5'-CAAGTGTGTTGGTCGCAGTT-3' Rev 5'-TCTAGCCCGACAAGCATACC-3'	700	E. coli KP37
mcr-3	fw 5'-AAATAAAAATTGTTCCGCTTATG-3' rev 5'-AATGGAGATCCCCGTTTTT-3'	900	*E. coli* 2013-SQ352
mcr-4	fw 5'-TCACTTTCATCACTGCGTTG-3' rev 5'-TTGGTCCATGACTACCAATG-3'	1100	*E. coli* DH5α
mcr-5	fw 5'-ATGCGGTTGTCTGCATTTATC-3' rev 5'-TCATTGTGGTTGTCCTTTTCTG-3'	1644	*Salmonella* 13-SA01718

Table 3. MIC values of all tested isolates were obtained by BDM according to the EUCAST criteria.

Serotypes (*n*=72)	Colistin MIC (mg/L)			Antimicrobial Resistance to Disc Diffusion								
	32	16	8	4	CN	K	SXT	EFT	AMP	PEF	MEM	CTX
S. Abony				2						1		
S. Anatum			1									
S. Enteritidis		1	9	9	1		1		3	2		
S. Hadar		1		1						2		
S. Havana		1										
S. Infantis		4	7	16	1		2		1	17		1
S. Kentucky				1						1		
S. Kikoma			1									
S. Kottbus		1	1									
S. Lexington		1										
S. Liverpool	1		1									
S. Mbandaka		1										
S. Newport		1		1								
S. Paratyphi B			1									
S. Thompson			1							1		
S. Typhimurium		1	3	1								
Salmonella Grup G1				1								
Salmonella II			1									
Salmonella Grup B	1									1		
Total	2	12	26	32	2 (2.8%)		3 (4,2%)		4 (5.6%)	25 (34.7%)		1 (1.4%)

CN: Gentamicin (30µg), K: Kanamycin (30µg), SXT: Sulphamethoxasol/trimethoprim (1.25/23.75 µg), EFT: Ceftiofur, AMP: Ampicilin (10µg), PEF: Pefloxacin (5 µg), MEM: Meropenem (10 µg), CTX: Cefotaxime (30 µg).

methoxasol/trimethoprim, gentamycin, cefotaxime, kanamycin, ceftiofur, and meropenem in *Salmonella* infections in humans and animals. The study found that resistance rates to pefloxacin, ampicillin, and sulfamethoxasol/trimethoprim were 34.7% (n=25), 5.6% (n=4), and 4.2% (n=3), respectively. Additionally, resistance rates to gentamycin, cefotaxime, kanamycin, ceftiofur, and meropenem were 2.8% (n=2), 1.4% (n=1), 0%, 0%, and 0%, respectively. When calculating the percentage of susceptible isolates, only those deemed to be fully sensitive were considered susceptible.

It was determined that the mcr genes investigated in this study were not responsible for the colistin resistance observed in the 72 isolates examined (Figure).

DISCUSSION

Compared with *E. coli*, few studies have investigated colistin resistance in *Salmonella* isolates. The study found that 24% (72/300) of *Salmonella* isolates were phenotypically resistant according to the EUCAST criteria using the microdilution method. This is consistent with the 21% phenotypic resistance observed in *Salmonella enterica* strains in Brazil (Morales et al., 2012). In Nigeria, *Salmonella* spp. was isolated from poultry, and in another study conducted on isolates, the phenotypic resistance rate was reported to be 11.7% (Ngbede et al., 2020). Surveillance studies conducted on *Salmonella* isolates from poultry in European Union member countries have reported a lower prevalence of colistin phenotypic resistance compared to that in the rest of the world. Specifically, the prevalence was reported to be 1.8% in broiler flocks and 8.1% in layer flocks (EFSA/ECDC, 2020; EFSA/

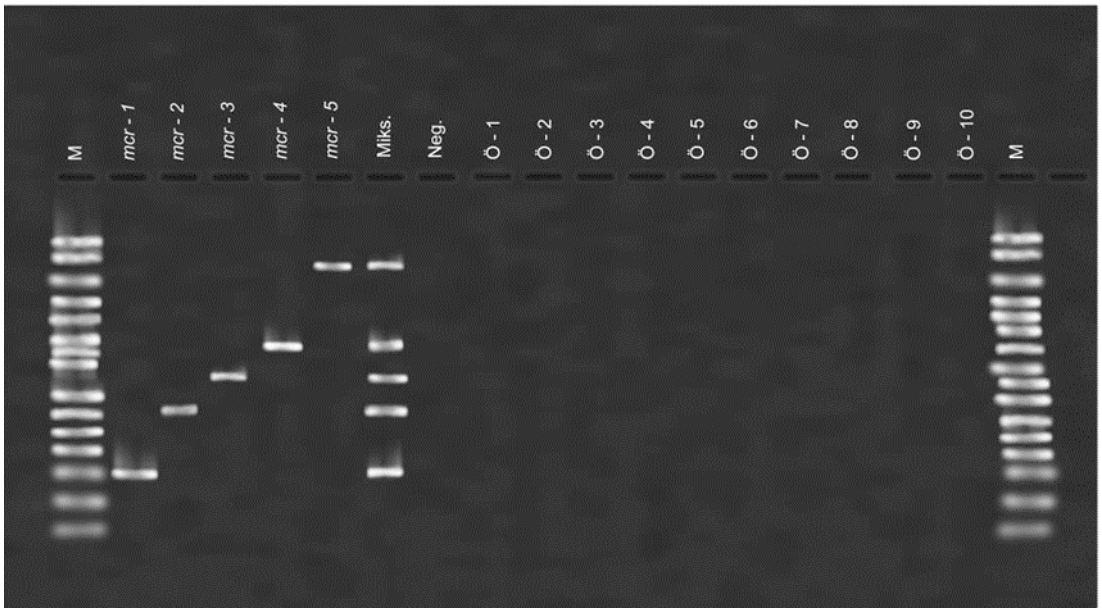

Figure 1. *mcr* 1-5 genes positive control strains and *mcr-negative* multiplex PCR samples. *mcr*1-320bp *E. coli* (2012-60-1176-27), *mcr*2-700bp *E. coli* KP37, *mcr*3-900 *E. coli* 2013-SQ352, *mcr*4-1100bp *E. coli* DH5α, *mcr*5-1644bp *Salmonella* 13-SA01718. M: 100 bp DNA Ladder (SM 0241, Thermo Fisher, ABD).

ECDC, 2022). However, a retrospective surveillance study of *Salmonella* strains isolated from poultry meat in Portugal reported a higher rate of 14.3% (Figueiredo et al., 2016). The phenotypic resistance rate to colistin in *Salmonella* strains isolated from poultry in Thailand, was reported to be 12.6% (Sakdinun et al., 2016). In contrast, studies conducted in Japan (Esaki et al., 2004) and Korea (Lim et al., 2009) reported much lower rates of 1.2% and 1%, respectively, which are similar to those reported in European Union countries. In summary, the reported rates of phenotypic colistin resistance in *Salmonella* strains vary significantly worldwide. The inability to detect mobile genes that encode resistance in isolates that are phenotypically resistant to colistin has been noted. This may be associated with lipid A modifications (Luo et al., 2017) and/or fluctuations in mRNA synthesis, resulting from mutations in certain chromosomal genes (Jovčić et al., 2020). Although chromosomal mutations are not horizontally transferable, it is important to consider *mcr*-negative colistin-resistant isolates (Luo et al., 2017). Research has shown that the quantity and stability of plasmids in transconjugant cultures carrying the *mcr*-1 and *mcr*-3 genes decreases over time (Yang et al., 2023). Studies have shown that in the absence of selective antibiotic pressure, plasmid stability decreases, and the ability of bacteria to adapt to the plasmid decreases (Nang et al., 2018). It is possible that changes in the plasmids in question may explain why genotypically mobile genes could not be detected in the strains showing phenotypic resistance in our study. Furthermore, the inconsistency in the phenotype-genotype relationship may be related to *mcr* genes and their variants, which were not investigated in this study (Gharaibeh et al., 2019).

Although colistin is not typically used to treat *Salmonella* infections, some *Salmonella* strains have been observed to be phenotypically resistant to colistin. However, we were unable to identify any of the investigated *mcr* genes associated with phenotypically determined colistin resistance. Similarly, the *mcr* gene was not found in the phenotypic colistin resistance detected in 47.5% of *Salmonella* isolates in China (Luo et al., 2017) and in 25% of food-origin *Salmonella* isolates in Turkey (Tok et al., 2023). Similar to our study, *mcr* (*mcr*-1-10) was not detected in any of the 210 phenotypic colistin-resistant *Salmonella* isolates from non-human sources tested in Brazil. It is also important to note that chromosomal mutations that had previously been identified as being associated with polymyxin resistance were only found in a limited number of isolates. Therefore, it is necessary to identify unknown genes that may contribute to resistance (Vieira et al., 2024). Nevertheless, chromosomal mutations and modifications (RfbN, LolB, and ZraR) in membrane lipopolysaccharide and multidrug pump (MdsC) pro-

teins have been identified in colistin-resistant but *mcr*-negative human *Salmonella* enterica strains (Fortini et al., 2022).

Therefore, it is necessary to conduct studies to monitor colistin resistance in animal, environmental, and human isolates using a one-health concept. In addition, new candidate mechanisms should be investigated to determine the prevalence of *mcr* determinants and colistin resistance.

In this study, we found that the resistance rates of colistin-resistant isolates to other antibiotics were low. Multiple resistance was found only in three isolates against the three drugs. Similarly, Gutierrez et al. (2020) did not find multiple resistance in *Salmonella* serotypes of poultry origin from litter in Florida. In a study comparing antibiotic resistance in fecal, carcass, and environmental samples from two poultry farming enterprises, organic and conventional, Bailey et al. (2020) emphasized a close relationship between resistance development and antibiotic use. The poultry production farms where the samples were collected in this study had very limited antibiotic use.

EUCAST (2018) and CLSI (2016) recommend using the disk diffusion test with a 5 µg pefloxacin disk as a reliable marker to determine fluoroquinolone susceptibility in typhoidal strains of *Salmonella enterica* (CLSI., 2016; Skov et al., 2015). Skov et al., (2015) reported that pefloxacin was a safe marker in non-typhoidal *Salmonella* strains. In this study, while colistin-resistant strains were highly sensitive to other antibiotics, pefloxacin resistance was observed in 34.72% of the cases. The significance of this outcome for human health is noteworthy because quinolone antibiotics are commonly used to treat *Salmonella* infections and resistance to quinolones can be transmitted between bacteria via plasmids.

CONCLUSION

Of the 300 isolates analyzed in this study, 72 (24%) were phenotypically resistant according to the EUCAST guidelines. However, none of these isolates contained *mcr* 1-5 genes. PCR and Whole Genome Sequencing (WGS) are considered reference tests for identifying the *mcr* genes in bacteria isolated from clinical, fecal, environmental, and food samples. As a recommendation for future studies, Whole Genome Analysis could be helpful in identifying all known or unknown colistin and quinolone resistance. *Salmonella* was found to have colistin resistance, which was not linked to *mcr* genes. Considering the zoonotic nature of *S. enterica*, other unknown mechanisms that may contribute to resistance need to be identified. Furthermore, the phenotypic mechanisms underlying colistin resistance in these strains should be investigated.

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CONFLICT OF INTEREST

This paper was prepared based on the Ph.D. thesis of the second author. Kırıkkale University, Institute of Health Sciences, Department of Veterinary Microbiology, Kırıkkale, Türkiye.

All authors disclose no conflicts of interest that may have influenced either the conduct or presentation of the research.

INFORMED CONSENT

In this study, the Local Ethics Committee of Kırıkkale University determined that there was no need for an ethics committee to review the animal experiments conducted at the university, as reported by the university in accordance with the decision numbered 14/03/2018 E.1876.

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