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Mobile Colistin Resistance Determinants among *Enterobacteriaceae* Isolated from Different Poultry Species

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Abstract

Antimicrobial resistance (AMR) is a global threat that requires serious attention, particularly when it is developed against colistin, which is considered one of the 'last resort' antibiotics in the poultry industry. This study aimed to investigate the AMR profile of *Enterobacteriaceae* isolates from different poultry species, detect colistin resistance and investigate the existence of *mcr* genes in multi and extreme-resistant isolates. A total of 233 birds, chickens, ducks, turkeys, and quails, of various ages and breeds were collected from several localities of the Sharkia governorate and analyzed bacteriologically. The disc diffusion and E-test assays scrutinized the patterns of antibiotic, multidrug-resistant (MDR), and colistin resistance. The PCR assay was carried out to detect the *mcr* variants. Bacteriological examination revealed the incidence of 42.3% (99/233) of different *Enterobacteriaceae* members with a high predominance of *E. coli*, *Salmonella*, and *Klebsiella* species. Disc diffusion findings disclosed that 78.78% of isolates were resistant to colistin but E-test detected 19.19% only. Observed colistin resistance was strongly linked to the distribution of plasmid *mcr*-operons. The *mcr* 1, 2, 3, 4, and 7.1 genes were detected in 42.1, 63.15, 57.89, 52.63, and 47.36% of the phenotypic resistant isolates, and about 36.84% harbored at least four *mcr* clusters. However, the *mcr*5 gene was not discovered. The statistical assessment revealed a significant association between colistin resistance and MDR ($p \leq 0.05$). Moreover, there was a strong correlation between *Mcr*-abundance and doxycycline, fosfomycin, beta-lactams, imipenem, and tobramycin resistances. In conclusion, this study highlights the alarming occurrence of colistin-resistant *Enterobacteriaceae* in various poultry aspects. An urgent strategy must be adopted to avert the spread of this phenomenon.

KEYWORDS
Colistin resistance, *Enterobacteriaceae*, *mcr* genes, Plasmid, Poultry species

INTRODUCTION

Gram-negative bacteria of the family *Enterobacteriaceae* are spread all over the environment, including in soil, water, plants, and in the intestines of animals. *Enterobacteriaceae* can cause disease by invading their host in a variety of ways, including motility, colonization factors, endotoxin, and enterotoxin (Sugawara *et al*., 2019). Chicken salmonellosis causes pasty diarrhea, loss of appetite, dehydration, growth retardation, blindness, and lameness. Hepatomegaly with necrotic foci, splenomegaly, pericarditis, panophthalmitis, persisting yolk sac, and arthritis are the primary gross lesions (Saif *et al*., 2008). Any type of bird, regardless of age, can become infected with *Salmonella*. High mortality rates at a young age can reach 80% or more, meanwhile rare mortality rates in older age over 3 weeks (Gast and Beard, 1992). *E. coli* can infect and harm many avian systems, leading to a variety of symptoms and localized or systemic diseases (Sabuj *et al*., 2019). *Klebsiella* has been isolated from dead embryos, omphalitis, yolk sac infections, dermatitis, cellulitis, inflamed respiratory mucosa, and ascites.

On the other hand, *Proteus*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, and *Shigella* are opportunistic pathogens that infrequently cause illnesses. *Salmonella* species and *Escherichia coli* are the main *Enterobacteriaceae* family pathogens that cause diseases in chickens (Yulistiani *et al*., 2019). Gram-negative bacteria of the family *Enterobacteriaceae* are a highly prevalent infectious agent that has increasingly shown a high rate of antimicrobial resistance (Cruz *et al*., 2011; Zhang *et al*., 2015).

Some members of the *Enterobacteriaceae* family, including *E. coli*, *Salmonella* spp., *Klebsiella* spp., and *Enterobacter* spp., have acquired colistin-resistance mechanisms; additional bacterial species' mechanisms are still unclear. The mechanisms of resistance are thought to be related to chromosomal mutations that cannot be transferred horizontally through gene transfer (Blair *et al*., 2015; Olaitan *et al*., 2016).

The plasmid-mediated *mcr* gene is the sole transferable resistance mechanism known to date. Due to the ease with which colistin-resistant genes can be transferred to susceptible strains, plasmid-mediated colistin poses a serious issue and causes widespread worry. Colistin resistance spreads horizontally thanks to the *mcr* genes. These plasmid-mediated genes were initially discovered in Chinese *E. coli* samples taken from pigs and meat (Zou *et al*., 2017). Isolates carrying the *mcr*1 gene display resistance to colistin without other resistance mechanisms. The existence of

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*mcr*1 in isolates is enough for colistin resistance without other resistance mechanisms, as isolates carrying this gene displayed a four- to eightfold increase in colistin MIC (Poirel *et al*., 2017). It is worth noting that the production of *mcr*1 leads to resistance to lysozymes (Sherman *et al*., 2016).

Before 2015, it was believed that chromosomal mutations caused colistin resistance, but since then, publications have documented resistance in alternative methods, including plasmid-mediated colistin resistance and ten *mcr* genes (*mcr*-1 to *mcr*-10) with minor variants (Tartor *et al*., 2021). However, studies focused on *Mcr*-resistant isolates from poultry have received little attention. Therefore, this study is designed to investigate the occurrence and resistance phenotypes of *Enterobacteriaceae* from different poultry species and correlate multidrug resistance [MDR; resistance to at least three tested antimicrobial groups] and extensive drug resistance [XDR; resistance to all tested antimicrobial groups except one or two] with plasmid-mediated *mcr* operons.

MATERIALS AND METHODS

Sample collection

A total of 233 apparently healthy, diseased, and freshly dead birds were collected from different localities at Sharkia governorate, the examined birds were represented as following 133 chickens) aged from 4-6 weeks,74 ducks (aged from 3-6 weeks), 16 quails (aged from 2 -3 weeks), and 10 turkeys aged (from 4-6 weeks).

The investigated birds were subjected to clinical examination then observable clinical signs and postmortem (PM) lesions were recorded.

Samples were taken from internal organs (liver, heart, intestine, spleen, cecum, lung) in addition to cloacal swabs. The internal organs from each bird were pooled together as one sample under aseptic conditions then labeled and transported in an ice box directly for bacteriological examination.

Bacterial isolation and identification

All samples were subjected to standard procedures for the isolation and identification of Enterobacterial isolates (Quinn *et al*., 2011), and were further identified using API20E identification kits from BioMérieux in Maryl'Etoile, France, and serotyped at the Animal Health Research Institute in Dokki, Giza, Egypt, using commercial antisera from Difco in Detroit, Michigan, USA, following the manufacturer's instructions.

Antimicrobial susceptibility testing (AST)

Antibacterial sensitivity tests were carried out on the obtained isolates using the disc diffusion method on Mueller-Hinton Agar (OXOID), according to the procedure recommended by Bauer *et al*., (1966). All isolates were tested for various routine antimicrobial drugs (OXOID), the tested antibiotics and their concentrations on µg/disc were as follows: Nalidixic acid (NA; 30), Norfloxacin (NX, 10), Ciprofloxacin (CIP, 5), Cefotaxime (CTX, 30), Amoxicillin-clavulanic acid (AMC; 30), Ampicillin (AMP;10), Imipenem (IMP, 10), Fosfomycin (FOS, 50), Tobramycin (TOB; 10), Chloramphenicol (C; 30), Tetracycline (TE, 30), Doxycycline (DO, 5), Sulfamethoxazole/ Trimethoprim (SXT, 25), and Colistin (CT;10). The inhibition zones, in millimeters, were measured in duplicate and scored as sensitive, intermediate, and resistant categories following the critical breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI, 2020).

Determination of minimum inhibitory concentration of colistin Resistance by Epsilometer test (E- test)

The E-test has been developed to provide a direct quantification of the antimicrobial susceptibility of microorganisms. Briefly, the isolates were checked to quantitatively determine colistin minimum inhibitory concentration (MIC). Colistin Ezy MIC strip (CL) (0.016–256 µg/mL) (Himedia Pvt Ltd, Mumbai, India as per manufacturer's instructions.

The results of E tests were interpreted by EUCAST-recommended colistin breakpoints for *Enterobacteriaceae* (sensitive ≤ 2, resistant > 2 µg/mL).

Molecular assay

Plasmid extraction

Following the manufacturer's instructions, plasmid DNAs were extracted from bacterial isolates using Plasmid DNA Miniprep Kits (Thermo Fisher Scientific, Waltham, MA, USA).

PCR amplification of *mcr*-genes

The colistin-resistant bacterial isolates were screened for the presence of *mcr* operons by adjusting the final volume of the reaction mixture to 25 µL consisting of 12.5µL of DreamTaq TM Green Master Mix (2X) (Fermentas, USA), 1µL of 100 pmole of each primer (Sigma, USA), 5 µLof template DNA and water nuclease-free up to 25 µL. Target genes, primers sequences, amplified product size, and annealing temperatures were illustrated in Ta-

Table 1. Oligonucleotide primers used in the present study.

Primer	Nucleotide sequence (5'→3')	Amplicon size (bp)	Annealing temperature (°C)	Amplification region (bp)
Mcr-1_FW *Mcr*-1_RW	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	309	55	35-343
Mcr-2_FW *Mcr*-2_RW	TGTTGCTTGTGCCGATTGGA AGATGGTATTGTTGGTTGCTG	567	65	494-1060
Mcr-3_FW *Mcr*-3_RV	TTGGCACTGTATTTTGCATTT TTAACGAAATTGGCTGGAACA	542	50	46-587
Mcr-4_FW *Mcr*-4_RV	ATTGGGATAGTCGCCTTTTT TTACAGCCAGAATCATTATCA	488	58	490-977
Mcr-5_FW *Mcr*-5_RV	TATCTCGACAAGGCCATGCTG GAATCTGGCGTTCGTCGTAGT	613	50	310-922
*Mcr*7.1_Fw *Mcr*7.1_RV	AGGGGATAAACCGACCCTGA TGATCTCGATGTTGGGCACC	335	55	669-1003

ble 1 as previously documented by Yang *et al*. (2018).

Visualization of PCR products

Electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in TBE (1x) buffer was used to separate the PCR products. Each gel slot had 15 µl of the product inserted for the gel analysis. Alpha Innotech, Biometra's gel documentation system captured photos of the gel, and computer software was used to analyze the data.

Statistical analysis

Data analysis was performed by SPSS version 22 for Windows. The chi-square test was used to evaluate the relationship between antibiotic resistance and species, age, breed, and disease status of the bird in addition to the association between resistance to some antibiotics and the presence of antimicrobial resistance genes.

Moreover, data were edited in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The antimicrobial resistance to different antibiotics as well as the colistin E-test was examined by the Fisher exact test. A multivariate mixed effects logistic regression model (PROC LOGISTIC; Stokes *et al*., 2012) was run with the level of significance set at $\alpha = 0.05$ to examine the effects of potential risk factors, including sample type, clinical history, species, and age category on the prevalence of microbes. Figures were fitted by the GraphPad Prism software 9.0 (GraphPad, US).

RESULTS

Postmortem examination and prevalence rate

The postmortem lesions of diseased and dead chickens included fibrinous pericarditis, peri-hepatitis, and congestion of internal organs such as the liver, lung, spleen, kidneys, heart, and intestine. The results of isolation showed 52/133 (39.09%) were positive for *Enterobacteriaceae*, meanwhile, the examined duck showed pasty vent, respiratory signs, and depression. About 34/74 (45.94%) samples were positive. The incidence of isolation concerning the breed, the incidence of isolation broiler was higher than Baladi in chickens but in ducks, Mullard showed the highest incidence of isolation (100%) followed by Muscovy (37%).

Clinically, the affected quails showed depression, huddling together, ruffling feathers, and some showed pasty vents. At necropsy, general congestion in internal organs (lung, liver, spleen, and intestine) some showed inflammation in air sacs, and the results of isolation showed 9/16 (56.25%) were positive for *Enterobacteriaceae*.

Clinically examined turkey showed high weight loss and depression. Postmortem examination revealed congestion of the lung and liver with an isolation percentage of 4/10 (40%).

Thus, the overall prevalence of *Enterobacteriaceae* isolates among examined poultry species was 42.3% (99/234) (Table 2).

The incidence of isolation about age, the highest incidence of isolation in turkeys was recorded in 6 weeks, and in chickens was 5 weeks as well as in ducks was in 5 and 6 weeks but in quail was in 2 weeks.

Table 2. Total prevalence of *Enterobacteriaceae* pathogens among examined different poultry species.

Bacterial pasthogens	Host (n=233)				Number (%)
	Chicken (n=133)	Duck (n=74)	Turkey (n=10)	Quail (n=16)	
E. coli					
O78	6	5	0	5	16 (6.86)
O111	3	4	0	1	8 (3.34)
O26	2	3	0	0	5 (2.14)
Klebsiella spp.					
K. pneumoniae	11	7	0	0	18 (7.72)
K. oxytoca	3	1	0	1	5 (2.14)
Salmonella spp.					
S. Enteritidis	3	2	3	0	8 (3.34)
S. Typhimurium	4	4	0	1	9 (3.86)
S. Kentucky	4	1	0	0	5 (2.14)
S. Hader	3	0	0	0	3 (1.28)
*S.*Infantis	1	0	0	0	1 (0.42)
Enterobacter cloacae	2	2	0	0	4 (1.71)
Citrobacter spp.					
C. freundii	1	3	1	0	5 (2.14)
C. koseri	3	2	0	1	6 (2.57)
*Proteus s*pp.					
P. mirabilis	3	0	0	0	3 (1.28)
P. vulgaris	3	0	0	0	3 (1.28)
Total	52	34	4	9	99 (42.48)

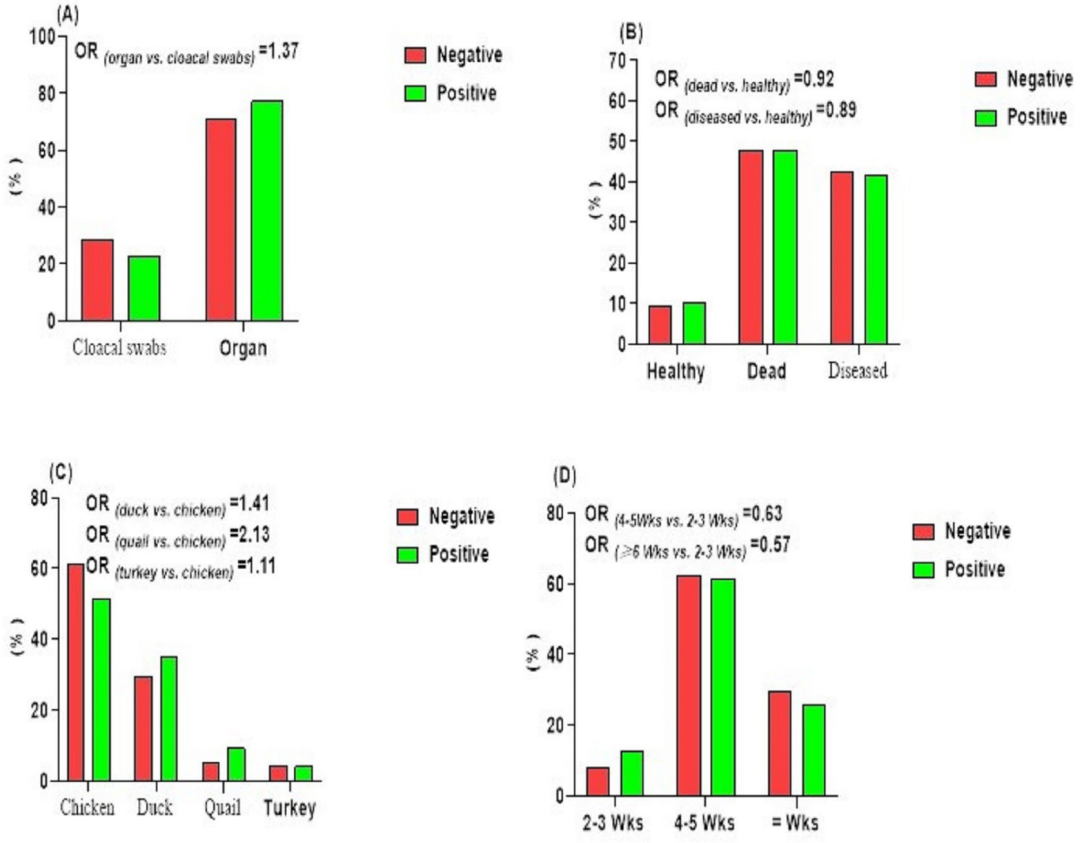

Fig. 1. Logistic regression analysis of different risk factors associated with the probability of microbial prevalence; OR, odds ratio.

The highest incidence of isolation was recorded in samples from dead birds (47%) followed by diseased birds (42%).

Results of logistic regression analysis of different risk factors associated with the probability of microbial prevalence indicated the organ had 37% higher odds of microbial prevalence than the cloacal swabs (OR=1.37 Figure 1-A). Also, the risk of microbial prevalence was higher increased by 41%, 2 times, and 11% in duck, quail, and turkey compared to chicken, the odds ratio was estimated to be 1.41, 2.13, and 1.11, respectively (Figure 1-C). About the clinical history, the likelihood of microbial prevalence decreased only by 8 and 11% in dead and diseased birds compared to the counterpart's healthy ones (OR=0.92 and 0.89, respectively; Figure 1-B). Concerning age, the birds in the age category 4-5 weeks and those more than or equal to 6 weeks had 37% and 43% lower odds of microbial prevalence compared to those in the age category 2-3Wks (OR=0.63 and 0.57, respectively; Figure 1-D).

Antimicrobial susceptibility pattern

Among 99 tested *Enterobacteriaceae* isolates by disc diffusion assay, almost isolates were susceptible to quinolones including norfloxacin (84.4%), ciprofloxacin (76.7%), and nalidixic acid (61.6%). Absolute resistance towards ampicillin (100%) followed by cefotaxime (84.8%), colistin (78.7 %), and chloramphenicol (75.5%) was detected.

Moreover, multidrug (MDR) and extreme (XDR) resistances were observed in 95.9 (95/99) and 4.1% (4/99), respectively, where all *E. coli*, *Salmonella* species, *Enterobacter cloacae*, and *Klebsiella pneumoniae* were resistant to at least five different antimicrobial agents tested and represented profound colistin resistance patterns.

On the contrary, *Proteus* and *Citrobacter* isolates were the most susceptible species to all antimicrobial agents used in our study.

Regarding E-test findings, only 19.19 % represented resistant patterns to colistin (Figure 2).

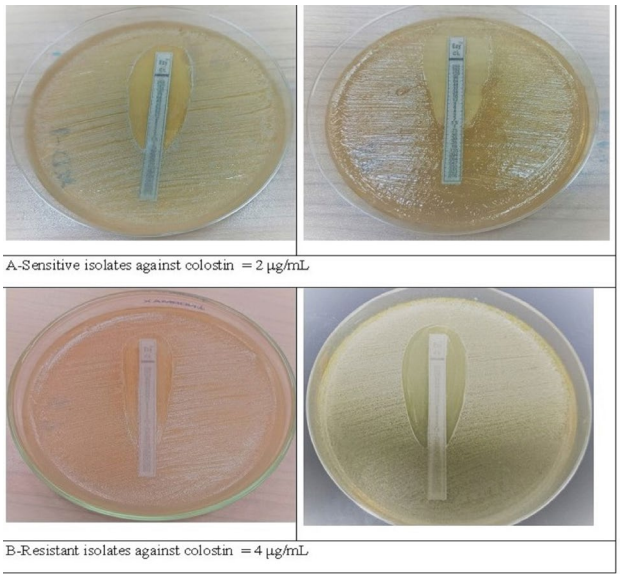

Fig. 2. Results of colistin Epsilometer MICs strip (E- test) against *Enterobacteriaceae* isolates (sensitive ≤ 2, resistant >2 µg/mL).

Statistical analysis revealed that there was no significant association between colistin antibiotic resistance by disc diffusion assay and E-test at p-value ≤0.05.

Antimicrobial resistance showed significant differences to all considered antibiotics as well ($p<0.01$). Also, significant differences were detected between chloramphenicol and colistin in favor of chloramphenicol (75% vs. 19%; $p<0.01$; Figure 3).

There was no significant association between different species and cefotaxime, colistin, imipenem, nalidixic acid, tetracycline, sulfamethoxazole/ trimethoprim, or norfloxacin resistances at p-value ≤0.05 but there were highly significant associations between different species and doxycycline, amoxicillin-clavulanic

acid, chloramphenicol, fosfomycin, and ciprofloxacin resistances at p-value ≤0.05. No statistics are computed because ampicillin is a constant. There was no significant association between different ages, diseases status and all used antibiotics resistance at p-value ≤0.05.

There was no significant association between different ages and colistin resistance at a p-value ≤0.05

There was a significant association between different districts and species in MDR results but there was no significant association between age, breed, and disease status and MDR at p-value ≤0.05.

Molecular screening for colistin-resistant isolates

All nineteen colistin-resistant *Enterobacteriaceae* isolates harbored *mcr* genes with different genotypic patterns. The *mcr* 1, 2, 3, 4, and 7.1 genes were detected in 42.1, 63.15, 57.89, 52.63, and 47.36% of the examined isolates (Figure 4), and about 36.84% harbored at least four *mcr* clusters. *Mcr*5 was absent in all isolates.

Statical analysis revealed that no significant differences were observed in the distribution of negative and positive cases of different genes (p>0.05; Figure 4). But there was a significant relationship between *mcr*1, 3, and 7 genes and cefotaxime resistance at p-value ≤0.05, significant relationships between the *mcr*2 gene and cefotaxime, amoxicillin-clavulanic acid, tobramycin resistance at p-value ≤0.05, and significant associations between *mcr*4 and doxycycline, amoxicillin-clavulanic acid, fosfomycin, and ciprofloxacin resistances at p-value ≤0.05.

DISCUSSION

Enterobacteriaceae are responsible for causing many nosocomial infections, and less commonly community-acquired infections, including urinary tract infections (UTI), respiratory infections, soft tissue infections, osteomyelitis, and endocarditis, among various poultry species (Ramirez and Giron 2022).

The results of this investigation demonstrate that representatives of six genera of *Enterobacteriaceae* isolates were isolated at a high rate. The genera were *E. coli* (29.30 %), *Salmonella* (26.26%), *Klebsiella* (23.23%), *Citrobacter* (11.11%), *Proteus* (6.06%), and *Enterobacter* species (4.04%). These findings are consistent with earlier studies (El-Demerdash *et al*., 2018; Ferreira *et al*., 2018) that isolated these species from several farms, but with varying

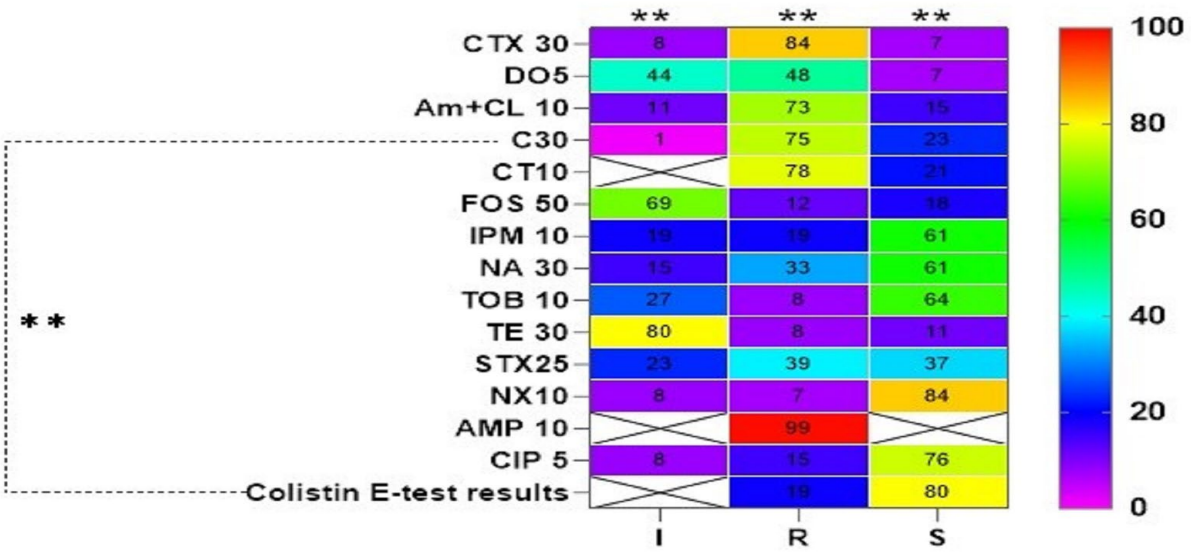

Fig. 3. Antimicrobial resistance to different antibiotics as well as colistin E-test; **p<0.01.

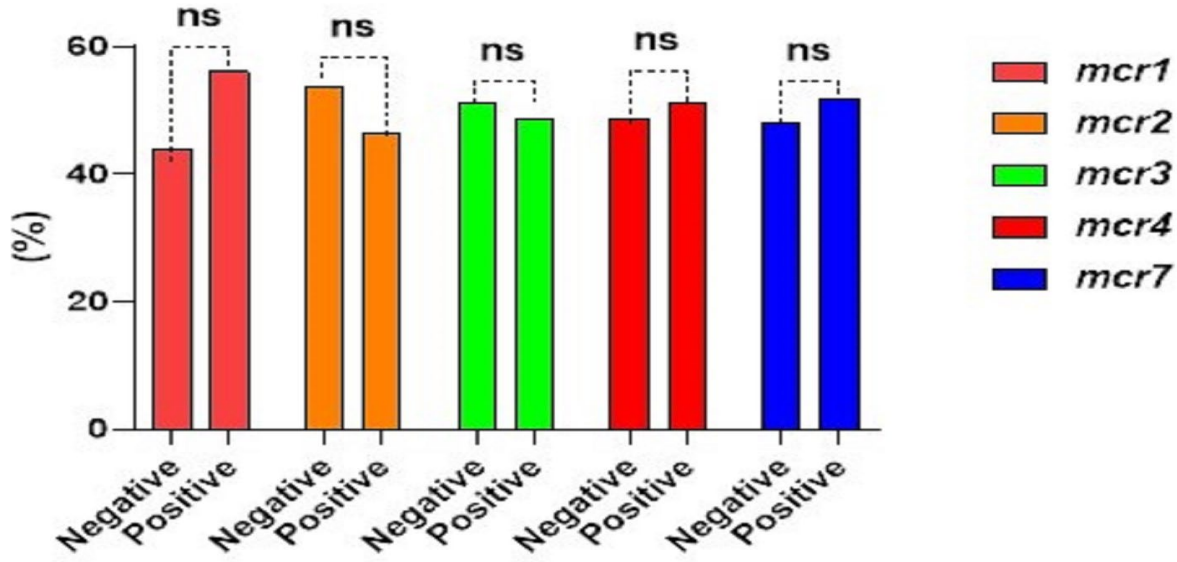

Fig. 4. Distribution of negative and positive cases of different genes; ns, non-significant.

percentages. The disparity in prevalence rates can be attributed to significant variations in sampling design, the differences in age and breeds of the chickens, turkeys, quails, and ducks, sample types, numbers, *Enterobacteriaceae* detection methods, and geographic location.

As previously reported by Nguyen *et al*. (2021) and Li *et al*. (2022), the serotyping results showed that *K. pneumoniae*, *Escherichia* O78, and *S.* Typhimurium were the main isolated serotypes.

Studies conducted by Roy *et al*. (2006) and Sharada *et al*. (2010) revealed that *E. coli* and *K. pneumoniae* are the major causes of high death and isolation percentages. Also, El-Demerdash *et al*. (2013) isolated *E. coli*, *Salmonella* spp., and *Klebsiella* spp. from freshly dead and/or morbid Japanese quails chicks aged between 2 days and three weeks with percentages of 38.75, 7.5, and 6.25%, respectively.

Resistance development is possibly associated with the long-term and widespread use of antimicrobials. In the present study, the antimicrobials used against the isolates produced varying reactions. The best overall potency was seen with quinolines. However, absolute resistance rates noticed with *Enterobacter* cloacae against ciprofloxacin (100%) are in accordance with the resistance rates observed by Benameur *et al.* (2018). In addition, high frequencies of resistance were found to cefotaxime (84.8%), chloramphenicol (78.7%), and amoxicillin-clavulanic acid (73.7%) as reported previously by El-Demerdash *et al*. (2018) and Islam *et al*. (2023).

Of interest, almost isolates tested exhibited multidrug (95.9%) and extreme resistance patterns (4.1%), which were higher than those recorded previously in Egypt (El-Demerdash *et al*., 2018), Japan (Ahmed *et al*., 2009), Ethiopia (Bushen *et al*., 2021), and Nigeria (Ezekiel *et al*., 2011) signifying that vast usage of antimicrobials on poultry farms might have contributed to this situation resulted in the presence of a profound extensive resistance incident (Shalaby *et al*., 2021).

Recently, the usage of colistin for therapy of serious infections caused by multi and extreme-resistant *Enterobacteriaceae* elevated in poultry farms among several countries. The extensive or inadequate use of colistin may lead to the development of the colistin resistance phenomenon (Gogry *et al*., 2021).

One of the most frequently utilized techniques in microbiology laboratories is the disc diffusion method (Falagas *et al*., 2005). The CA-SFM, BSAC, and product literature criteria are all insufficient for detecting colistin resistance, according to Alfouzan *et al*. (2018). Several studies have demonstrated that using disc diffusion for evaluating colistin susceptibility is unreliable.

To confirm colistin-resistant isolates, the MIC measurement is necessary. The dilution procedures continue to be the gold standard, although many clinical laboratories find it challenging to conduct them as routine testing. The E-test is a quick and reliable alternative technique for assessing the susceptibility of colistin (Behera *et al*., 2010).

In this research, there was no significant correlation between disc diffusion and E-test. By disc diffusion, the MICs by E-test were lower. As colistin-resistant percent by disc diffusion assay reached 78.78% but E-test detected 19.19% only. E-test did not reveal any false-resistant or false-susceptible results (Maalej *et al*., 2011).

Genes known as *mcr* genes emerged for mobilized colistin resistance. These genes produce phosphoethanolamine transferase enzymes that attach a phosphoethanolamine (PEtN) moiety to lipid A in the outer membrane of Gram-negative bacteria, reducing their net negative charge and enabling colistin resistance (Hussein *et al*., 2021).

Herein, the high rate of *mcr* genes in all 19 colistin-resistant *Enterobacteriaceae* isolates was unexpected. Significantly high percentages of *K. pneumoniae* were found to have *mcr* genes, particularly *mcr*2, in line with other earlier studies (Imtiaz *et al*., 2021; Gharaibeh *et al*., 2022). Savin *et al*. (2020) and Yang *et al*. (2023) recorded that *mcr*1 was the predominant gene in the colistin-resistant phenomenon while we detected it in the lowest incidence.

Interestingly, all colistin-resistant *Salmonella* isolates (n= 4) harbored three *mcr* variants with different patterns and *mcr*5 was absent in all *Enterobacteriaceae* isolates, consistent with Luk-In *et al*. (2021)

The key factor influencing the variation in *mcr* detection was the dissemination of IncHI2A and IncHI2 plasmids (Yang *et al*., 2023).

The prevalence of *mcr* genes on plasmids makes them spread easily to a wide range of organisms, including those of clinical significance, and pose serious risks to public health (Snyman *et al*., 2021).

An association between *Mcr* existence and antimicrobial resistance patterns was noted. As all *Mcr*-harboring isolates were resistant to β-lactams drugs. Bastidas-Caldes *et al*. (2023) attributed this finding to *mcr* genes coexisting most frequently with *bla* genes, especially in *E. coli*. Moreover, there were significant associations between *mcr*4 and our isolate's resistance to tetracyclines, carbapenems, and phosphonic antibiotics. These reflect the level of antibiotic resistance in the different poultry species which serve as reservoirs for the propagation of *mcr* genes as well as other plasmid-mediated antimicrobial resistance loci like ESBL, integrons, and carbapenemases (Bastidas-Caldes *et al*., 2023).

CONCLUSION

The profound abundance of *mcr* genes on plasmid supports the probability of transmitting resistant bacteria from poultry to humans or from one bird to another. Therefore, continuous monitoring and antibiotic stewardship strategies in poultry farms are essential.

CONFLICT OF INTEREST

The authors affirm that they have no conflicts of interest.

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