OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Fluoroquinolones Resistance Pattern of *Escherichia coli* from Apparently Healthy Broiler Chickens in Egypt

Mohamed S. Hussein[1*], Hanan A. Fahmy[2], Ashraf A. Abd El Tawab[3]

[1]Department of Bacteriology, Kafr El-Sheikh Laboratory, Animal Health Research Institute, Agricultural Research Center (ARC), Egypt; [2]Department of Biotechnology, Animal Health Research Institute, Dokki, Giza, Agricultural Research Center (ARC), Egypt; [3]Department of Bacteriology, Immunology and Mycology, Faculty of Veterinary Medicine, Benha University, PO Box 13736, Moshtohor, Toukh, Egypt.

Abstract | Antimicrobial resistance in *Escherichia coli* is increasingly becoming a matter of serious concern in veterinary and human health sectors worldwide. The objective of this study was to investigate the phenotypic and genotypic characteristics of quinolones and fluoroquinolones resistance in *Escherichia coli* recovered from apparently healthy broiler chickens in Egypt. A total of 150 cloacal swabs collected from broiler chickens from farms and bird markets were subjected to bacteriological and biochemical examination. The results revealed that 92% (138/150) of samples were found positive for *E. coli*. The antimicrobial susceptibility testing of 72 confirmed *E. coli* isolates against seven quinolones and fluoroquinolones antibiotics revealed that 62 (86.1%) were resistant to at least one antibiotic, with highest resistance rates observed against first generation (86.1% for nalidixic acid, and 81.9% for flumequine), and lowest rate against levofloxacin (33.3%). A total of 24 isolates, dsplaying high resistance to at least 5 fluroquinolone antibiotics, were then screened for plasmid-mediated quinolone resistance (PMQR) genes *qnr*A, *qnr*B, *qnr*S, *qep*A and *aac(6')-Ib-cr* by PCR. The results revealed that 22 isolates (91.7%) harboured at least one PMQR gene, with *qnr*S being the most frequent (83.3%). The *qep*A, *qnr*B and *aa (6')-Ib-cr* genes occurrence was 54.2%, 16.7% and 4.2% respectively, while *qnr*A was not detected in any isolate. The high prevalence of fluoroquinolones resistance, and transferable fluoroquinolones resistance determinants in *E. coli* from apparently healthy broilers in this study could pose a serious public health hazard, which highlights the need for effective monitoring and surveillance programs and cooperation between the sectors related to the epidemiology of these determinants.

Keywords | Antimicrobial resistance, Broiler chickens, Commensals, *Escherichia coli,* Fluoroquinolones

Received | July 22, 2021; **Accepted** | August 02, 2021; **Published** | January 15, 2022
***Correspondence** | Mohamed S Hussein, Department of Bacteriology, Kafr El-Sheikh Laboratory, Animal Health Research Institute, Agricultural Research Center (ARC), Egypt; **Email:** vetsaber@yahoo.com
Citation | Hussein MS, Fahmy HA, Abd El Tawab AA (2022). Fluoroquinolones resistance pattern of *escherichia coli* from apparently healthy broiler chickens in egypt. Adv. Anim. Vet. Sci. 10(3): 472-479.
DOI | http://dx.doi.org/10.17582/journal.aavs/2022/10.3.472.479
ISSN (Online) | 2307-8316

INTRODUCTION

Antimicrobial resistance poses a serious threat to human and animal health worldwide (Berendonk et al., 2015). The growing emergence of resistance in pathogenic bacteria constitutes a direct threat to humans and animals. However, there is mounting evidence that commensal and environmental bacteria are reservoirs of resistance determinants that can be transferred to pathogenic bacteria (Juricova et al., 2021). Commensal *Escherichia coli* (*E. coli*), in particular, is widely recognized as an indicator for tracking antimicrobial resistance in monitoring programs, and serves as a model for studying the emergence of antimicrobial resistance in animals, for a wide range of bacteria

(Hesp et al., 2021). In addition, it reflects the risk for consumers (Kaesbohrer et al., 2012). *E. coli* is a part of the normal microflora in poultry intestine. Nonetheless, certain strains could spread into various internal organs and cause a systemic fatal disease (colibacillosis) with great economic loss (Ibrahim et al., 2019). Furthermore, a recent report demonstrated that commensal *E. coli* isolates originating from broiler chickens can quickly transfer its antibiotic resistance determinants to human intestinal microbes (Lambrecht et al., 2019). Antimicrobials are used in poultry to prevent or treat infectious diseases and to promote growth, and are mainly administered through food or water to the entire group (Mehdi et al., 2018). Quinolones (Qs) and fluoroquinolones (FQs) are synthetic antibiotics that have been used against many Gram-positive and Gram-negative bacteria in humans and animals (Ruiz, 2019). As a result of the use, high levels of FQs resistance emerged in *E. coli* in chickens (Ferreira et al., 2018; Mahmud et al., 2018; Moawad et al., 2018; Pourhossein et al., 2020; Temmerman et al., 2020; Seo and Lee, 2021) and in humans as well (Kotb et al., 2019). Thus, FQs ended up designated as "Highest Priority Critically Important Antimicrobials" (WHO, 2019). Quinolones act by targeting bacterial DNA gyrase (topoisomerase II), and topoisomerase IV, derailing the process of bacterial DNA synthesis (Hooper and Jacoby, 2016). While FQs resistance is mainly attributable to mutations in *gyr*A and *par*C genes of the quinolone resistance determining region (QRDR), the increasing reporting of plasmid-mediated quinolone resistance (PMQR) has been causing concerns globally over its dissemination (Poirel et al., 2018). Moreover, acquisition of PMQR determinants may promote QRDR mutations, ending up with increased in the overall FQs resistance levels (Hooper and Jacoby, 2015). The resistance determinants acquired on plasmids act by three different mechanisms; target protection (*qnr*A, *qnr*B qnrC, *qnr*D, *qnr*E, *qnr*S, and *qnr*VCl); antibiotic efflux (*Qep*A, *Oqx*AB); and antibiotic modification (*aac(6') Ib-cr*) (Ruiz, 2019). Several studies around the world have recently reported on quinolone resistance genes in commensal *E. coli* (Ferreira et al., 2018; Mahmud et al., 2018; Pourhossein et al., 2020; Seo and Lee, 2021). Even though wet markets and live bird shops have been suspected to be a potential source of multi-drug resistant *E. coli* (Sarker et al., 2019; Effendi et al., 2021), and despite the flourishing poultry farming and live bird markets in Kafr El-Sheikh, an agricultural governorate in north Egypt, reporting on the subject is still scarce in region. Therefore, this study investigated the phenotypic and genotypic (PMQR) characteristics of Qs and FQs resistance in commensal *E. coli* from apparently healthy broiler chickens from farms and live bird markets in Kafr El-Sheikh governorate, Egypt.

MATERIAL AND METHODS

Ethical approval

Sample collection was performed according to the guidelines of the Animal Health Research Institute, Egypt, and in accordance with all international guidelines for use of animals.

Samples collection

A total of 150 cloacal swabs were aseptically collected from apparently healthy broiler chickens from farms (81 samples of 20-25 day old) and live bird markets (69 samples of 40-45 days old) distributed throughout different localities in Kafr El-Sheikh governorate, Egypt, in the period between October 2018 April, 2019. The samples were placed in buffered peptone water (BPW) (Lab M Limited, Lancashire, UK) and transported to the laboratories of Animal Health Research Institute for further examinations.

Isolation and identification of *E. coli* from apparently healthy broiler chickens

The collected samples were incubated at 37°C for 24h for pre-enrichment (in BPW). By using sterile cotton-tipped swabs, enriched samples were then streaked onto MacConkey agar (Oxoid, Basingstoke, UK) and incubated at 37°C for 18 to 20 h. The suspected *E. coli* colonies (pink color) were streaked onto Eosin Methylene Blue agar (Merck, Germany). After overnight incubation at 37°C, typical *E. coli* colonies (a greenish metallic sheen with a dark center) were further identified according to their phenotypic criteria, cultural characters and biochemical testing (Edwards and Ewing, 1986). Confirmed *E. coli* strains were stored at -80°C in tryptic soy broth (TSB) with 30% glycerol until further use. In total, 138 non-repetitive avian fecal *E. coli* isolates were confirmed and then used for antibiotic susceptibility testing.

Antibiotic susceptibility testing of *E. coli* isolates from broiler chickens

Using disk diffusion method, as described by (Bauer et al., 1966), antibiotic susceptibility of the 72 *E. coli* isolates was tested against seven different Qs and FQs; nalidixic acid (NA) 30 µg, ciprofloxacin (CIP) 5 µg, norfloxacin (NOR) 10 µg, moxifloxacin (MO) 5µg, enrofloxacin (ENR) 5 µg, levofloxacin (LEV) 5µg, and flumequine (UB) 30 µg (Thermo Scientific™ Oxoid, UK). The zones of inhibition were examined and recorded as sensitive, intermediate or resistant, according to according CLSI (2018) interpretative criteria. *E. coli* strain ATCC 25922 was used as control.

Molecular detection of PMQR Genes in *E. coli* isolates from broiler chickens

A total of 24 FQs-resistant *E. coli* isolates (highly resistant phenotypes, displaying resistance to at least 5 fluroquino

Table 1: The primers used in the amplification of PMQR genes from *E. coli* isolated from apparently healthy broiler chickens.

Target	Primer Sequence	Annealing.	Amplified product	Reference
*qep*A	F: CGTGTTGCTGGAGTTCTTC	50°C 40 sec.	403 bp	(Cattoir et al., 2008)
	R: CTGCAGGTACTGCGTCATG			
aac(6′)-Ib-cr	F: CCCGCTTTCTCGTAGCA	52°C 30 sec.	113 bp	(Lunn et al., 2010)
	R: TTAGGCATCACTGCGTCTTC			
*qnr*A	F: GATAAAGTTTTTCAGCAAGAGG	57°C 40 sec.	543 bp	(Broszat et al., 2014)
	R: ATCCAGATCGGCAAAGGTTA			
*qnr*B	F: ATGACGCCATTACTGTATAA	53°C 40 sec.	562 bp	(Yang and Yu, 2019)
	R: GATCGCAATGTGTGAAGTTT			
*qnr*S	F: ATGGAAACCTACAATCATAC	48°C 40 sec.	491 bp	(Vien et al., 2009)
	R: AAAAACACCTCGACTTAAGT			

Table 2: The prevalence of *E. coli* in the cloacal swabs from apparently healthy broiler chickens.

Origin of the samples	Number of tested samples	Positive samples	
		NO	%
Broiler chicken farms	81	73	90.1
Live bird markets	69	65	94.2
Total	150	138	92

The percentage was calculated according to the corresponding number of samples.

Table 3: Antibiogram profile of the *E. coli* isolates from cloacal swabs from apparently healthy broiler chickens.

Samples location	Age (day)	NO tested	Sensitivity pattern	NA	UB	CIP	NOR	ENR	LEV	MO
Broiler chicken farms	20-25	36	R	29	27	20	15	24	10	13
			I	3	2	11	5	8	15	10
			S	4	7	5	16	4	11	13
Live bird markets	40-45	36	R	33	32	27	27	28	14	17
			I	0	0	6	3	3	11	10
			S	3	4	3	6	5	11	9
Total		72	R	62	59	47	42	52	24	30
			I	3	2	17	8	11	26	20
			S	7	11	8	22	9	22	22

R=Resistant, I= Intermediate, S=Sensitive, NA=nalidixic acid, CIP=ciprofloxacin, NOR=norfloxacin, ENR=enrofloxacin, UB=flumequine, LEV=levofloxacin, MO=Moxifloxacin

Table 4: The prevalence of quinolone and fluroquinolone-resistant *E. coli* from apparently healthy broiler chickens.

Name of quinolone antibiotic	Number and percent of resistant isolates					
	Broiler chicken farms		Live bird markets		Total	
	NO	%	NO	%	NO	%
Nalidixic Acid	29	80.6	33	91.7	62	86.1
Flumequine	27	75	32	88.9	59	81.9
Ciprofloxacin	20	55.6	27	75	47	65.3
Norfloxacin	15	41.7	27	75	42	58.3
Enrofloxacin	24	66.7	28	77.8	52	72.2
Levofloxacin	10	27.8	14	38.9	24	33.3
Moxifloxacin	13	36.1	17	47.2	30	41.7

Table 5: The prevalence of PMQR genes in 24 fluroquinolone-resistant *E. coli* isolates from apparently healthy broiler chickens.

Samples location	NO of Samples tested	Detected PMQR genes									
		*qnr*A		*qnr*B		*qnr*S		aac (6 ')-Ib-cr		*qep*A	
		NO	%	NO	%	NO	%	NO	%	NO	%
Broiler chicken farms	12	0	0	1	8.3	9	75	1	8.3	6	50
Live bird markets	12	0	0	3	25	11	91.7	0	0	7	58.3
Total	24	0	0	4	16.7	20	83.3	1	4.2	13	54.2

lone antibiotics), 12 isolates of broiler farms origin, and 12 isolates of live bird markets origin, were used. The DNA was extracted from these isolates using QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Single PCR reactions were then used for amplification of each of PMQR gene (*qnrA*, *qnr*B, *qnr*S, *qep*A and *aac(6')-Ib-cr*) using specific primers (Table 1). Each PCR reaction was performed in a 25 µL reaction mixture containing 5 µL of template DNA, 12.5 µL of EmeraldAmp GT PCR Master Mix (TAKARA BIO INC.™, Japan), 1 µL of forward primer(20 pmol), 1 µL of reverse primer (20 pmol) and 5.5 µL of PCR grade water. The primers sequences, annealing temperatures, and size of amplified product for the investigated genes are shown in Table 1. PCR products were resolved on 1% agarose gel with ethidium bromide dye and the gel was visualized under a UV transilluminator (Biometra Goettingen, Germany).

RESULTS

The prevalence of *E. coli* among the examined samples

Out of 150 investigated cloacal swabs from apparently healthy broiler chickens, 138 (92%) were found positive for *E. coli* (Table 2), with slightly higher incidence in samples from live bird markets (94.2%) than those from farms (90.1%).

Phenotypic resistance of *E. coli* isolates to quinolones and fluoroquinolones

The resistance pattern of *E. coli* isolated from apparently healthy broiler chickens (Table 3, Table 4, and Figure 1) showed that 86.1% of isolates were resistant to at least one of the tested Qs or FQs antibiotics. The results revealed that 84.7% were resistant to FQs (one or more antibiotic other than nalidixic acid), and 29.2% were resistant to all tested Q and FQs. The highest resistance rates were observed against first generation (86.1% for nalidixic acid, and 81.9% for flumequine). The rates of resistance to enrofloxacin, ciprofloxacin, norfloxacin and moxifloxacin were 72.2%, 65.3%, 58.3%, and 41.7% respectively. The lowest resistance rate was observed against levofloxacin (33.3%).

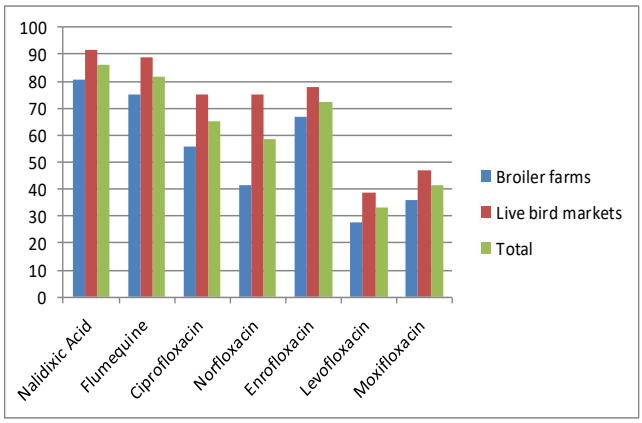

Figure 1: Prevalence of quinolone and fluoroquinolones resistance in *E. coli* from apparently healthy broiler chickens.

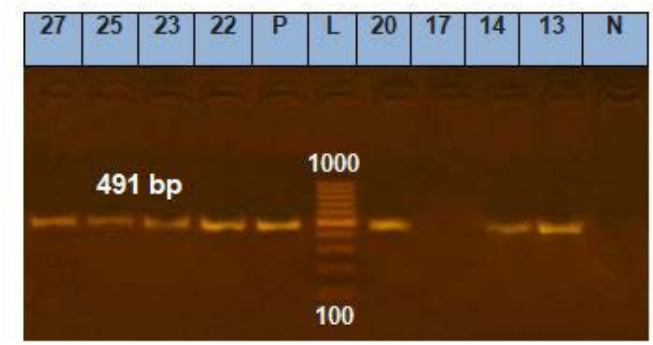

Figure 2: PCR amplification of the 491 bp fragment of *qnr*S gene from 8 *E. coli* isolates. P is control positive, N is control negative, and L is DNA ladder.

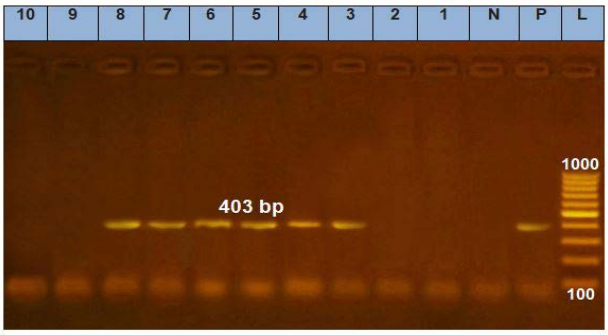

Figure 3: PCR amplification of the 403 bp fragment of *qep*A gene from 10 *E. coli* isolates. P is control positive, N is control negative, and L is DNA ladder.

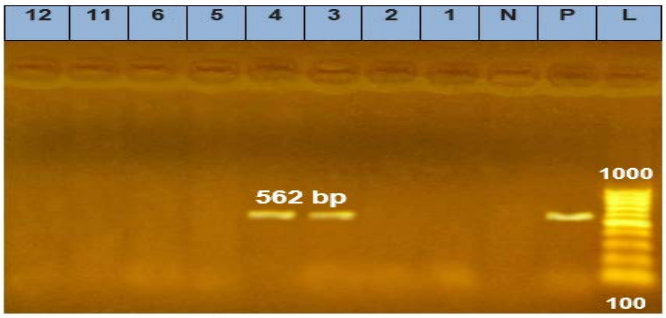

Figure 4: PCR amplification of the 562 bp fragment of *qnr*B gene from 8 *E. coli* isolates. P is control positive, N is control negative, and L is DNA ladder.

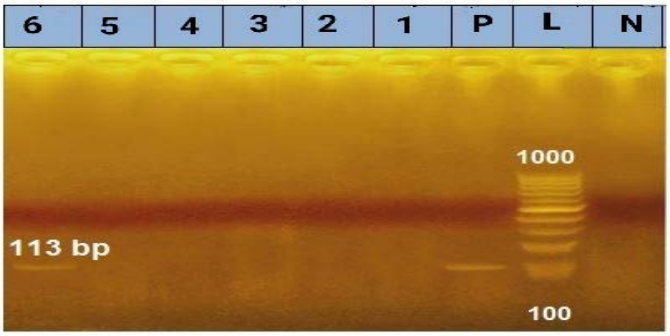

Figure 5: PCR amplification of the 113 bp fragment of *aac(6')-Ib-cr* gene from 6 *E. coli* isolates. P is control positive, N is control negative, and L is DNA ladder.

Prevalence of PMQR genes

Out of 24 phenotypically-resistant isolates, 22 (91.7%) harbored at least one PMQR gene. The *qnr*S gene was detected in (20/24, 83.3%) of tested isolates, which was the most frequent gene, while *qep*A incidence was 54.2%. The *qnr*B gene was detected in (4/24, 16.7%) of resistant isolates. Only one isolate (4.2%) was found positive for *aac(6')-Ib-cr* gene, while *qnr*A was not detected in any tested isolate (Table 5, Figures 2, 3, 4, and 5).

DISCUSSION

Even though some *E. coli* strains are pathogenic to chickens and cause colibacillosis (Younis et al., 2017), most *E. coli* strains are still a part of the normal intestinal microflora. However, the serious threat of these commensals lies in its role as a reservoir of resistance determinants, from which pathogenic bacteria could acquire resistance via horizontal gene transfer (Juricova et al., 2021).

In the present study, the overall prevalence of *E. coli* in cloacal swabs from apparently healthy broiler chickens was 92% , which was close to results by Mohamed et al. (2014) (92.6%) and Ferreira et al. (2018) (90.5%). Higher recovery rate (100%) was recorded by (Rahman et al., 2011), and slightly lower incidence was reported by Moawad et al. (2018) (87.5%), while much lower recovery rate reported by Hardiati et al. (2021) (55.6%). These differences may be attributable to the level of hygienic standards and husbandry, mechanical vectors and biosecurity (Gompo et al., 2019). Besides, host genetic factors (Berghof et al., 2019), and the detrimental impact of antibiotic use on intestinal *E. coli* could affect the results. Our findings showed higher occurrence of *E. coli* in samples from live bird markets than those from farms. This may be due to overcrowding during transportation, confining the birds in highly contaminated environment with birds of different species and origins, with receiving them on the same old litter which accumulates the microbes, and, in addition, the bacteria circulating in the place from the intestinal content of the slaughtered birds. Moreover, the birds of older age may have more chance of contracting the microbe (Gompo et al., 2019).

For decades, quinolones and fluoroquinolones have been effectively used against many serious Gram-negative bacterial infections in humans and animals (Ruiz, 2019), including colibacillosis in chickens (Vanni et al., 2014). Under the selection pressure, high levels of FQ-resistant *E. coli* have been reported worldwide from poultry (Ferreira et al., 2018; Mahmud et al., 2018; Moawad et al., 2018; Pourhossein et al., 2020; Temmerman et al., 2020; Seo and Lee, 2021). In the present study, *E. coli* displayed high rates of resistance to most of tested Qs and FQs. Recent reports from Egypt supported our findings, recording high levels of FQs resistance in *E. coli* from broiler chickens (Awad et al., 2016; El-Shazly et al., 2017), food of animal origin (Abdelkarim et al., 2020) and from humans as well (Kotb et al., 2019), highlighting the widespread of FQs resistance in Egypt in different sectors. Our results did not come as a surprise, as almost all farms in Egypt have been reportedly using antibiotics (Kimera et al., 2020). We observed highest resistance against first generation (86.1% for NA, and 81.9% for UB), which was consistent with some recent reports (Awad et al., 2016; Younis et al., 2017; Kim et al., 2020; Pourhossein et al., 2020; Hardiati et al., 2021). However, lower resistance against first generation was recorded by Talavera-González et al. (2021). The resistance against second generation FQs; ENR, CIP, and NOR in the current study (72.2%, 65.3%, and 58.3% respectively) was close to results from previous findings (Ammar et al., 2015; Agabou et al., 2016; Abo-Amer et al., 2018; Amer et al., 2018; Kim et al., 2020; Effendi et al., 2021), while higher rates were reported by El-Shazly et al. (2017), and lower rates were recorded by Khalaf et al. (2020). These variations in resistance rates may be attributed to the varied levels of Qs and FQs use in animal production in different regions (Mehdi et al., 2018; Roth et al., 2019), and anthropological and socioeconomic factors (Collignon et al., 2018). To our knowledge moxifloxacin and levofloxacin are not in use in the veterinary sector in Egypt. Resistance rates against them in this study (41.7% and 33.3% respectively) could be

due to cross-resistance among antibiotics of the same class (FQs) and/or exchanging resistance determinants with microbes from working staff through horizontal gene transfer. Our findings revealed higher resistance rates to almost all tested Qs and FQs in live bird markets samples than their corresponding rates in farms. This may be due to the possible transfer of the circulating resistant microbes and/or their resistance genes between birds from different origins (Talavera-González et al., 2021) and between birds and human staff (Lambrecht et al., 2019) in highly contaminated environment with low hygienic standards, leading to dissemination of resistance to *E. coli* which has a great capacity to accumulate resistance genes through horizontal gene transfer (Poirel et al., 2018). Furthermore, drug-resistant bacteria in animals and the environment proliferate as birds get older, due to the selective pressure of antibiotic use during the rearing period, leading to an increase in the overall resistance of bacteria (Han et al., 2020).

FQs resistance dissemination is mainly attributed to transferable mechanisms by mobile genetic elements which are usually expressed as PMQR (Ruiz, 2019). Even though PMQR genes cause reduction in Qs and FQs susceptibility which does not reach the breakpoints, additive effect is observed by accumulation of two or more genes (Rodríguez-Martínez et al., 2016). Moreover, PMQR may promote the selection of high-level resistance strains with mutations on the chromosome (Liu et al., 2012). The present study showed high prevalence (91.7%) of PMQR genes among FQ-resistant *E. coli* isolates, which is close to reports by Ammar et al. (2015) (100%) Egypt. Lower prevalence, however, was recorded by (Ferreira et al., 2018) in Brazil (23%), (Kim et al., 2020) in Korea (15.2%), and (Mahmud et al. (2018) in Bangladesh (72.22%). The *qnrS* gene was the most frequent PMQR gene (83.3%), which is consistent with findings from previous studies (Ammar et al., 2015; Mahmud et al., 2018). In contrast, low *qnr*S prevalence was reported by Ferreira et al. (2018), Pourhossein et al. (2020), Seo and Lee, (2021), Kim et al. (2020).

Our results revealed 54.2% occurrence of *qep*A, which is close to results by Pourhossein et al. (2020) (53.40%). In contrast, Agabou et al. (2016) and (Kim et al., 2020) did not detect the gene in any tested isolate. The *qnr*B was detected in 16.7% of samples in the current study, which is higher than those reported by Agabou et al. (2016) (0%), Seo and Lee, (2021) (3.8%), and Kim et al. (2020) (0%) and lower than findings by Ferreira et al. (2018) (21.5%). The current study displayed low *aac(6')-Ib-cr* occurrence (4.2%), which was close to previous findings (Seo and Lee, 2021). However, a higher rate (22.2 %) was reported by Agabou et al. (2016), and lower rate were recorded by Ferreira et al. (2018) (0.5%). The *qnr*A was not detected in any tested isolate in the current results, which is similar to previous findings (Agabou et al., 2016; Mahmud et al,, 2018). In contrast, the gene was detected by Pourhossein et al. (2020) (15.5%), Seo and Lee (2021) (6.6%) and Kim et al. (2020) (12.7%).

The differences in the occurrence of PMQR genes are often attributed to variations in the levels of Qs and FQs use in each region and the period of time. However, the levels of hygienic standards and biosecurity may affect the results, as there's evidence that even farms that do not use FQs are still at risk of acquisition of resistance determinants through contamination of production system premises with FQ-resistant *E. coli* from other sources (Taylor et al., 2016). Besides, there's evidence that the older the birds get, the more resistance determinants they tend to accumulate (Han et al., 2020).

The absence or low prevalence of PMQR in some of the tested isolates, despite displaying high phenotypic resistance, could be attributed to chromosomal mutation, which is the primary mechanism for FQs resistance (Temmerman et al., 2020).

CONCLUSION

In conclusion, the high prevalence of FQs resistance in *E. coli* from broiler chickens in Egypt, with phenotypes of high resistance to multiple FQs antibiotics, along with the high occurrence of transferable FQs resistance determinants (PMQR genes), in our study, highlight the need of monitoring FQs use in poultry and routine screening for these genetic determinants in the sector. Besides, the increasing relevance of broiler chickens as a meat source facilitates the repeated exposure of the public to FQ-resistant *E. coli* in live birds or their contaminated meat products, which, in turn, enhances the transfer of FQs resistance determinants to human pathogens. Therefore, cooperation between veterinary, human health and environmental research institutions is highly recommended for better understanding of the epidemiology of FQs resistance emergence and dissemination, and for effective control plans.

ACKNOWLEDGEMENTS

The authors would like to thank the Bacteriology Department of Kafr El-sheikh Laboratory, Animal Health Research Institute, for their help with the laboratory work.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

MSH collected samples, did laboratory work, data analy-

sis, and drafted the manuscript. AAA designed the study, helped with laboratory work and reviewed the manuscript. HAF helped with molecular investigation and reviewed the manuscript.

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