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Characteristics of Fluoroquinolone-Resistant *Escherichia coli* from Broiler Chickens with Colibacillosis

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ABSTRACT

Avian pathogenic *Escherichia coli* causes high mortalities and substantial economic losses in the poultry industry worldwide, a problem that intensified with the increasing antibiotic resistance. In this study, we characterized fluoroquinolones resistance in *E. coli* from broiler chickens with colibacillosis. A total of 150 visceral organs collected from broiler chickens with postmortem colibacillosis lesions were subjected to bacteriological and biochemical examination. Out of them, 89 samples (59.3%) were found positive for *E. coli*. The most predominant serogroups were O125 (23.6%), O44 (16.6%), O127 (15.7%), and O18 (11.2%). The antimicrobial susceptibility testing of the recovered *E. coli* isolates against seven quinolone antibiotics revealed that 88.8% of the isolates were resistant to at least one tested antibiotic, while 25.8% were resistant to all of them. The highest resistance rates were observed against flumequine (77.5%) and nalidixic acid (73%), while the lowest resistance rate was observed against levofloxacin (29.2%). A total of 12 isolates displaying phenotypic resistance to fluoroquinolones were then screened for plasmid-mediated quinolone resistance (PMQR) genes *qnr*A, *qnr*B, *qnr*S and *aac (6')-Ib-cr* by PCR. The results showed that 66.7% of the isolates were positive for *qnr*S gene, while *aac (6')-Ib-cr*, *qnr*A and *qnr*B genes were not detected in any isolate. The high occurrence of fluoroquinolones resistance and fluoroquinolones resistance genes in avian pathogenic *E. coli* is alarming, and urgently requires effective monitoring of the use of fluoroquinolones in the broiler sector, with regular screening for these resistance genes to evaluate the scale of their threat to veterinary practice and public health.

1. INTRODUCTION

Escherichia coli (*E. coli*) is a common inhabitant of the intestinal tract of humans, animals, and birds. However, some strains, known as avian pathogenic *E. coli* (APEC), could spread into chickens internal organs after inhalation of fecal dust causing extra-intestinal infections (septicemia, peritonitis, air sacculitis, pericarditis, perihepatitis, and others), which as a whole are called colibacillosis, a leading cause for high mortalities and great economic losses in the poultry production sector (Kim et al., 2020). In addition to economic losses, some APEC isolates share similarities in phylogenetics and virulence profiles with human neonatal meningitis and uro-pathogenic *E. coli* strains and have the capability to cause meningitis and urinary tract infections in humans (Kathayat et al., 2021). Furthermore, the tendency of *E. coli* to accumulate resistance genes through horizontal gene transfer and their role as a reservoir of resistance determinants made it a great challenge and serious threat to both veterinary practice and human health sectors globally (Juricova et al., 2021). Fluoroquinolones (FQs) are synthetic bactericidal antimicrobial agents that have been used in the treatment of great varieties of serious bacterial infections, and are effectively used against *E. coli* and other Gram-negative bacteria in humans and animals (Seo and Lee, 2021). They have been widely used as a treatment option for avian colibacillosis (Hricová et al., 2017; Mahmud et al., 2018). Quinolones target the bacterial DNA gyrase (topoisomerase II), and topoisomerase IV, impeding bacterial DNA synthesis (Hooper and Jacoby, 2015). FQs resistance is attributed to amino acid changes in the active sites of these target enzymes which occur as a result of point mutations in the quinolone resistance determining regions (QRDR) of *gyr*A and *par*C genes (Hooper and Jacoby, 2016). However, there has been a global concern over resistance acquired through transferable resistance determinants, which are called plasmid-mediated quinolone resistance (PMQR) (Ferreira et al., 2018). The PMQR includes three mechanisms: target protection through Qnr peptides (with multiple identified families like *qnr*A, *qnr*B, *qnr*C, *qnr*D, and *qnr*S); efflux pumps (mainly OqxAB and QepA); and drug target modification by acetylation such as *aac(6`)-Ib-cr*, a variant of aminoglycoside acetyltransferase (*aac(6`)-*

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Ib) (Rodríguez-Martínez *et al*., 2016). Even though PMQR determinants confer low resistance levels in comparison with chromosomal mutations, they could achieve the clinical CLSI breakpoints of FQs resistant isolates by the additive effect of two or more PMQR genes. Besides, these determinants were thought to promote mutations at the QRDR, resulting in an overall high resistance level in the isolate harboring them (Rodríguez-Martínez et al., 2016). With the FQs still approved for use in poultry production worldwide (except USA) (Roth et al., 2019), high resistance levels against these agents have been globally reported in the broiler sector (Rafique et al., 2020; Temmerman et al., 2020; Hardiati et al., 2021; Seo and Lee, 2021). However, the scarcity of the reports on the issue in Egypt demanded further investigations. The present study was conducted to shed some light on the occurrence of FQs resistance, and prevalence of PMQR genes in APEC strains from broilers chickens in Kafr el-sheikh, Egypt.

2. MATERIAL AND METHODS

2.1. Collection of samples

A total of 150 samples (84 livers, and 66 tracheal swabs) were collected from septicemic broiler chickens older than 15 days, showing symptoms and postmortem lesions of colibacillosis, from farms distributed in different locations in Kafr El-Sheikh governorate, Egypt, in the period between June 2018 and April 2019. The samples were collected in sterile tubes containing buffered peptone water (BPW) (Lab M Limited, Lancashire, UK) and then transferred immediately to the laboratories of Animal Health Research Institute (AHRI) in Kafr El-Sheikh city

2.2. Isolation and identification of E. coli

After surface sterilization of liver samples using Bunsen burner and heated spatula, bacteriological loops from liver depths were taken and placed in BPW, while tracheal swab tips were directly immersed in BPW tubes. The samples were then incubated at 37 °C for 24 h for pre-enrichment. The enriched samples were then examined for isolation and identification of *E. coli* according to the techniques described by Swayne (1998). In total, 89 *E. coli* isolates were recovered.

2.3. Serological identification of E. coli isolates

By using polyvalent and monovalent rapid diagnostic *E. coli* antisera (Denka Seiken Co., Ltd. Tokyo, Japan), and according to methods described by Lee et al. (2009), the confirmed 89 *E. coli* isolates were serotyped in the laboratories of Animal Health Research Institute, Dokki, Giza, Egypt.

2.4. Antibiotic susceptibility testing of the recovered E. coli isolates

The antibiotic susceptibility of the recovered 89 *E. coli* isolates was tested using disk diffusion method (Bauer et al., 1966), against seven quinolones (Qs) and FQs: ciprofloxacin (CIP) 5 µg, nalidixic acid (NA) 30 µg, moxifloxacin (MO) 5µg, norfloxacin (NOR) 10 µg, levofloxacin (LEV) 5µg, enrofloxacin (ENR) 5 µg, and flumequine (UB) 30 µg (Thermo Scientific™ Oxoid, UK). The measured inhibition zones were then recorded as resistant, intermediate, or sensitive according to the interpretative criteria by CLSI (2018), with *E. coli* ATCC 25922 used as control.

2.5. Molecular detection of PMQR genes in E. coli isolates

A total of 12 *E. coli* isolates that were resistant to FQs (showing high resistance to at least 2 tested quinolone antibiotics) were screened for *qnr*A, *qnr*B, *qnr*S, *and aac(6')-Ib-cr* genes. After DNA extraction using QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions, single PCR reactions were performed to amplify the aforementioned PMQR genes. Each PCR reaction was conducted in a 25 µL reaction mixture containing 12.5 µL of Emerald Amp GT, 5 µL of template DNA, PCR Master Mix (TAKARA BIO INC.™, Japan), one µL of reverse primer (20 pmol), one µL of forward primer (20 pmol), and 5.5 µL of PCR grade water. The sequences of the primers, annealing temperatures, and size of the PCR product for the examined genes are shown in table (1). All the PCR products were then resolved on 1% agarose gel with ethidium bromide. The gel was then visualized under a UV transilluminator (Biometra Goettingen, Germany) ((Xie et al., 2014).

Table (1) the primers used in the amplification of PMQR genes from *E. coli* from septicemic broiler chickens

Target	Primer Sequence (5-----3)	Annealing	Amplified product	Reference
aac(6')-Ib-cr	F: CCCGCTTTCTCGTAGCA	52˚C	113 bp	(Lunn et al., 2010)
	R: TTAGGCATCACTGCGTCTTC	30 sec.		
*qnr*A	F: GATAAAGTTTTTCAGCAAGAGG	57˚C	543 bp	(Broszat et al., 2014)
	R: ATCCAGATCGGCAAAGGTTA	40 sec.		
*qnr*B	F: ATGACGCCATTACTGTATAA	53˚C	562 bp	(Yang and Yu, 2019)
	R: GATCGCAATGTGTGAAGTTT	40 sec.		
*qnr*S	F: ATGGAAACCTACAATCATAC	48˚C	491 bp	(Vien et al., 2009)
	R: AAAAACACCTCGACTTAAGT	40 sec.		

3. RESULTS

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

Out of 150 investigated broiler chickens, 89 (59.3%) were found positive for *E. coli* (Table 2).

Table (2) The prevalence of *E. coli* in septicemic broiler chickens

Sampling organ	Number of tested samples	Positive samples No.	%
liver	84	51	60.7
trachea	66	38	57.6
Total	150	89	59.3

The percentage was calculated according to the corresponding number of samples

3.2. Results of serotyping of E. coli isolates

The results of serotyping of *E. coli* isolates (Table 3) showed that the most predominant serotypes were O125 (23.6%), O44 (16.6%), O127 (15.7%), and O18 (11.2%)

Table (3) Serotyping results of *E. coli* isolates from septicemic chickens

E. coli serotype	No.	%
O125	21	23.6
O166	8	9
O142	9	10.1
O18	10	11.2
O44	15	16.6
O158	6	6.7
O127	14	15.7
O86	2	2.2
O55	4	4.5

The percentage was calculated according to the total number of *E. coli* isolates (n=89)

3.3. Phenotypic resistance of E. coli isolates to quinolones and FQs

The resistance patterns of *E. coli* isolated from septicemic broiler chickens (Table 4) showed that 88.8% of isolates were resistant to at least one of the tested FQs antibiotics, while 25.8% were resistant to all tested Qs and FQs. The highest resistance rates were observed against flumequine (77.5%) and nalidixic acid (73%). The rate of resistance to both enrofloxacin and ciprofloxacin was 71.9%, while that against norfloxacin was 64%. The lowest resistance rates were observed against levofloxacin (29.2%) and moxifloxacin (44.9%).

Table (4) the pattern of antimicrobial resistance of *E. coli* isolates from septicemic broiler chickens

quinolone antibiotic	Resistance pattern					
	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Nalidixic Acid (NA) 30 µg	17	19.1	7	7.9	65	73
Flumequine (UB) 30 µg	12	13.5	8	9	69	77.5
Ciprofloxacin (CIP) 5 µg	8	9	17	19.1	64	71.9
Norfloxacin (NOR) 10 µg	24	27	8	9	57	64
Enrofloxacin (ENR) 5 µg	11	12.4	14	15.7	64	71.9
Levofloxacin (LEV) 5µg	30	33.7	33	37.1	26	29.2
Moxifloxacin (MO) 5µg	26	29.2	23	25.8	40	44.9

3.4. Prevalence of PMQR genes

Out of 12 phenotypically-resistant *E. coli* isolates, 66.7% (8/12) harbored *qnr*S gene. The *aac(6')-Ib-cr*, *qnr*A, and *qnr*B genes were not detected in any tested isolate (Table 5, Figure 1)

Table (5) the prevalence of PMQR genes in 12 fluoroquinolone-resistant *E. coli* isolates from septicemic broiler chickens

E. coli serotype	Phenotypic resistance pattern	Detected PMQR genes
O125	NA-UB-CIP-NOR-ENR-LEV-MO	- ve
O125	NA-UB	*qnr*S
O166	NA-UB-NOR-ENR- LEV	*qnr*S
O142	NA-UB-ENR	- ve
O18	NA-UB-ENR	*qnr*S
O44	NA-UB- -ENR- LEV-MO	*qnr*S
O44	NA-UB-CIP-NOR-ENR-LEV-MO	*qnr*S
O158	NA-UB-CIP-NOR-ENR	- ve
O127	NA-UB-CIP-NOR-ENR-LEV-MO	*qnr*S
O127	NA-UB-CIP-ENR- LEV	*qnr*S
O86	NA-UB-ENR-MO	*qnr*S
O55	NA-UB-CIP-ENR	- ve

NA=nalidixic acid, CIP=ciprofloxacin, NOR=norfloxacin, ENR=enrofloxacin, UB=flumequine, LEV=levofloxacin, MO=Moxifloxacin

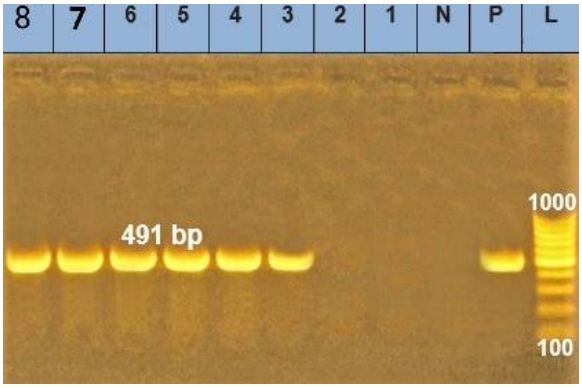

Fig (1) PCR amplification of the 491 bp fragment of *qnr*S gene from 8 *E. coli* isolates. Lane P is control positive. Lane N is control negative. Lane L is 100 - 1000bp DNA Ladder. Lanes 3-8 are positive *E. coli* isolates. Lanes 1 and 2 are negative *E. coli* isolates

4. DISCUSSION

APEC strains are linked to a wide range of systemic and localized infections in poultry, a role that is facilitated by a powerful virulence profile that enables them to cause an invasive systemic disease known as colibacillosis (Younis et al., 2017). More importantly, the emerging of resistance to almost all of the antimicrobial classes in these patho-types aggravated the problem, leaving us with very limited options for treatment, which may be unsuccessful (Awad et al., 2016; El-Shazly et al., 2017). In the present study, the occurrence of *E. coli* in septicemic broiler chickens was 59.3% , which was consistent with results by Abd El Tawab et al. (2021) (56%). Higher rate, however, was recorded by Khalaf et al. (2020) (70%), and lower incidence was reported by Ammar et al. (2015) (20%), Younis et al. (2017) (36.5%), (Amer et al., 2018) (35 %), Abd El Tawab et al. (2015) (37.1%) and Awad et al. (2016) (29%). The most predominant serotypes were O125 (23.6%), O44 (16.6%), O127 (15.7%), and O18, which is consistent with findings by Amer et al. (2018) and Abd El Tawab et al. (2021). However, A wide variety of different predominant *E. coli* serotypes were also reported from broiler chickens in Egypt (Abd El Tawab et al., 2015; Ammar et al., 2015; Awad et al., 2016; Younis et al., 2017) and abroad (Ibrahim et al., 2019; Kim et al., 2020). The variations in *E. coli* incidence rates could be due to factors related to the flock immunity, the degree of stress resulting from housing system and environmental conditions, ammonia and dust, the extent of spreading of some predisposing bacterial (e.g. Mycoplasma) and viral diseases (e.g. Newcastle and Infectious Bronchitis viruses or their vaccines) (Guabiraba and Schouler, 2015). In addition, host genetic components may contribute to some extent (Berghof et al., 2019). Fluoroquinolones have been recognized as one of the effective options against *E. coli* infections in chickens (**Hricová *et al*., 2017)**, and are still allowed for use in the poultry sector almost all over the world (Roth et al., 2019). As a result, high resistance rates of this critically important antibiotic class have been globally reported in APEC from the broiler sector (Hardiati et al., 2021; Kim et al., 2020; Seo and Lee, 2021; Temmerman et al., 2020). In the current study, *E. coli* isolates displayed high resistance rates against Qs and FQs, with 88.8% of the tested isolates were found resistant to at least one of the tested quinolone antibiotics. Recent findings from Egypt backed up our results (Abd El Tawab et al., 2015; Ammar et al., 2015; Awad et al., 2016; El-Shazly et al., 2017), highlighting the declining value of FQs as a treatment option in the broiler sector in Egypt. The highest resistance rates were observed against flumequine (77.5%) and nalidixic acid (73%). Higher resistance rates to these first-generation quinolones were recorded by Awad et al. (2016) and Hardiati et al. (2021), while lower rates against nalidixic acid were reported by Younis et al. (2017) (49.3%), and Talavera-González et al. (2021) (26.9%). The rates of resistance to second generation in the current study (ENR, CIP, and NOR, 71.9%,71.9%, and 64% respectively) were consistent with findings by Abd El Tawab et al. (2015) (60% for ENR), Amer et al. (2018) (75% for ENR). Higher rates, however, were reported by Abd El Tawab et al. (2021) (82.1% for both NOR and CIP), El-Shazly et al. (2017) (82% for

CIP), Hardiati et al. (2021) (88% for CIP; 72% for ENR). Lower rates were recorded by Khalaf et al. (2020) (8% for NOR), Younis et al. (2017) (36.9% for NOR), and Abo-Amer et al. (2018) (59% for CIP). The lowest resistance rates were observed against levofloxacin (29.2%) and moxifloxacin (44.9%). A higher finding for Levofloxacin was recorded by Younis et al. (2017) (42.4%). These variations in resistance rates could be a reflection of the differences in antimicrobial use in each locality and period, along with the differences in methodology between the studies (Temmerman et al., 2020). While moxifloxacin and levofloxacin are not reported to be in use in the broiler sector in Egypt, we assume that resistance to them could be due to cross-resistance between different quinolone antibiotics, or due to low standard biosecurity systems which facilitate contamination with moxifloxacin and levofloxacin resistant microbes (Taylor et al., 2016). Although PMQR mechanisms confer lower resistance levels than the chromosomal mediated ones (Temmerman et al., 2020), the concern over these determinants is mainly due to their role in FQs resistance dissemination to other animal hosts and humans (Rodríguez-Martínez et al., 2016). This study showed that 66.7% of the tested isolates were positive to at least one PMQR gene, which is nearly close to results obtained by Ahmed et el. (2013) (64.4%) in the same geographic region, despite a wide time gap between the two reports. lower PMQR occurrence was reported by Mostafa et al. (2014) (30%) Kim et al. (2020) (15.2%) and Temmerman et al. (2020) (18%), while a higher finding was recorded by Ammar et al. (2015) (100%).The current study showed 66.7% incidence of *qnr*S, while *aac(6')-Ib-cr*, *qnr*A, and *qnr*B genes were not detected in any tested isolate. In Egypt, Ammar et al. (2015) reported higher *qnr*S prevalence (100%), while Ahmed et el. (2013) reported *qnr*S in the rate of 15.1% and also found *aac(6')-Ib-cr*, *qnr*A and *qnr*B genes. Seo and Lee (2021) detected all four genes; *qnr*S (9.4%), *qnr*A (6.6%), *qnr*B (3.8%), and *aac(6')-Ib-cr* (1.9%), while Kim et al. (2020) detected only *qnr*A and *qnr*S (12.7% and 2.5% respectively). Even though the exact origins of PMQR genes are still questionable, some reports mentioned that *qnr* genes may have originated from the chromosomes of some environmental microorganisms, especially from water sources before dissemination to animal and human microbes (Rodríguez-Martínez et al., 2016). Therefore, The hygienic standards and biosecurity may play a role in these forementioned variations in the prevalence of PMQR genes (Taylor et al., 2016). We observed that some isolates were negative for PMQR genes despite being highly resistant to most of the tested FQ antibiotics, which may be due to mutations in the QRDR.

5. CONCULOSION

In conclusion, the high resistance levels to FQs in APEC strains of broiler chicken origin, along with the high incidence of the transmissible PMQR genes is alarming, and make the efficacy of FQs use questionable in the sector, and pose a public health hazard through the possible transmission of these resistant isolates and their determinants to humans through the direct and indirect contact with the birds. Therefore, ensuring the prudent use of FQs and emphasizing the implementation of all preventive measures, infection control policies, vaccinations, high standard biosecurity, and hygiene procedures along with using other treatment options to minimize the unnecessary FQs administration are highly recommended measures.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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