Phenotypic and Genotypic Characterization of Antibiotic Resistance in *Escherichia* coli Strains Isolated from Broiler Chickens with Colibacillosis in Isfahan Province, Iran

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Authors
Mohammad Horri,*DVM*[1]
Majid Gholami-Ahangaran, *PhD*[2]*

[1]Graduated from Veterinary Medicine Faculty, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran
[2]Department of Poultry Diseases, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

***Correspondence**
Department of Poultry Diseases, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran
Email: gholami.m@iaushk.ac.ir

ABSTRACT

Backgrounds: The aim of this study was to evaluate genotypes and phenotypes of antibiotic resistance in *Escherichia coli (E. coli)* strains isolated from poultry farms in Isfahan province, Iran.

Materials & Methods: In this study, 50 *E. coli* strains isolated from pericarditis and perihepatitis lesions of broilers in Isfahan (central Iran) were selected. After microbiological and biochemical tests and confirmation of bacterial colonies, the colonies were purified. The pure colonies were cultured on Müeller-Hinton culture medium and then subjected to antibiotic susceptibility testing. In the next step, DNA was extracted from the purified bacteria, and the *qnrA* and *sul1* genes were amplified with specific primers.

Findings: The results showed that 85% of *E. coli* isolates were resistant to at least two antibiotics, and 6% of *E. coli* strains were resistant to all 13 antibiotics used in this study. *E. coli* isolates showed the highest resistance to enrofloxacin (70%) and the lowest resistance to gentamicin (6%). Examination of resistance genes showed that about 54% of enrofloxacin-resistant *E. coli* strains contained the *qnrA* gene, and 48% of sulfonamide-resistant *E. coli* strains contained the *sul1* gene.

Conclusion: In this study, some resistant strains lacked the resistance genes studied, indicating the importance of other resistance genes in inducing resistance against sulfonamides and fluoroquinolones. Also, the lack of resistance in some strains harboring *qnrA* and *sul1* genes indicates the importance of gene expression in mediating resistance, and that the presence of resistance genes alone is not sufficient to induce antibiotic resistance in *E. coli* strains.

Keywords: *Escherichia coli*, Antibiotic resistance, Antibiotic resistance genes.

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Introduction

Escherichia coli (*E. coli*), as a member of the *Enterobacteriaceae* family, is part of the normal flora of the human and animal gastrointestinal tract. *E. coli* is a Gram-negative bacillus that grows easily on MacConkey culture medium and produces purple lactose-positive colonies [1]. The mammalian gastrointestinal tract acquires *E. coli* shortly after birth. Most *E. coli* strains are harmless but could lead to secondary and opportunistic infections outside the gastrointestinal tract and cause diseases such as urinary tract infection, wound infection, pneumonia, meningitis, and sepsis in humans [2]. In terms of epidemiology and pathogenicity, *E. coli* is the cause of important diseases in humans and animals, including intestinal diseases such as hemorrhagic enteritis, neonatal diarrhea, and neonatal sepsis [3]. Many virulence factors have been identified in *E. coli* strains, such as capsules, fimbriae, endotoxins, structures responsible for bacterial colonization, enterotoxins, verotoxins, and other secretory substances that are involved in bacterial adhesion and lesion formation on the mucosal surfaces. In addition, host factors such as age, immune status, diet, and exposure to high microbial load are also involved in the pathogenesis of this bacterium [1]. The presence of this bacterium in the environment generally depends on the level of urbanization, human and animal factors, climate, and rainfall. In microbiological examinations, the presence of *E. coli* is a reliable indicator of fecal contamination of water and indicates the risk of water-borne diseases [4, 5].

Colibacillosis is one of the most important diseases in poultry, to which chickens of different ages are highly susceptible, but the infection is more common in young chickens than in adults. The disease occurs more often at the age of 4 to 9 weeks. Among the obvious clinical signs of this disease are respiratory problems, mortality of less than 5%, and prevalence of more than 50% along with polyserositis detected in autopsy findings [1]. Decreased feed conversion efficiency, decreased carcass quality, increased carcass removal in slaughterhouses, and increased treatment costs due to the acquired resistance of bacteria to common antibiotics are other characteristics of this disease. Antibacterial drugs are widely used to reduce the damage caused by colibacillosis in poultry [6]. Extensive use of antimicrobial drugs in recent years has led to the emergence of antibiotic resistance. The genetic basis for the development of antibiotic resistance is well known, and the molecular sequences responsible for resistance could be transferred between one species or between different types of bacteria through mobile genetic components such as plasmids, integrons, and transposons. Antibiotic resistance, especially multidrug resistance, and the potential of human bacteria to receive resistance factors from animal bacteria have raised concerns about the use of antimicrobials in the treatment of colibacillosis in poultry [7].

To evaluate antibiotic resistance, phenotypic examinations are commonly used based on the presence or absence of a growth inhibition zone around commercial antibiogram discs [8]. However, molecular tests based on the amplification of a fragment of the genome involved in antibiotic resistance against a class of antibiotics have recently attracted much attention.

Objectives: In this study, phenotypic and genotypic antibiotic resistance patterns against three widely used antibiotics in the poultry industry (fluoroquinolones, aminoglycosides, and sulfonamides) were evaluated and compared to obtain the correlation of genotypic and phenotypic tests.

Materials and Methods

Sampling: In this cross-sectional study,

50 strains were selected from the total number of *E. coli* strains isolated from avian colibacillosis cases during one year from April 2020 to April 2021. The selected strains were isolated from pericarditis and perihepatitis lesions on serosal surfaces of the heart and liver, respectively.

Identification of *E. coli* strains: To identify *E. coli* bacteria, the samples were cultured linearly with a sterile swab on MacConkey culture medium and incubated at 37 °C for 24 hrs.

To confirm lactose-positive colonies (pink) as *E. coli*, five suspected colonies were cultured linearly on EMB (Eosin methylene blue) agar culture medium and incubated at 37 °C for 24 hrs. Lactose positive colonies creating a metallic green luster on EMB medium were identified primarily as *E. coli*. Then IMVIC differential tests were performed on the colonies. The suspected colonies were identified as *E. coli* in microbial culture with positive results in indole (I) and methyl red (MR) tests and negative results in Voges–Proskauer (VP) and citrate (C) tests, respectively [9].

DNA extraction and confirmation of *E. coli* strains: Overnight grown bacterial colonies were used to extract DNA. The colonies were put in a test tube containing one mL of distilled water, boiled for 10 min, and then centrifuged at 1000 rpm for five min. About 5 μL of each supernatant was used in PCR screening. After DNA extraction, molecular confirmation of bacterial colonies was carried out according to the 16S rRNA gene of *E. coli* as described by Sabat et al. (2000) [10].

The primer set of ECP79F (forward, targeting bases 79 to 96; 59-GAAGCTT GC TTCTTTGCT-39) and ECR620R (reverse, targeting bases 602 to 620; 59-GAGCCCGGGG ATTT CACAT-39) were used for molecular identification of *E. coli* [10]. *E. coli* ATCC 25922 was used as a reference and quality control organism in PCR method to confirm the

E. coli band.

Determination of antibiotic-resistant strains: To evaluate the antibiotic susceptibility of *E. coli* strains, disc diffusion method (Kirby-Bauer method) was used by employing commercial antibiotic discs (Padtan-Teb; Iran), including enrofloxacin (5 μg), difloxacin (10 μg), florfenicol (30 μg), gentamicin (10 μg), ampicillin (10 μg), chloramphenicol (30 μg), doxytetracycline (30 μg), lincospectin (300 μg), colistin (10 μg), oxytetracycline (30 μg), amoxicillin (25 μg), neomycin (30 μg), and sulfonamide plus trimethoprim. To perform this experiment, the criteria proposed by the Clinical and Laboratory Standards Institute (CLSI) were used (CLSI, 2018). For this purpose, a suspension of pure bacterial culture equivalent to 0.5 McFarland (1.5×10^8 CFU/mL) was prepared. The bacteria were then cultured on Müeller-Hinton agar medium and then incubated at 37 °C for 24 hrs after placement of antibiotic discs. The diameter of the growth inhibition zone was measured and interpreted according to the standard antibiogram protocol.

Detection of antibiotic resistance genes: The presence of genes associated with resistance to fluoroquinolones (*qnr A*), sulfonamides (*sul1*), and aminoglycosides (*aac-3-IV*) was examined using PCR technique. The primers were used according to Tavakol et al. (2008) [11] (Table 1).

PCR reactions were performed in a total volume of 25 μL, including 2.5 μL of PCR buffer 10X, 0.2 mM dNTPs (Fermentas, Germany), 1.5 mM $MgCl_2$, 0.5 μm of primers, 1.0 IU of Taq DNA polymerase (Fermentas, Germany), and 5 μL (200 ng) of DNA.

PCR amplification reactions were carried out using a DNA thermocycler (Eppendorf Mastercycler, Eppendorf-Netheler-Hinz GmbH, Biorad, Germany) under the following thermal cycling conditions: an initial denaturation at 95 °C for 3 min, followed by 35 cycles of

Table 1) Characteristics of antibiotic resistance genes

Antibiotic	Gene	Sequence (3→5)	Amplicon Length
Quinolones	*qnrA*	F:GGGTATGGATATTATTGATAAAG R:CTAATCCGGCAGCACTATTTA	670
Sulfonamides	*sul1*	F:TTCGGCATTCTGAATCTCAC R:ATGATCTAACCCTCGGTCTC	822
Aminoglycosides	*aac-(3)-IV*	F: CTTCAGGATGGCAAGTTGGT R:TCATCTCGTTCTCCGCTCAT	286

denaturation at 94 °C for one min, annealing (at 50 °C for *qnr* A, 47 °C for *sul*1, and 55 °C for aac-3-IV) for 90 s, and extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels and stained with ethidium bromide. A 100 bp DNA marker (Fermentas, Germany) was used.

Findings

Identification of *E. coli*: All 50 selected strains were initially confirmed by purple color on MacConkey culture medium; metallic green luster on EMB; and indole-positive, MR-positive, VP-negative, and citrate-negative results in IMViC test. All 50 *E. coli* strains were then confirmed by PCR test via the amplification of the 16S rRNA gene of *E. coli*.
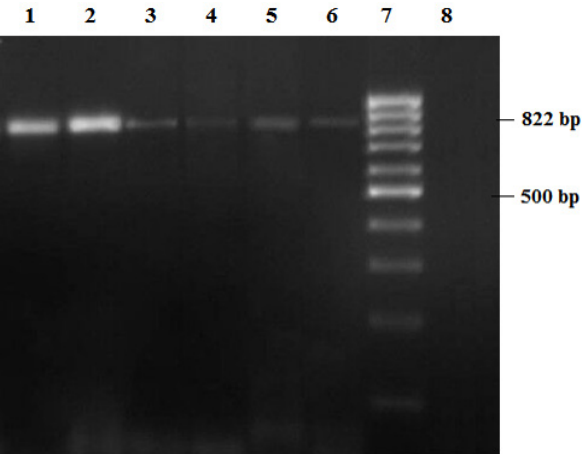
Antibiotic resistance phenotypes: Isolated strains showed the lowest antibiotic resistance to gentamicin (6%) and the highest resistance to enrofloxacin (70%). In this study, 85% of the strains were resistant to at least two antibiotics, and 6% of the strains were resistant to all 13 antibiotics used in the antibiogram test. Antibiotic resistance results are shown in Table 2.

Antibiotic resistance genotypes: Electrophoresis of PCR products showed that 15, 21, and one *E. coli* strains harbored the *sul1*, *qnrA*, and *aac-(3)-IV* genes detected by amplification of 822 (Figure 1), 670 (Figure 2), and 286 (Figure 3) bp fragments of *E. coli*, respectively. In other words, 30, 42, and 2%
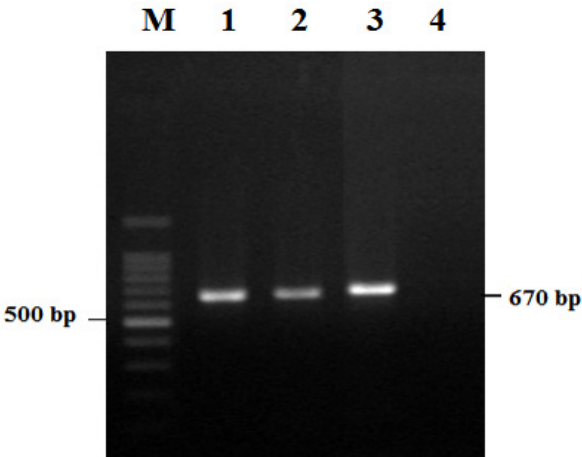
Table 2) Percentage of antibiotic resistance in *E. coli* strains isolated from chickens

| Antibiotics	Resistance		Intermediate		Sensitive	
	Frequency	Percent	Frequency	Percent	Frequency	Percent
Enrofloxacin (5 µg)	35	70	10	20	5	10
Sulfonamide plus trimetoprim	25	50	5	10	20	40
Florfenicol (30 µg)	22	44	4	8	24	48
Gentamicin (10 µg)	3	6	7	14	40	80
Oxytetracycline (30 µg)	33	66	3	6	14	28
Lincospectin (300 µg)	15	30	10	20	25	50
Doxycycline (30 µg)	30	60	10	20	10	20
Difloxacin (10 µg)	15	30	5	10	30	60
Chloramphenicol (30 µg)	23	46	9	18	18	36
Neomycin (30 µg)	18	36	6	12	26	52
Colistin (10 µg)	29	58	6	12	15	30
Ampicillin (10 µg)	20	40	5	10	25	50
Amoxicillin (25 µg)	23	46	5	10	22	44

of *E. coli* strains contained the *sul1*, *qnrA*, and *aac-(3)-IV* genes, respectively. In this study, 48% (12 strains) of sulfonamide-resistant *E. coli* strains contained the *sul1* gene, and 54.2% (19 strains) of enrofloxacin-resistant *E. coli* strains harbored the *qnrA* gene. Furthermore, 33% (one strain) of gentamicin-resistant *E. coli* strains carried *aac-(3)-IV* (Table 3). Also, three (6%) of the 50 *E. coli* strains contained the *sul1* gene but did not show the sulfonamide resistance phenotype. Also, two (4%) of the 50 *E. coli* strains contained the *qnrA* gene but did not have the enrofloxacin resistance phenotype. In addition, 52% (13 strains) of sulfonamide-resistant strains lacked the *sul1* gene, and 45.8% (16 strains) of enrofloxacin-resistant strains lacked the *qnrA* gene.

Figure 2) Electrophoresis of PCR product related to *qnrA* gene amplification (M: marker, columns 1 to 3: positive samples with amplification of a 670 bp fragment, column 4: negative control).

Figure 1) Electrophoresis of PCR product related to *sul1* gene amplification (columns 1 to 6: positive samples with amplification of an 822 bp fragment, column 7: marker, column 8: negative control).
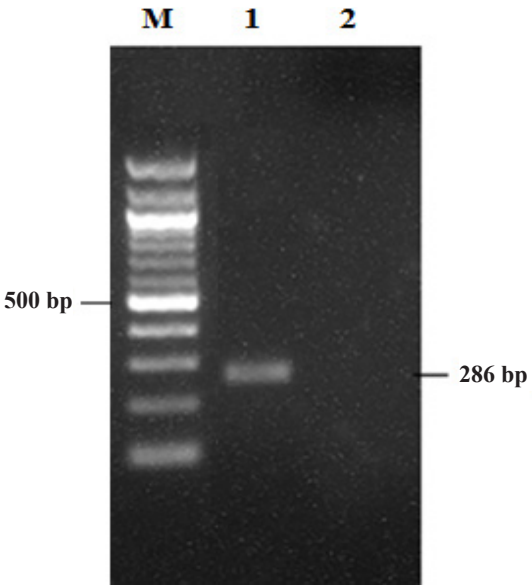
Figure 3) Electrophoresis of PCR product related to *aac-(3)-IV* gene amplification (M: marker, column 1: positive sample with amplification of a 286 bp fragment, column 2: negative control).

Table 3) Frequency of resistance genes in *E. coli* strains isolated from chickens with suspected colibacillosis

Target Genes	*sul1*	*qnrA*	*aac(3)-IV*
Percent (frequency) of detection (n=50)	30 (15 strains)	42 (21 strains)	2 (one strains)
Percent (frequency) of detection in sulfonamide + trimethoprim-resistant strains (n=25)	48 (12 strains)	NE*	NE
Percent (frequency) of detection in enrofloxacin-resistant strains (n=35)	NE	54.2 (19 strains)	NE
Percent (frequency) of detection in gentamycin-resistant strains (n=3)	NE	NE	33.3 (one strain)

*NE: Not examined

Discussion

Colibacillosis is one of the most common infectious diseases in birds, and broad-spectrum antibiotics are commonly used to control and treat this infection [1]. Studies have shown that following long-term use of various antibiotics, high levels of antibiotic resistance develop in this bacterium (*E. coli*), which ultimately leads to the ineffectiveness of common antibiotics [3]. The results obtained from antibiograms of *E. coli* strains isolated from chickens with suspected colibacillosis in Isfahan showed that a high percentage of isolates were resistant to common antibiotics used in the poultry industry and medicine. According to the results, more than 70% of the isolates were resistant to enrofloxacin, and more than 50% of them were resistant to sulfonamide + trimethoprim, indicating the high resistance of *E. coli* isolates to antibiotics widely used in the poultry industry in Isfahan, Iran. Although the pattern of antibiotic resistance is different in different geographical areas and at different times [4], a review of previous studies in Iran in the provinces of Fars, Tehran, Khuzestan, and Azerbaijan shows that this pattern is similar in most parts of Iran. This indicates the development of antibiotic resistance in *E. coli* strains. Jafari et al. (2016) reported the highest antibiotic resistance of *E. coli* strains isolated from poultry in Ahvaz to enrofloxacin (90%) [12]. Rafiei-Tabatabai and Nasirian (2003) in Tehran reported the highest resistance against tetracycline (94%), sultrime (56%), and enrofloxacin (44%) [13]. Zahraei-Salehi and Farashi-Bonab (2006) in Tabriz reported the highest resistance against doxycycline (88%), but resistance to enrofloxacin (76%) was in the third place [14]. Widespread use of enrofloxacin alone and simultaneously with some antibiotics in poultry farms throughout Iran could be the reason for this high resistance. In addition, studies in other countries have also reported the highest antibiotic resistance of *E. coli* strains against this antibiotic. Gregova et al. (2012) in Slovakia [15] and Yang et al. (2004) in China [16] have reported the highest antibiotic resistance of *E. coli* strains isolated from poultry against enrofloxacin.

In the present study, the lowest resistance was observed to gentamicin (6%) and then to lincospectin (30%). Other studies in Iran have also reported the lowest resistance to lincospectin. Jafari et al. (2016) in Ahvaz (68%) [12], Zahraei-Salehi and Farashi-Bonab (2006) in Tabriz (15%) [14], Bozorgmehri et al. (2007) in Masjed Soleiman (1.5%) [17], and Saberfar et al. (2008) by collecting samples from across the country (79%) have reported the lowest resistance to lincospectin [18]. The lowest antibiotic resistance to gentamicin and lincospectin in the present study may be due to the lower use of these two antibiotics in the poultry industry because these antibiotics are injectable and less commonly used in poultry. The apparent difference between the results of the present and mentioned studies may be due to differences in time, place, and *E. coli* strains [4], but it should be noted that the high resistance to enrofloxacin, sulfonamides, florfenicol, and doxycycline may be due to the common use of these antibiotics in the control of infectious diseases in poultry. Also, repeated administration of common antibiotics without performing antibiotic susceptibility testing in recent years has led to the emergence and spread of resistant microorganisms.

Antibiotic resistance genes play a major role in inducing resistance among microorganisms; thus, they have received much attention in recent years to identify resistant strains. Therefore, in the present study, the frequency of genes encoding resistance to fluoroquinolone and sulfonamide as important antibiotics in

veterinary medicine was investigated. In the current study, 21 (42%) out of 50 *E. coli* strains harbored *qnrA,* and 15 (30%) isolates contained *sul1*. In this regard, Ponce-Rivas et al. (2012) showed that about 52% of *E. coli* strains harbored the *qnrA* gene and in total, about 73% of the isolates harbored one of the *qnr* resistance genes; in many isolates, the *qnr* gene was detected along with class 1 integrons [19]. In the *Enterobacteriaceae* family, resistance to sulfonamides is controlled by three genes (*sul1, sul2,* and *sul3*). The *sul1* gene is part of class I integrons, which are the most abundant integrons isolated from *Enterobacteriaceae.* Therefore, *sul1* plays the most prominent role in inducing resistance to sulfonamides [12]. The antibiogram test results in this study showed that the resistance to sulfonamides was 50% (25 of 50 strains), while out of 25 sulfonamide-resistant *E. coli* strains, only 12 strains (48%) contained the *sul1* gene. The absence of *sul1* gene in 52% of sulfonamide-resistant strains indicates that *sul1* alone is not responsible for sulfonamide resistance, and other genes such as *sul2* and *sul3* may also be involved. However, previous studies have shown that *sul1* is more common among sulfonamide resistance genes, and the importance of *sul1* gene in inducing sulfonamide resistance in *E. coli* strains has already been demonstrated [12]. On the other hand, the presence of *sul1* in three sulfonamide-sensitive *E. coli* strains indicates that the presence of this gene alone is not sufficient to induce resistance, and suitable conditions must be provided for its expression. These results are consistent with the findings of another study by Mojavar-Rostami et al. (2017). They reported about 45% resistance to sulfadiazine in *E. coli* strains isolated from broiler chickens with colibacillosis in Urmia. In their study, about 57% of *E. coli* strains harbored the *sul1* gene, and five strains carrying the *sul1*

gene showed no resistance to sulfonamide [6]. According to the present study results, it seems that although *sul1* plays a major role in inducing resistance to sulfonamides, but various factors are involved in the expression of this gene and the development of resistance.

In this regard, 69.4 and 53.9% of *E. coli* strains in Mazandaran and West Azerbaijan have been shown to be resistant to sulfonamides, respectively [8]. Also, the prevalence of *sul1* gene in *E. coli* strains isolated from poultry has been reported to be 23% in Portugal [20], 41% in Tunisia [21], 26.6% in Thailand [22], and 27% in Vietnam 27% [23]. The long history of the use of sulfonamide in the treatment of poultry diseases and the possibility of using this antibiotic simultaneously with other common antibiotics in the poultry industry could be the reason for the spread of sulfonamide resistance genes among *E. coli* strains.

The high frequency of sulfonamide and quinolone resistance and the detection of *sul1* gene in only 48% of sulfonamide-resistant *E. coli* strains and *qnrA* gene in 54% of quinolone-resistant *E. coli* strains indicate that in addition to the studied genes, other genes are also involved in inducing resistance to sulfonamides (e.g., *sul2* and *sul3*) and quinolones. The development of antibiotic resistance requires the presence and proper expression of resistance genes to lead to the emergence of resistance phenotype. Therefore, to monitor antibiotic susceptibility in clinical cases or in an epidemic area, phenotypic evaluation of antibiotic resistance is more reliable than detection of resistance genes. However, the high prevalence of resistance genes among the isolates in the present study indicates the development of resistance in *E. coli* strains. The presence of resistance genes and antibiotic resistance in this bacterium could be used as an indicator to assess the

level of antibiotic resistance in humans and animals. Strains carrying resistance genes could serve as a source of resistance genes in poultry products and even humans.

This study results show that antibiotic resistance in *E. coli* strains isolated from chickens with colibacillosis is very high; in other words, resistance to common antibiotics used in the poultry industry is high. High resistance among strains raises two concerns. First, high antibiotic resistance makes antibiotics ineffective in controlling infectious diseases in poultry, leading to high drug losses and costs. Second, high antibiotic resistance leads to the transfer of resistance genes to human pathogens, which complicates the treatment of infectious diseases in humans and raises serious public health concerns.

Conclusion
It is recommended that antibiotic susceptibility testing be performed in cases of infectious diseases and that appropriate drugs be selected based on antibiotic susceptibility testing results. In addition, it is necessary to observe the existing requirements for prescribing antibiotics by observing the dose and duration of drug use, and to use safe biological compounds instead of antibiotics used in veterinary medicine as much as possible.

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