HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Isolation and Characterization of Cefotaxime and Ciprofloxacin Co-Resistant *Escherichia coli* in Retail Chicken Carcasses

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Abstract: Transmission of antimicrobial-resistant bacteria to humans through the food chain is of great importance for public health. In this study, it was aimed to isolate and characterize the cefotaxime and ciprofloxacin-resistant *Escherichia coli* in retail chicken meat samples sold in Hatay. The isolates were subjected to phylogenetic group typing and antimicrobial susceptibility testing. The genetic relatedness of the isolates was determined using Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) technique. The isolates were also screened for the presence of both antimicrobial and plasmid-mediated quinolone resistance (PMQR) genes by PCR. Cefotaxime and ciprofloxacin co-resistant *E. coli* isolates with diverse genetic origins were recovered in 42.3% (22/52) of retail chicken carcasses. The *E. coli* isolates belonged to the phylogenetic group D2 were dominant (40.9%, 9/22), followed by B1 (27.3%, 6/22), $B2_3$ (18.2%, 4/22), and A1 (13.6%, 3/22), respectively. Based on dendrogram analysis, the ERIC-PCR method differentiated the isolates into 10 clusters (I-X). The multidrug resistance (MDR) was observed in 81.8% (18/22) of the isolates. PMQR determinants were not identified in any isolates tested. Molecular analysis revealed one or more β-lactamase-encoding genes in all isolates as a single or in combination: bla_{CTX-M}-bla_{TEM} (n=5), bla_{CMY-2} (n=5), bla_{CTX-M} (n=5), bla_{CMY-2}-bla_{SHV} (n=3), bla_{CMY-2}-bla_{TEM} (n=3), and bla_{CTX-M}-bla_{CMY-2} (n=1). This study highlights that retail chicken meat is an important reservoir of cefotaxime and ciprofloxacin co-resistant *E. coli* isolates. It is necessary to evaluate their contribution to the community and hospital infections.

Keywords: *Antimicrobial resistance, Chicken carcasses, ERIC-PCR, Escherichia coli, Phylogenetic typing.*

Perakende Tavuk Karkaslarından Sefotaksim ve Siprofloksasin Eş Dirençli *Escherichia coli* İzolasyonu ve Karakterizasyonu

Özet: Antimikrobiyal dirençli bakterilerin gıda zinciri yoluyla insanlara bulaşması halk sağlığı açısından büyük önem taşımaktadır. Bu çalışmada Hatay'da satışa sunulan perakende tavuk eti örneklerinde sefotaksim ve siprofloksasine dirençli *Escherichia coli*'nin izolasyonu ve karakterizasyonu amaçlandı. İzolatlar filogenetik grup tiplendirmesine ve antimikrobiyal duyarlılık testlerine tabi tutuldu. Ayrıca izolatlar arasındaki genetik yakınlığı belirlemek için Enterobacterial Repetitive Intergenic Consensus Polimeraz Zincir Reaksiyonu (ERIC-PZR) tekniği kullanıldı. Plazmit aracılı kinolon direnci (PMQR) ile diğer direnç genleri PCR ile araştırıldı. Perakende tavuk karkaslarının %42.3'ünden (22/52) farklı genotipe sahip sefotaksim ve siprofloksasine dirençli *E. coli* izole edildi. İzolatlar arasında dominant filogenetik grup D2 (%40.9, 9/22) olup; bunu sırasıyla B1 (%27.3, 6/22), $B2_3$ (%18.2, 4/22) ve A1 (%13.6, 3/22) filogrupları izledi. Dendrogram analizine dayalı olarak, ERIC-PCR yöntemi izolatları 10 kümeye (I-X) ayırdı. İzolatların %81.8'inde (18/22) çoklu ilaç direnci (MDR) belirlendi. PMQR genleri izolatların hiçbirinde tespit edilmezken, diğer sınıftan antimikrobiyallere dirence aracılık eden çok sayıda gen saptandı. İzolatlarda β-laktamaz sentezinden sorumlu genlerin tek veya kombine olarak bulunduğu görüldü: bla_{CTX-M}-bla_{TEM} (n=5), bla_{CMY-2} (n=5), bla_{CTX-M} (n=5), bla_{CMY-2}-bla_{SHV} (n=3), bla_{CMY-2}-bla_{TEM} (n=3), and bla_{CTX-M}-bla_{CMY-2} (n=1). Bu çalışma, perakende tavuk etinin, sefotaksim ve siprofloksasine dirençli *E. coli* izolatları için önemli bir rezervuar olduğunu göstermiştir. Toplum ve hastane enfeksiyonlarına katkılarının değerlendirilmesi gerekmektedir.

Anahtar Kelimeler: *Antimikrobiyal direnç, ERIC-PCR, Escherichia coli, Filogenetik tiplendirme, Tavuk karkas.*

Introduction

The misuse and overuse of antibiotics in food animals for different purposes (treatment, prophylaxis, feed additive, etc.) have led to the selection and spread of antibiotic-resistant *Escherichia coli* strains (Ramos et al., 2020**)**. Poultry meat production and consumption have increased significantly worldwide and are expected to increase in the coming decades (Klaharn et al., 2022). Poultry meat is considered a potential vehicle for foodborne pathogens and resistant bacteria, making it a major public health problem worldwide (Gonçalves-Tenório et al., 2018). On the other hand, this situation also has a high global impact on human health and socioeconomic burden (Buzby et al., 2009; Parisi et al., 2020; WHO, 2015). Bacterial contamination has been shown to occur at every stage of the production chain from farm to table (Ananchaipattana et al., 2012; Heyndrickx et al., 2002). However, contamination mostly occurs during slaughtering processes; plucking, evisceration, and chilling have been shown to be the most important operations. It is therefore strongly suggested that an improvement in hygiene practices throughout the food chain is required to reduce the risk of foodborne pathogens and resistant bacteria from poultry meat products (Klaharn et al., 2022). Resistant *E. coli* isolates can cause intestinal and extra-intestinal infections as a result of the consumption of contaminated chicken meat and ready-to-eat chicken meat products (Davis et al., 2018).

Ciprofloxacin, a quinolone class antibiotic, inhibits DNA replication, while cefotaxime, a third-generation cephalosporin antibiotic, prevents bacterial cell wall synthesis, thereby hindering bacterial proliferation. Both antibiotics play a crucial role in clinical practice, used to combat various bacterial infections. However, excessive and inappropriate usage can lead to the development of resistance, limiting treatment options and posing a serious public health concern. This study aimed to search for the presence of ciprofloxacin and cefotaxime co-resistant *E. coli* isolates in retail chicken carcasses and to perform their molecular characterization.

Material and Methods

Ethic Statement

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Chicken Carcasses

From March to July 2023, retailed chicken carcasses were purchased from different markets in Hatay and its districts. The chicken carcasses were transported on ice within six hours to the laboratory for examination.

Isolation of *E. coli* resistant to cefotaxime and ciprofloxacin

Chicken carcasses were thoroughly rinsed with 500 ml buffered peptone water (BPW) and incubated for 18-20 hours at 37 °C. Following the incubation period, 100 µl of the culture was taken and transferred to 10 ml EC broth and incubated for 20-22 hours at 44 °C. A loopful of this culture was taken and plated on MacConkey medium containing 4 µg/ml ciprofloxacin and 8 µg/ml cefotaxime and incubated for 22–24 hours at 37 °C (CLSI, 2022). One of the typical brick red-coloured colonies were selected and identified with classical biochemical tests. Isolates identified as *E. coli* were stored at -80 °C in LB broth containing 20% glycerol until antimicrobial susceptibility testing and molecular analysis are performed.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities of *E. coli* isolates were performed and evaluated in line with the Clinical Laboratory Standards Institute (CLSI) using disk diffusion method (CLSI, 2022). The following antibiotic disks were used for the determination of susceptibilities of the isolates: ampicillin (AM, 10 µg), cefoxitin (FOX, 30 µg), amoxicillin-clavulanic acid (AMC, 10/20 µg), cefotaxime (CAZ, 30 µg), cefepime (FEB, 30 µg), meropenem (MEM, 10 µg), gentamicin (CN, 10 µg), tobramycin (TOB, 10 µg), amikacin (AK, 10 µg), tetracycline (TE, 30 µg), sulfamethoxazole-trimethoprim (SXT, 1.25/23.75 µg), ciprofloxacin (CIP, 5 µg) and chloramphenicole (C, 30 µg). *E. coli* ATCC 25922 was used as a control strain for antimicrobial susceptibility testing.

Determination of MIC values of CIP^R-CTX^R *E. coli* isolates

MIC values of ciprofloxacin (0.002-32 µg/ml) and cefotaxime (0.016-256 µg/ml) resistant isolates for these antimicrobials were determined by E-test.

Phylogenetic typing

Phylogenetic types of CIP^R-CTX^R *E. coli* isolates were determined using primers targeting *chuA*, *yjaA* and *TSPE4.C2* genes by Clermont et al. (2000). Determination of phylogenetic groups was done based on the profiles of these three genes (*chuA*/*yjaA*/*TSPE4.C2*): A0 (−/−/−), A1 (−/+/−), B1 (−/−/+), $B2_2$ (+/+/−), $B2_3$ (+/+/+), D1 (+/−/−) and D2 (+/−/+) by Escobar-Páramo et al. (2004).

Investigation of resistance genes in *E. coli* isolates

Resistance genes mediating resistance to aminoglycoside *(aac(3)-IV, aadA, strA/B, aadB, aphA1,* and *aphA2),* trimethoprim-sulfamethoxazole *(sul1, sul2, sul3, dhfrI, dhfrIII, dhfrV, dhfrIX, and dhfrXIII),* chloramphenicol *(catI, catII,* and *catIII),* cefotaxime *(bla_{CTX-M}, bla_{SHV}, bla_{TEM}),* tetracycline *(tetA, tetB, tetC, tetD, tetE,* and *tetG),* amoxicillin-clavulanic acid and cefoxitin *(bla_{CMY-2})* were searched by PCR as previously reported (Kozak et al. 2009; Monstein et al. 2007; Ng et al. 2001; Zhao et al. 2001). Screening of PMQR genes in ciprofloxacin-resistant isolates (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib* and *qepA* genes) were investigated as per Cavaco et al. (2009), Kim et al. (2009), and Park et al. (2006).

Genotyping of isolates by Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR)

Molecular genotyping of *E. coli* isolates was performed by ERIC-PCR using specific primers (Versalovic et al., 1991). The bands for each isolate were counted using the zero-one manual method, the data was then entered into the

following site: http://insilico.ehu.es/dice_upgma/, dendrograms were plotted.

Results

Of the 52 tested chicken carcass samples, 22 (42.3%) ciprofloxacin and cefotaxime co-resistant *E. coli* isolates were recovered. Based on the CLSI clinical cut-off values for CIP^R (cut-off ≥4 µg/ml) and CTX^R (cut-off ≥16/ml) in *Enterobacterales*, and all isolates (n = 22) exhibited MICs above the threshold values. All CIP^R-CTX^R *E. coli* isolates were resistant to AMP, but susceptible to ME, FEB and AK. The highest resistance rate was detected against SXT (72.7%, 16/22), followed by TE (59.1%, 13/22), C (59.1%, 13/22), AMC (54.5%, 12/22), FOX (45.5%, 10/22), CN (36.4%, 8/22), and TOB (18.2%, 4/22). Regarding the ERIC-PCR profiles, the isolates were differentiated into ten clusters (I-X). ERIC-PCR profiles of representative E. coli isolates were given in Figure 1.

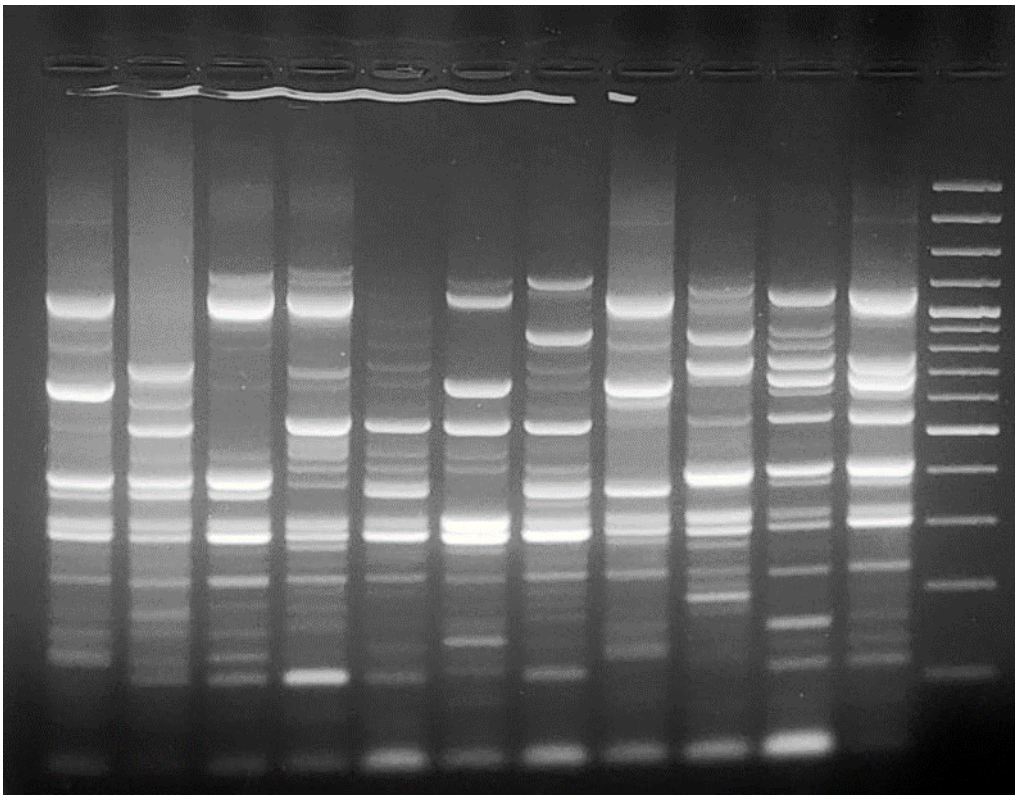

Figure 1. ERIC-PCR profiles of representative *E. coli* isolates.

PMQR genes were not among the isolates. All CIP^R-CTX^R isolates carried at least one of the beta-lactamase genes. The most predominant beta-lactamase gene was observed as bla_{CMY-2}, which was detected in 12 (54.5%) isolates. Among the beta-lactamase genes examined, bla_{CTX} in 11 (50%) isolates, bla_{TEM} in 8 (36.4%) isolates, and bla_{SHV} in 3 (13.6%) isolates was detected. The tetracycline resistance was associated with *tetA* and *tetB* genes, of which *tetA* was present in 12 isolates, and *tetB* in seven isolates. Among gentamicin-resistant isolates (n=8), *aac(3)-IV* (n=5) and *aadB* (n =3) were the only genes detected. In addition, several aminoglycoside resistance genes including *aadA*, *strA/B*, *aph1*, and *aph2* were detected in these isolates as well. The *catI* (n=6) and *catII* (n=4) genes were only genes associated with chloramphenicol-resistance, however, none of the isolates carried *catIII*. The *sul1*, *sul2*, and *sul3* genes encoding sulfonamide resistance were found in nine, 12, and five *E. coli* isolates, respectively. Additionally, the genes responsible for trimethoprim resistance [*dhfrI* (n=9), *dhfrV* (n=3), *dhfrIX* (n=3) and *dhfrXIII* (n=5)] were also detected in SXT-resistant isolates (Figure 2).

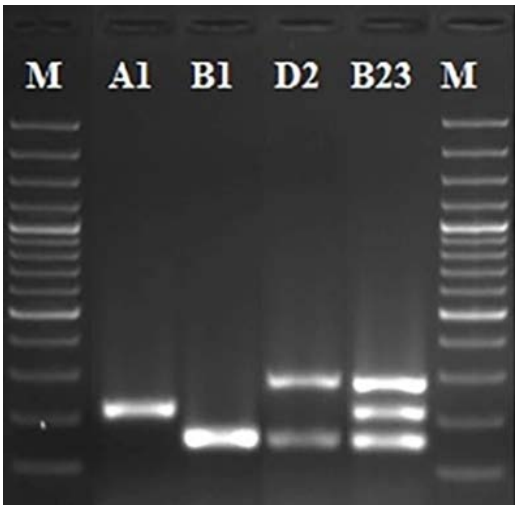

Figure 3. Phylogenetic groups determined among *E. coli* isolates

The phylogenetic analysis showed that the predominant phylogroup was D2 (n=9), followed by B1 (n=6), $B2_3$ (n=4), and A1 (n=3) in *E. coli* isolates (Figure 3).

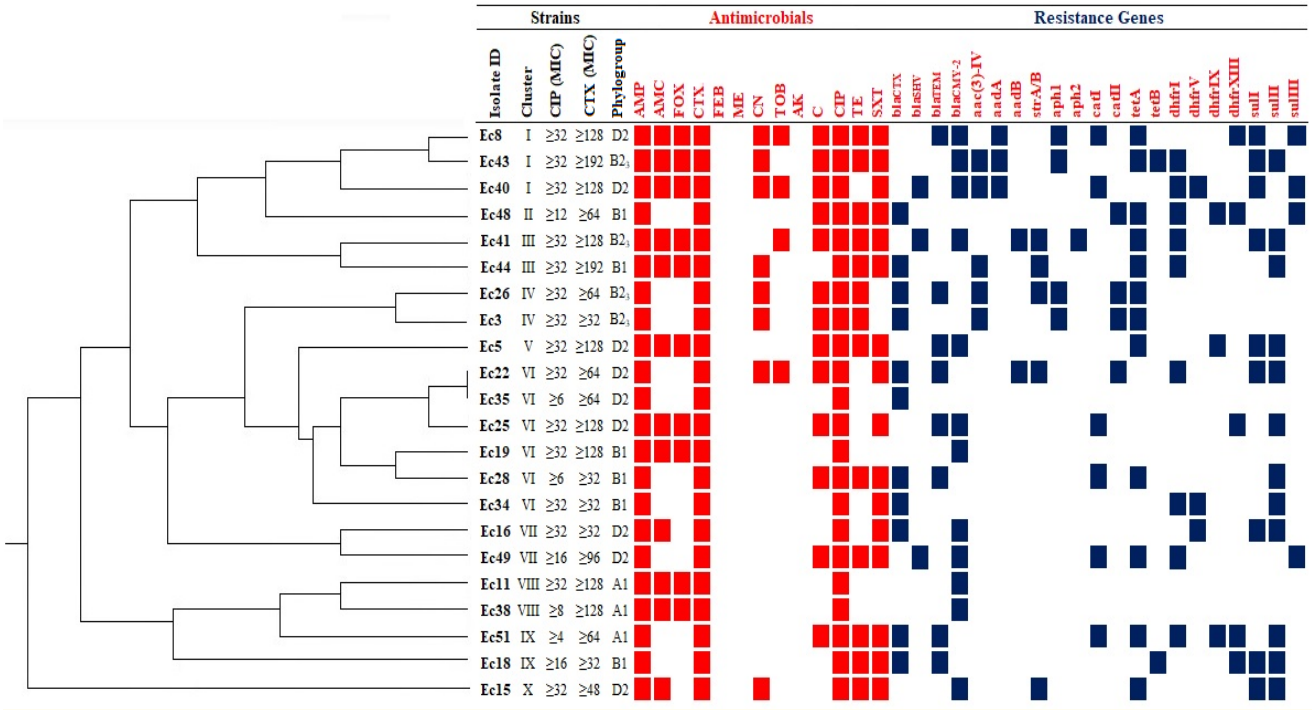

Figure 2. The dendrogram constructed based on ERIC-PCR profiles.
The antimicrobial resistance and its related genes are indicated with red and blue squares, respectively. AMP; ampicillin, AMC; amoxycillin-clavulanic acid, FOX; cefoxitin, CTX; cefotaxime, FEB; cefepime, ME; meropenem, AK; amikacin, TOB; tobramycin, CN; gentamicin, C; chloramphenicol, TE; tetracycline, CIP; ciprofloxacin, SXT; trimethoprim-sulfamethaxole.

Discussion

In the current study, ciprofloxacin and cefotaxime co-resistant *E. coli* strains with diverse genetic origins were isolated in 42.3% (22/52) of retail chicken carcasses samples sold in Hatay province. The findings of this study indicated that chicken carcasses were a growing source of MDR bacteria that pose significant public health threats due to the transfer of these resistant bacteria from food-producing animals and their products to the community. This highlighted the increasing problem of CIP^R-CTX^R *E. coli* in chicken carcasses for public health (Xu et al. 2014; Şahin et al. 2020).

The ERIC-PCR profiles of CIP^R-CTX^R *E. coli* isolates demonstrated a high genetic heterogeneity, indicating that contamination of chicken retail meat with these resistant isolates originated from different sources and the high transmission potential of these isolates (Xu et al. 2014). A similar observation was reported by Şahin (2020), who determined high clonal diversity among CIP^R isolates based on pulsed-field gel electrophoresis (PFGE). On the other hand, Wasyl et al. (2014) explained diverse *XbaI*-PFGE profiles in commensal quinolone-resistant *E. coli* isolates by antimicrobial pressure causing the selection of random *gyrA* and *parC* mutants.

In the present study, most of the CTX^R and CIP^R *E. coli* isolates had MDR profiles (81.8%, 18/22). In addition to ciprofloxacin and cefotaxime resistance, higher resistance rates to AMP (100%), SXT (72.7%), TE (63.6%), C (59.1%), AMC (54.5%), FOX (45.5%), and CN (36.4%) were determined, of which some were widely used antimicrobials in food-producing animals. In a study conducted by Şahin (2020), CIP^R *E. coli* isolates were found to show higher resistance rates to AMP (94.9%), SXT (76.3%), and TE (69.5%). Moreover, Xu et al. (2014) in China reported higher resistance rates to SXT (96.3%), TE (92.5%), C (90%), and CN (46.3%) in CIP^R-CAZ^R *E. coli* strains isolated from whole chicken carcasses. This could be explained by the fact that the high capacity of *E. coli* to accumulate resistance genes through horizontal gene transfer (Vanstokstraeten et al., 2023). The fact that CIP^R-CTX^R isolates also develop resistance to other classes of antimicrobials, makes treatment options for infections caused by these factors very limited, and can significantly lead to high mortality and financial burden (WHO, 2021).

In previous studies conducted in the region, *bla*$_{CTX-M}$ was reported as the dominant type in ESBL-producing *E. coli* from poultry meat, and being alone or mostly together with *bla*$_{TEM}$ gene (Kürekçi et al., 2019; Pehlivanlar Önen et al., 2015). In contrast to our study, most of the isolates (54.5%, 12/22) carried *bla*$_{CMY-2}$, which was alone in five isolates and in seven isolates in combination with other beta-lactamase genes. Similarly, Bilge et al. (2020) reported a high prevalence of the plasmid-mediated AmpC beta-lactamase gene, *bla*$_{CMY}$ (65%, 13/20), among cephalosporin-resistant *E. coli* isolates obtained from chicken meats. Besides, Aslantaş (2020) reported a higher prevalence of plasmid-mediated AmpC type beta-lactamase (pAmpC), *bla*$_{CMY-2}$, in broiler flocks in the region, but to a lesser extent *bla*$_{CTX-M}$. This indicates the changing epidemiology of ESBL-producing *E. coli* in broiler flocks.

Point mutations in the *gyrA* and *parC* genes in Gram-negative bacteria, including *E. coli*, are the main mechanism of quinolone resistance. Furthermore, several PMQR

mechanisms have been reported, some of which encode efflux pumps (*QepA*, *OqxAB*), quinolone-modifying enzymes (acetyltransferase *aac(6')-Ib-cr*), and protective proteins (QnrABCDS) (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*) (Imkamp et al., 2023). In this study, the isolates were not examined for mutations on *gyrA* and *parC* genes. However, only PMQR genes were searched in the isolates and no PMQR gene was found in any isolate. Similarly, Şahin (2020) reported that CIP^R *E. coli* strains obtained from chicken meat samples were negative for PMQR genes. Additionally, in another study, a low-level presence of the PMQR genes was reported in ESBL-producing *E. coli* strains from chicken meat samples (Kürekci et al., 2018). The authors reported the presence of only *qnrS* (9.6%) and *qnrB* (15.4%) genes in ESBL-producing *E. coli* strains, but did not detect other PMQR genes (*qnrA*, *qnrC*, *qnrD*, and *aac(6')-Ib-cr*). Therefore, the ciprofloxacin resistance observed in this study could be attributed to point mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes.

Based on phylogenetic grouping, *E. coli* isolates have been divided into four main groups; virulent strains that cause extraintestinal infections are generally in groups B2 and D, while most of the commensal isolates belong to groups A and B1. In this study, while most of the isolates belonged to the D2 (40.9%, 9/22) and $B2_3$ (18.2%, 4/22) phylogenetic groups, which include virulent strains, the rest of the isolates belonged to B1 (27.3%, 6/22) and A1 (18.2%, 4/22) phylogenetic groups, which includes commensal strains. Soufi et al. (2009) reported a high prevalence (91%) of virulence-associated genes in the *E. coli* isolates belonging to phylogenetic groups B2 and D. In contrast to our study, it has been reported that there are *E. coli* isolates belonging to low virulent phylogroups among those obtained from chicken meat origin. In China, Wu et al. (2015) reported phylogroup B1 (33.5%) in *E. coli* isolates from broiler carcasses and Xu et al. (2014) reported group A (59.4%) in CIP^R-CTX^R *E. coli* isolates from retail broiler carcasses as predominant group. Kürekci et al. (2018) reported the dominance of phylogroup D among ESBL-producing *E. coli*, but the presence of phylogroup B2 only in a few isolates. In Spain, Egea et al. (2012) reported that B1 and A1 accounted for more than 60% of ESBL-producing *E. coli* recovered raw poultry meat (chicken and turkey). Soufi et al. (2009) attributed the high prevalence of virulence-related genes to the fact that these genes are encoded by pathogenicity islands, which favour the spread of pathogenicity determinants in different ecosystems.

In conclusion, the findings of this study indicate that chicken carcasses are a reservoir of cefotaxime and ciprofloxacin co-resistant isolates with several resistance mechanisms against different classes of antimicrobials, and emphasize the necessity for careful regulation of antibiotic usage. The presence of resistant *E. coli* strains that could be transmitted to humans through contaminated chicken meat and products increases the risk of foodborne infections. It was identified that bacterial contamination intensifies, particularly during slaughtering processes, highlighting the crucial need for improving hygiene standards. Since these isolates have different resistant mechanisms and different genotypes, their pathogenicity should be investigated, and their contributions to community and hospital infections should be evaluated.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author on reasonable request.

Ethical Statement

The study doesn't require ethical approval from Animal Experiments Local Ethics Committee.

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Competing Interests

The authors declared that there is no conflict of interest.

Author Contributions

Concept: ÖA, AMK, MBN
Design: ÖA, AMK, MBN
Supervision/Consultation: ÖA, AMK, MBN
Data Collection and/or Processing: ÖA, AMK, MBN
Analysis and/or Interpretation: ÖA, AMK, MBN
Literature Search: ÖA, AMK, MBN
Writing Manuscript: ÖA, AMK, MBN
Critical Review: ÖA, AMK, MBN

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