


Evaluation of genotypical antimicrobial resistance in *ESBL* producing *Escherichia coli* phylogenetic groups isolated from retail poultry meat

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Abstract

The aim of this study was evaluation of resistance-encoding genes distribution among extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolated from retail poultry meat. A total of 123 raw poultry breasts meat samples from different batches (sample per batch) were taken from randomly selected six Lithuanian retail markets. The study showed high contamination with *E. coli* (92.7%), and more than half (54.0%) of the *E. coli* isolates were ESBL-producing *E. coli*. ESBL-producing *E. coli* isolates were resistant to beta-lactams (except cefotetan), monobactams (100%), quinolones (54.5%), and aminoglycosides (33.3%). Most (85.0%) strains were attributed to phylogenetic group B1 and expressed *bla*CTX-M genes associated with resistance to β -lactam antibiotics, while *bla*TEM was identified more often (70.6%) in phylogenetic group A and *bla*SHV in phylogenetic group D (50.0%). A subgroup, B1, had the highest antimicrobial resistance rate (74.7%) and more resistance genes were detected in this subgroup. Correlations between phylogenetic groups and the genes that encoded resistance to β -lactams were not detected. These research findings shows some link between phylogenetic group and antimicrobial resistance genes. Also it shows a high risk of spreading antimicrobial resistance encoding genes to human commensal or potential pathogenic bacteria.

Practical applications

Escherichia coli is common commensal bacteria associated with antimicrobial resistance to wide range of antimicrobials which uses in human medicine also. Genes, encoding antimicrobial resistance, could be easily spread among animals, humans or moved from animals to humans at slaughter or food processing. Even resistant bacteria damaged or killed during food processing, it is possible genetic sequences remain intact. High hygiene standards should be followed at slaughter houses and during food preparation process to avoid potential hazardous genetic material spreading to food processing workers and consumers. It could lead treatment failure in medicine, especially immunocompromised people.

1 | INTRODUCTION

Escherichia coli is a commensal bacteria in the intestines of poultry, cattle, and pigs that are used for food production. Food of animal origin can be contaminated with *E. coli* during animal slaughter and carcass processing (Johnson, Kuskowski, & Smith, 2005). In cases of imbalances of gut microbiota, *E. coli* may cause extraintestinal infections, and antimicrobials are most frequently chosen for treatment. Intensive use of antimicrobial agents in food animal husbandry leads to frequent meat

contamination with antimicrobial-resistant *E. coli* (Hammerum & Heuer, 2009). The human gut could be colonized with *E. coli* of animal origin and resistance to commonly used antimicrobials may cause infections with limited therapeutic options. This may lead to treatment failure and result in serious consequences for the patient. Furthermore, *E. coli* of animal origin may act as a donor of antimicrobial resistance genes for other pathogenic *E. coli*. Therefore, the intensive use of antimicrobial agents in animals' food may add to the burden of antimicrobial resistance in humans. Bacteria from food animals that are resistant to

antimicrobial agents regarded as highly or critically important in human therapy (e.g., aminoglycosides, fluoroquinolones, and third- and fourth-generation cephalosporins) are of great concern. Usage of broad-spectrum beta-lactam antimicrobials increases the rapid spread of extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae* worldwide (Bush, 2008). There are some reports describing retail chicken meat contamination by ESBL-producing *Enterobacteriaceae* in the Netherlands and Spain (Da Silva & Mendonca, 2012; Pilar, Lorena, & Eva, 2012). A close resemblance has been found between strains and ESBL genes among ESBL-producing *E. coli* (ESBL-EC) from chicken meat and from humans; this makes chicken meat a reliable source of ESBL-EC in humans. ESBL genes are usually located on plasmids, which may also carry virulence factors (Hawkey & Jones, 2009; Woodford, Turton, & Livermore, 2011). Huge geographic differences in prevailing ESBL genes are observed worldwide; however, despite wide spreading of these genes, local differences clearly exist. Several studies have described the presence of ESBL-EC in animals and meat, most likely caused by the usage of the third-generation cephalosporin ceftiofur in animals' food. Since the late 1990s, ESBL-EC have been detected in retail meat and production animals in Europe, Asia, Africa, and the United States (Blanc, Mesa, & Saco, 2006; Jensen, Hasman, & Agerso, 2006; Jouini, Vinue, & Slama, 2007). Virulence factors are also important in the epidemiology and pathophysiology of *E. coli* infections. Phylogenetic groups are associated with virulence factors and reliably cause various infections (Clermont, Bonacorsi, & Bingen, 2000; Gordon, Clermont, & Tolley, 2008). Virulent strains causing extraintestinal infections mainly belong to groups B2 and D, while strains from group A and B1 are less often recovered from extraintestinal body sites.

The aim of this study was evaluation of resistance-encoding genes distribution among ESBL-EC phylogenetic groups isolated from raw poultry meat.

2 | MATERIALS AND METHODS

2.1 | Sampling scheme

A total of 123 retail raw poultry breasts meat samples from different batches (sample per batch) were taken from randomly selected six Lithuanian retail markets. Samples were taken from April 2015 to June 2015. Sterile cotton swabs with transport media (TRANSWAB, PolysciencesINC.) used for sample collection. Samples transported on ice ($4 \pm 1^\circ\text{C}$) and processed at the same day at the Institute of Microbiology and Virology of Lithuanian University of Health Sciences.

2.2 | Microbiological analysis

Aliquots of the initial sample were plated directly onto chromogenic Brilliance ESBL-agar plate (Oxoid, Wesel, Germany) for quantification of ESBL-forming *E. coli*. Plates were incubated for 24 hr at $37^\circ\text{C} \pm 1.0^\circ\text{C}$ aerobically. Presumptive identification of *E. coli* was based on the growth and morphological characteristics. One positive colony were selected for subsequent characterization and further identifica-

tion using standard biochemical tests Microgen GNA (Microgen Bioproductions, Camberley, UK).

2.3 | Antimicrobial susceptibility testing

Antimicrobial testing carried out by commercial broth microdilution panel GN-2F (Thermo Scientific, Sensititra, Ashford, UK) and ARIS 2X device (Thermo Scientific, Sensititre, Ashford, UK) according manufacturer's instruction. Sensititre® plates GN-2F contains following antimicrobials: amikacin, ampicillin, ampicillin/sublactam, aztreonam, cefazolin, cefepime, cefotetan, ceftiofur, cefepime, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, gatifloksacin, gentamicin, imipinem, meropenem, nitrofurantoin, piperacillin, piperacillin/tazobactam, ticarcillin/clavulanic acid, tobramycin, trimethoprim/sulfamethoxazole. Interpretation of results was carried out using manufacturer's software (SWIN®) adapted to clinical breakpoints of the EUCAST and CLSI. The quality control strain *E. coli* ATCC 35218 was included in each assay for validation purposes.

2.4 | Molecular testing

DNA for molecular testing extracted after bacterial lysis according to the extraction protocol prepared by the Community Reference Laboratory for Antimicrobial Resistance with slight modifications (CRL, 2009). Briefly, a few colonies taken from the Mueller Hinton Agar surface and transferred to phosphate buffered saline (pH 7.3). The content was centrifuged for 5 min at 10^4 rcf. Supernatant was discarded and the pellet resuspended in Tris-EDTA (TE) buffer. The suspension was heated at 100°C for 10 min. Boiled suspension was transferred directly on ice and diluted by 1:10 in TE. The PCR method was used for resistance encoding genes detection (Table 1). PCR Mastermix components, Dream Taq Green PCR Master Mix, Nuclease free water and DNA marker "Gene Ruler" were provided by Thermo Scientific (Lithuania). Primers of antimicrobial susceptibility gene were obtained from Metabion International AG (Germany). PCR protocols was described by Community Reference Laboratory for Antimicrobial Resistance (CRL, 2009).

2.5 | Phylogenetic typing

ESBL-producing isolates were assigned to phylogenetic groups by PCR and based on *chuA*, *yjaA*, and *TspE4.C2* gene combination. The isolates were assigned to one of these groups A0, A1, B1, D1, and D2 (Clermont et al., 2000). Primers of gene obtained from Metabion International AG (Germany). PCR standard protocols described by Community Reference Laboratory for Antimicrobial Resistance (CRL, 2009). Sterile water was used as a template.

2.6 | Statistical analysis

Statistical analysis was performed using "R 1.8.1" package (<http://www.r-project.org/>). Chi-square test and Fisher's exact test was calculated for comparison between categorical variables. Results were considered statistically significant if $p < .05$.

TABLE 1 Primers used for PCR protocols

Primer name	Sequence (5'-3')	PCR product size (bp)	Ann. temp (°C)
<i>cmlA</i> -F	TTGCAACAGTACGTGACAT	293	55
<i>cmlA</i> -R	ACACAACGTGTACAACCAG		
<i>sul1</i> -F	TTCGGCATTCTGAATCTCAC	822	60
<i>sul1</i> -R	ATGATCTAACCTCGGTCTC		
<i>sul2</i> -F	CGGCATCGTCAACATAACC	722	60
<i>sul2</i> -R	GTGTGCGGATGAAGTCAG		
<i>sul3</i> -F	GAGCAAGATTTTTGGAATCG	880	60
<i>sul3</i> -R	CATCTGCAGCTAACCTAGGGCTTTGGA		
CTX-MF	ATGTGCAGYACCAGTAARGT	593	58
CTX-MR	TGGGTRAARTARGTSACCAGA		
Dfr1-F	ACGGATCCTGGCTGTTGGTTGGACGC	254	58
Dfr1-R	CGGAATTCACCTTCCGGCTCGATGTC		
DfrA7-F	AAAATTTTCATTGATTCTGCA	471	58
DfrA7-R	TTAGCCTTTTTTCCAAATCT		
<i>tetA</i> -F	GTGAAACCCAACATACCCC	740	62
<i>tetA</i> -R	GAAGGCAAGCAGGATGTAG		
<i>tet</i> (B)-F	CCTTATCATGCCAGTCTTGC	627	57
<i>tet</i> (B)-R	ACTGCCGTTTTTTCGCC		
<i>bla</i> TEM-F	GAGTATTCAACATTTTCGT	857	58
<i>bla</i> TEM-R	ACCAATGCTTAATCAGTGA		
<i>bla</i> SHV-F	CAAAACGCCGGTTATTC	937	58
<i>bla</i> SHV-R	TTAGCGTTGCCAGTGCT		
<i>aac</i> (3)II-F	TGAAACGCTGACGGAGCCTC	369	55
<i>aac</i> (3)II-R	GTCGAACAGGTAGCACTGAG		
<i>aacA4</i> -F	ATGACTGAGCATGACCTTGCG	487	65
<i>aacA4</i> -R	TTAGGCATCACTGCGTGTTCG		
<i>aadA</i> -F	GTGGATGGCGCCTGAAGCC	525	54
<i>aadA</i> -R	AATGCCAGTCGGCAGCG		
<i>aphA1</i> -F	AAACGTCTTGCTCGAGGC	500	55
<i>aphA1</i> -R	CAAACCGTTATTCATTCGTGA		
ChuA.1	GACGAACCAACGGTCAGGAT	279	55
ChuA.2	TGCCGCCAGTACCAAAGACA		
YjaA.1	TGAAGTGTGAGGAGAC GCTG	211	55
YjaA.2	ATGGAGAATGCGTTCCTCAAC		
TspE4C2.	GAGTAATGTCGGGGCATTCA	152	55
TspE4C2	CGCGCCAACAAAGTATTACG		

3 | RESULTS

One hundred and fourteen *E. coli* strains were isolated from 123 poultry samples and 54.4% grew on ESBL agar with typical colony color. All

62 ESBL-EC were attributed to the phylogenetic groups suggested by Clermont et al. (2000) (Table 2, Figure 1).

All ESBL-EC were resistant to aztreonam and cefpodoxime. The A and B phylogenetic group ESBL-EC were resistant to the following

TABLE 2 *E. coli* phylogenetic groups based on genetic markers combinations

Phylogenetic subgroup	Number in subgroup	Genes		
		ChuA	YjaA	TSPE4.C2
A0	6	–	–	–
A1	13	–	+	–
B1	23	–	–	+
D1	8	+	–	–
D2	12	+	–	+

Group B1 was the most prevalent group in this study, but no reliable statistically significant differences ($p > .05$) were assessed between the A0 and A1 and the D1 and D2 phylogenetic subgroups. Therefore, the A, D phylogenetic groups and B1 subgroup were further analyzed.

antibiotics: ampicillin, piperacillin, and ampicillin/sublactam. These strains were also resistant to 1st–3rd generation cephalosporines: ceftazolin, ceftriaxone, and cefuroxime. Strains that belonged to the D phylogenetic group showed less resistance to the antimicrobials used in this study except for quinolones (ciprofloxacin). The resistance to piperacillin was statistically reliable ($p < .05$) in phylogenetic groups A and B, that is, groups A and B resistance was higher than in group D. A third (28.6–38.1%) of the strains attributed to any phylogenetic group were resistant to gentamycin. All strains were sensitive to amikacin and gatifloxacin as well as carbapenems, cefotetan, and ceftoxitin (Figure 2).

The ESBL-EC responded differently to the other antibiotics used in this study. An *E. coli* B1 phylogenetic subgroup showed higher resistance to the most antimicrobials, but statistically the data were not significant ($p > .05$). ESBL-EC strains assigned to the A phylogenetic group, were more resistant to the cephalosporins ceftazolin, ceftazidime, ceftriaxone, and cefuroxime ($p > .05$) than strains in group D.

All resistant strains carried genes associated with antimicrobial resistance. *bla*TEM and *bla*SHV distribution was statistically significant ($p < .05$) in phylogenetic groups A and D (Figure 3). ESBL-EC phylogenetic group D showed higher rates of the following resistance-encoding genetic determinants: *sul2* ($p < .05$), *tetA*, *bla*SHV ($p < .05$), *cmIA* ($p < .05$), and *aphA1*. Regarding antimicrobial resistance genes,

*bla*TEM ($p < .05$), *tetB*, and *cmY2* were detected in group A strains whereas the *dfrA1*, *aadA*, and *ctxM* genes were found significantly more often ($p < .05$) in phylogenetic subgroup B1.

Analysis of genetic determinant differences among the phylogenetic groups did not show a reliable correlation. Phylogenetic groups A and B for one ESBL-EC showed 0.26 resistance to antibiotics encoding gene, meanwhile in phylogenetic group D showed 0.22 resistance to antibiotics encoding gene. The statistical reliability ($p < .05$) among resistance-encoding genetic determinants and the corresponding antibiotic class are shown in Figure 4. In Figure 4 we see that the statistically identified relations are between the *bla*SHV and *ctxM* genetic determinants and ceftazidime and cefepime, *aacA* and gentamicin, and *dfrA* and SXT.

4 | DISCUSSION

The study identified high levels of *E. coli* contamination in poultry meat. High contamination of poultry meat by intestinal microflora was also reported in Finland and Iceland (Lyhs, Ikonen, & Pohjanvirta, 2012; Thorsteinsdottir, Haraldsson, & Fridriksdottir, 2010). More than half (54.4%) of the isolates were ESBL-EC and were resistant to β -lactam class antibiotics, raising concern about the amount of consumed antimicrobials. This situation is not so bad, as compared with the rate of 94% ESBL-EC from poultry in the Netherlands and it is worse than the 44% ESBL-EC in Sweden (Borjesson, Egervarn, Lindblad, & Englund, 2013; Voets, Fluit, Scharringa, & Schapendonk, 2013). Such results are related to the usage of antimicrobials in the feed of animals intended for food production. Some scientists have linked phylogenetic groups with antimicrobial resistance. According to published data, *E. coli* phylogenetic group distribution varies by country. Phylogenetic groups A and B1 are mostly identified in Denmark (Jakobsen, Kurbasic, & Skjøt-Rasmussen, 2010), while 50.7% of all strains assigned to phylogenetic group D are in Finland (Lyhs et al., 2012). Subgroup A1 is mostly identified in poultry in Brazil (Carlos, Pires, & Stoppe, 2010). Our research, in contrast, mostly identified *E. coli* phylogenetic groups B1 and A. Commensal *E. coli* are assigned for these phylogenetic groups, which are not pathogenic. The virulent D group comprised one-third (32.2%) of all the samples in our research. It is difficult to compare the

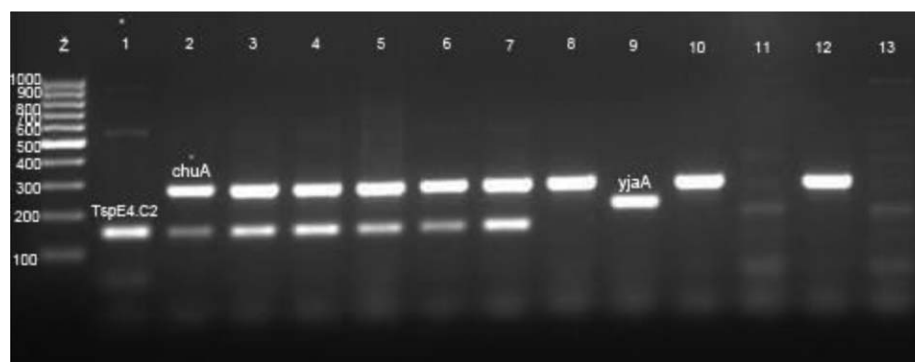


FIGURE 1 The electrophoretic profile of the phylogenetic group identified in ESBL producing *E. coli* isolates. P.S. Ž, DNA marker 100 bp; 1 strip, B1 phylogenetic group; 2–7 strips, D2 phylogenetic subgroup; 8,10,12 strips, D1 phylogenetic subgroup; 9 strip, A1 phylogenetic subgroup; 11,13 strips, A0 phylogenetic subgroup

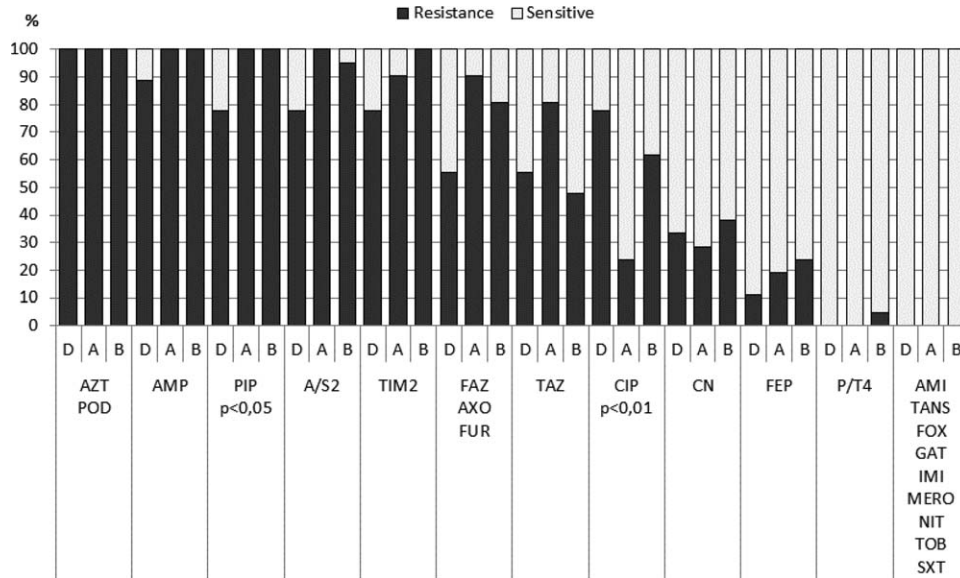


FIGURE 2 Antimicrobial resistance in *E. coli* strains isolated from poultry meat. P.S., AZT-aztreonam; POD, cefpodoxime; AMP, ampicillin; PIP, piperacillin; A/S2, ampicillin/sublactam; TIM2, ticarcillin/clavulanic acid; FAZ, ceftazidime; AXO, ceftriaxone; FUR, cefuroxime; TAZ, ceftazidime; CIP, ciprofloxacin; CN, gentamicin; FEP, cefepime; P/T4, piperacillin/tazobactam; AMI, amikacin; TANS, cefotetan; FOX, cefoxitin; GAT, gatifloxacin; IMI, imipinim; MERO, meropenem; NIT, nitrofurantoin; TOB, tobramycin; SXT, trimethoprim/sulfamethoxazole

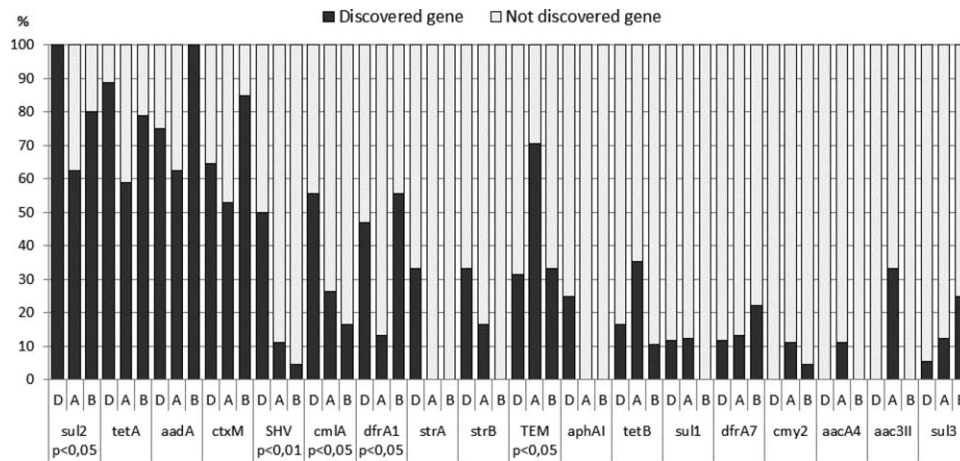


FIGURE 3 Distribution of genetic determinants in *E. coli* strains isolated from poultry meat

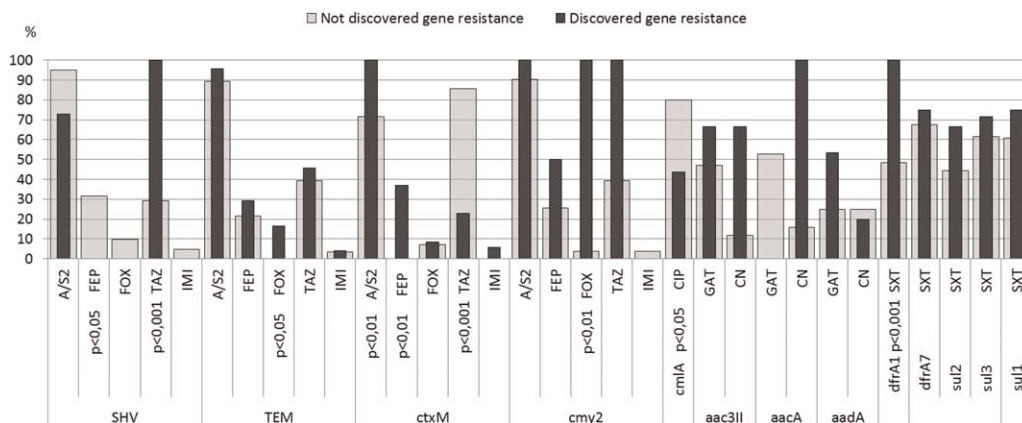


FIGURE 4 Statistical reliance among resistance to antibiotics and resistance encoding genetic determinant

prevalence of *E. coli* phylogenetic groups in various countries, because the age of the birds was not recorded. Bird age is an important factor because the B group is most common during hatching and the A phylogenetic group is dominant in later age (Pasquali, Lucchi, & Braggio, 2015). Phylogenetic group differences among human feces isolates in different countries have also been reported (Gordon, 2013). Our research showed that ESBL-EC have high resistance patterns, not only to the β -lactam antimicrobials most frequently prescribed to animals and humans in Europe, but also to other classes of antibiotics as well. It shows *E. coli* is reservoir for spreading antimicrobial resistance in the community. ESBL-EC were resistant not only to ampicillin and a first-generation cephalosporin (cefazolin), but to the second- and third-generation cephalosporins (cefuroxime, ceftazidime, cefpodoxime, and ceftriaxone). Phylogenetic subgroup B1 *E. coli* showed the most frequent resistance to antimicrobials. These *E. coli* strains were more often resistant to piperacillin, ticarcillin/clavulanic acid, and gentamicin. The resistance to ciprofloxacin might be linked to increased chinolons consumption in Europe (Economou & Gousia, 2015; Vieira, Collignon, & Aarestrup, 2011). Unfortunately, there is no published epidemiological data about ESBL-EC phylogenetic group distribution in Lithuanian poultry farms or slaughterhouses personal.

Earlier dominant genes, which caused ESBL production, were *bla*-TEM and *bla*SHV; nevertheless, lately the *bla*CTX-M gene became dominant in many countries of the world (Branger, Zamfir, & Geoffroy, 2005; Dierikx, van der Goot, & Smith, 2013), which was identified in our research as well. The *bla*CTX-M gene was identified in more than half (67.5%) of ESBL-EC isolated from poultry. This gene was most common in other scientists' reports (Overdeest, Willemsen, & Rijnsburger, 2011; Reich, Atanassova, & Klein, 2013). We often identified this genetic determinant in the *E. coli* B1 phylogenetic subgroup, where isolates were resistant to cefepime and ampicillin/sublactam. Typically, *bla*CTX-M genes are detected not only in poultry but in humans as well (Maciucă, Williams, & Tuchilus, 2015; Overdeest et al., 2011). This confirms the assumption that poultry meat could be a reservoir for *E. coli* bacteria, which can be transmitted to humans. The literature does not indicate that this gene would be identified exclusively more often than other genes in B1 subgroup, but during our research, the *bla*CTX-M gene was repeatedly detected from all the genes identified in this phylogenetic group. Other studies reported that the *bla*CTX-M gene was identified more frequently in the D1 subgroup (Branger et al., 2005). Both the *bla*TEM and *bla*SHV genes were identified in the D1 group in our study and *bla*SHV genetic determinants were identified in strains resistant to ceftazidime. In our study, the quantity of the *bla*TEM and *bla*SHV genes varied ($p > .05$), but different results were obtained by other researchers too. Therefore, the spread of genes varies by geographic location. In our study, gene distribution differed among the phylogenetic groups ($p < .05$): *bla*SHV was common to phylogenetic group D, while *bla*TEM was common to phylogenetic group A. The distribution of *bla*SHV genes significantly differs among phylogenetic groups, but no correlation reported in the literature. To obtain more reliable results about gene distribution in phylogenetic groups, more research is needed in different geographical regions. The

quantity of genes that encoded antimicrobial resistance was not significantly different amongst the ESBL-EC isolates. Such results show that spreading resistance genes by horizontal gene transmission could be quick and independent.

5 | CONCLUSIONS

In this study, we have shown high (92.7%) retail poultry meat contamination by *E. coli*, where 54.4% produced ESBL. ESBL-EC were mostly resistant to monobactams, quinolones, and aminoglycosides. Resistance to β -lactam antibiotics was mostly caused by the genetic determinant *bla*CTX-M (85.0%) identified in the B1 phylogenetic subgroup, while *bla*TEM was more often (70.6%) identified in the A phylogenetic group, and *bla*SHV (50.0%) in the D phylogenetic group. The majority of strains were attributed to the B1 subgroup in the research. These *E. coli* isolates showed more resistance encoded by genetic determinants. No statistical correlation among the phylogenetic groups and quantity of β -lactam resistance genes was detected ($p > .05$). Our study results showed link between phylogenetic group and antimicrobial resistance genes. These research findings show high potential risk to the poultry slaughterhouse workers and meat consumers. Food processing and consuming could lead antimicrobial resistance genes transmission to human commensal or potential pathogenic bacteria.

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