Antimicrobial Resistance among Commensal *Escherichia coli* from Broilers in Turkey

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ABSTRACT

In this study it was aimed to determine the antimicrobial susceptibility, resistance mechanisms, the prevalence of class 1 and 2 integrons and phylogenetic groups in 300 *Escherichia coli* isolates from poultry farms in Turkey. The antimicrobial susceptibility of the isolates was determined by disk diffusion method, the occurence of antimicrobial resistance genes, class 1 and 2 integrons and phylogenetic grouping were investigated by polymerase chain reaction (PCR). In ciprofloxacin resistant *E. coli* isolates, *gyr*A and *par*C mutations were detected by mismatch amplification mutation assay-PCR (MAMA-PCR). All extended spectrum β-lactamase (ESBL) and plasmidic AmpC β-lactamase (pAmpC) positive *E. coli* isolates were investigated for presence of ESBL and pAmpC genes, and following DNA sequencing. Antimicrobial test results revealed that 86.7% of the isolates had multidrug resistance (MDR) phenotype. MDR profiles were observed in all of the integron-positive isolates, whereas 81.4% of non-integron-carrying isolates were MDR ($p < 0.001$). Phylogenetic analysis showed that integron carrying isolates mainly belonged to D (54.5%), A (32.5%), B1 (10.6%) and B2 (2.4%). The results indicate a high level of MDR and prevalence of class 1 and 2 integrons among commensal *E. coli* from Turkish broiler flocks. Urgent measures should be taken to promote prudent use of antimicrobials and to limit use of antimicrobials in broiler flocks.

Keywords: *Escherichia coli*; Broiler; Integron; Microbial Drug Resistance.

INTRODUCTION

The increasing trend of antimicrobial resistance in pathogens isolated from both humans and animals has become a serious concern for public health throughout the world (1). Presence of antimicrobial resistant bacteria in animals has negative consequences due to transmission of resistant bacteria from animal to human through direct contact, food chain and contamination of the environment (2). Bacteria develop resistance to antimicrobials through acqusition of resistance genes or spontaneous mutations. Horizantal transfer of genes encoding different resistance mechanisms is mostly carried out through the mobile genetic elements such as plasmids, transposons and integrons (3).

Integrons are genetic elements that can acquire gene cassettes by a site-specific recombination and ensuring their expression (4). To date, five types of integrons have been described based on amino acid sequences of integrase genes that play an important role for the integration and the excision of gene cassettes (5). Mobility of integrons is provided by self-transmissible elements such as plasmids and transposons (6). Among integron classes, class 1 integron is the most encountered and well-charaterized, as well as frequently reported among the members of Enterobacteriaceae (5).

Important role of integrons for the transfer of antimicrobial resistance genes among gut microbiota of poultry have been well documented by many studies (7-9). However, there is no information on the occurence of integrons in *E. coli* from commercial broiler flocks in Turkey. Therefore, the

aims of this study were to gain insight on phenotypic and genotypic resistance profiles of *E. coli* strains isolated from broiler flocks in Turkey. Searching for the presence of class 1 and 2 integrons were also included in this study.

MATERIALS AND METHODS

Ethical statement

The study was approved by the Animal Ethical Committee of Mustafa Kemal University (2013-7/2).

Sampling and bacterial isolation

A total of 300 cloacal swabs were collected from broiler farms in Southern Turkey, using Amies transport medium, between 2013 and 2014.

Cloacal swabs were inoculated onto Eosin Methylen Blue (EMB) agar and incubated aerobically at 37˚C for 18-24 h. From each plate, one typical *E. coli* colony for each sample was selected and subcultured onto Blood Agar supplemented with 5% defibrinated sheep blood. Pure cultures were identified using biochemical tests (urease, oxidase, IMVIC tests and growth characteristics in Triple Sugar Iron) and confirmed by polymerase chain reaction (PCR) using *E. coli* spesific 16S RNA primers. The strains found positive by both biochemical tests and PCR were stored at -20˚C on Tryptic Soya Broth containing 20% glycerol for subsequent analysis.

Antimicrobial Susceptibility Testing

Antimicrobial susceptilities of the isolates to 14 antimicrobials were determined using disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2012) guideline (10). The antimicrobials used were: ampicillin (10 μg), amoxicillin + clavulanic acid (30 μg), nalidixic acid (30 μg); ciprofloxacin (5 μg), chloramphenicol (30 μg), gentamicin (10 μg); streptomycin (10 μg); kanamycin (30 μg), tetracycline (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), trimethroprim (5 μg) and sulfamethoxazole (300 μg). *E. coli* ATCC 25922 strain was used as control strain. The isolates that were resistant to three or more antimicrobials from different classes were defined as multidrug resistant (MDR). Penicillins and cephalosporins were deemed as separate classes.

DNA extraction

DNA extraction was made as previously described (11). Briefly, selected single colonies were grown in LB broth. An overnight culture (200 µl) was mixed with 800 µl of RNase/DNase free water and heated at 100˚C for 10 min. Then, the resulting solution was centrifuged at 13 000 g for 10 min and the supernatant was used as the DNA template and stored at -20˚C until use.

Phylogenetic grouping

Detection of phylogenetic *E. coli* groups was performed by using triplex PCR method described by Clermont *et al*. (12). The isolates were assigned to one of phylogroups based on presence and absence of *chu*A, *yja*A genes and TSPE4.C2 fragment.

Determination of antimicrobial resistance genes

The resistance genes studied was selected based on their frequency of occurrence in resistant *E. coli* strains (11, 15-18). Accordingly, 28 genes conferring resistance to different classes of antimicrobials (beta-lactams, aminoglycosides, tetracycline, phenicols, trimethoprim and sulfonamides) were selected to search their distributions in *E. coli* isolates.

Detection of class I and class II integrons

Class 1 and class 2 integrons were investigated as previously described Levesque *et al*. (13) and White *et al*. (14).

Detection of *E. coli* mutations in *gyr*A and *par*C mutations

In ciprofloxacin resistant *E. coli* isolates, mismatch amplification mutation assay-PCR (MAMA-PCR) were used to detect mutations in the quinolone resistance determining region (QRDR) of *gyr*A and *par*C genes as previously described by Qiang *et al*. (19).

Statistical analysis

Data were analyzed by using IBM SPSS Statistics package Version 23 (IBM Corp., Armonk, NY, USA). Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Antimicrobial susceptibility testing

With the exception of a few of the antibiotics tested, a high rate of resistance to different antibiotics was observed among the *E.coli* isolates tested (Table 1). All together, 96.3% of the isolates were resistant to at least one of the antimicrobials

tested. Highest resistance percentages were found against nalidixic acid (85.3%), ampicillin (80.3%), sulfamethoxazole (79.7%), tetracycline (78%), trimethoprim (77%), and ciprofloxacin (76%), whereas lowest resistance rate were detected against cephalosporins (3.7% for cefotaxime, 2.0% for ceftazidime, and 1.0% for cefoxitin). Resistance rates for the remaining antimicrobials were 58.3% to streptomycin, 48.7% to chloramphenicol, 38.7% to kanamycin, 22% to gentamicin, and 20.3% to amoxicillin–clavulanic acid.

The majority of the isolates (86.7%) showed resistance to three or more antimicrobial classes. All the integron carrying isolates exhibited MDR patterns, whereas 80.8% of the isolates that did not harbour integrons, displayed MDR patterns. In particular, among the integron carrying isolates, MDR to eight, seven, six, five and four antimicrobial classes were observed in five (4.1%), 31 (25.2%), 46 (37.4%), 31 (25.2%) and 10 (8.1%) isolates, respectively. Out of the 177 integron negative isolates, MDR to eight, seven, six, five, four and three antimicrobial classes were seen in 3 (1.7%), 33 (18.6%), 55 (31.1%), 18 (10.2%), 17 (9.6%) and 17 (9.6%) isolates, respectively. Resistance to one and two classes of antimicrobials were detected in 11 (3.7%) and 12 (4%) isolates, respectively. Only eleven isolates were susceptible to all class of antimicrobials.

Distribution of resistance genes

PCR and DNA sequencing results are presented in Table 2. PCR and DNA sequencing results revealed the presence of beta-lactamase encoding genes in 228 (76%) *E. coli* isolates. The beta-lactamase encoding genes include: $bla_{\text{TEM-1b}}$ in 220 isolates, $bla_{\text{CMY-2}}$ in two isolates, $bla_{\text{CTX-M-15}}$ in two isolates, $bla_{\text{CTX-M-3}}$ in one isolate, $bla_{\text{CTX-M-1}}$ in one isolate, and $bla_{\text{SHV-12}}$ in two isolates.

Of the six tetracycline resistance genes tested, only *tet*A and *tet*B were found in 226 isolates (96.6%) of the 234 tetracycline positive isolates. The distribution were as follows: 98 (43.4%) *tet*A, and 14 (6.2%) *tet*B, the combination of *tet*A and *tet*B were found in 114 (50.4%) isolates. All isolates were negative for *tet*C, *tet*D, *tet*E and *tet*G.

Of the 146 chloramphenicol resistant isolates, 131 (89.7%) isolates contained *cat* genes. The most frequent resistance gene was *cat*I (96.2%), followed by *cat*II in three isolates and both *cat*I and *cat*II in two isolates. *cat*III was not detected in the isolates.

Among the 231 trimethoprim resistant isolates, 196 (84.8%) isolates contained *dfr*A genes alone or in combination. Overall, *dfr*A1 was detected in 104 isolates, *dfr*A5 in 43 isolates, *dfr*A13 in 4 isolates, *dfr*A1 - *dfr*A5 in 35 isolates, dfrA1 - *dfr*A5 - *dfr*A13 in 6 isolates, *dfr*A1 - *dfr*A13 in 3 isolates and *dfr*A5 - *dfr*A13 in one isolate. None of the isolates were found to have *dfr*A7 and *dfr*A9.

The *sul1* and *sul2* genes were largely present among the isolates. Of 239 sulfonamide-resistant isolates, 230 contained *sul* genes, and *sul*2 was the most common *sul* gene, detected in 93 isolates alone, in 85 isolates detected together with *sul*1 gene. *sul*1 in 16 isolates, *sul*2-*sul*3 in 13 isolates, *sul*1-*sul*2-*sul*3 in eight isolates and *sul*1-*sul*3 in three isolates were detected.

Table 1: Antimicrobial resistance profiles of *E. coli* isolates

Antimicrobial agents tested	Integron presence		Total n (%)	P value
	Integron Positive n (%)	Integron Negative n (%)		
Nalidixic acid	119 (96.7)	137 (77.4)	256 (85.3)	< 0.001
Ampicillin	99 (80.5)	142 (80.2)	241 (80.3)	0.955
Sulphanamid	119 (96.7)	120 (67.8)	239 (79.7)	< 0.001
Tetracycline	96 (78.0)	138 (80.0)	234 (78)	0.986
Trimethoprim	122 (99.2)	109 (61.6)	231 (77)	< 0.001
Ciprofloxacin	110 (89.4)	118 (66.7)	228 (76)	< 0.001
Streptomycin	115 (93.5)	60 (33.9)	175 (58.3)	< 0.001
Chloramphenicol	49 (39.8)	97 (54.8)	146 (48.7)	0.011
Kanamycin	60 (48.8)	56 (31.6)	116 (38.7)	0.003
Gentamicin	40 (32.5)	26 (14.7)	66 (22)	< 0.001
Amoxicillin/clavulanic acid	35 (28.5)	26 (14.7)	61 (20.3)	0.773
Cefotaxime	7 (5.7)	4 (2.3)	11 (3.7)	1.00
Ceftazidime	3 (2.4)	3 (1.7)	6 (2)	0.692
Cefoxitin	2 (1.6)	1 (0.6)	3 (1)	1.00

Table 2: Genetic context of resistance genes in studied *E. coli* isolates

Antimicrobial	Number of phenotypic resistant isolates	PCR/DNA sequencing results	Genes detected
Trimethoprim	231	*dfr*A1	104
		*dfr*A5	43
		*dfr*A13	4
		*dfr*A1, *dfr*A5	35
		*dfrA*1, *dfr*A5, *dfr*A13	6
		*dfr*A1, *dfr*A13	3
		*dfr*A5, *dfr*A13	1
Tetracycline	234	*tet*A	98
		*tet*B	14
		*tet*A, *tet*B	114
Sulphanamid	239	*sul*1	16
		*sul*2	93
		*sul*3	12
		*sul*1, *sul*2	85
		*sul*1, *sul*3	3
		*sul*1, *sul*2, *sul*3	8
		*sul*2, *sul*3	13
Chloramphenicol	146	*cat*I	126
		*cat*II	3
		*cat*I, *cat*II	2
Beta-lactams		*bla*TEM-1b	220
		*bla*CTX-M-15	2
		*bla*CTX-M-3	1
		*bla*CTX-M-1	1
		*bla*SHV-12	2
		*bla*CMY-2	2
Streptomycin		*aad*A	71
		*aad*A-*str*A/B	89
		*str*A/B	16
Gentamicin		*aad*B	23
		aac(3)-IV	5
Kanamycin		*aph*A1	86

Aminoglycoside resistance genes was identified in 290 isolates. Of aminoglycoside resistance genes, *aad*A in 71 isolates, *aad*A-*str*A/B in 89 isolates, *str*A/B in 16 isolates, *aad*B in 23 isolates, *aac*(3)-IV in 5 isolates and *aph*A1 in 86 isolates were detected.

Two hundred and twenty eight ciprofloxacin isolates were examined by MAMA PCR and mutations were found in 216 (94.7%) isolates. The results revealed 12 different combination according to mutations in *gyr*A and *par*C genes (Table 3). The most common mutations encountered were *gyr*A83-*gyr*A87-*par*C80 in 83 isolates, *gyr*A83-*gyr*A87 in 56 isolates and *gyr*A87-*par*C80 in 33 isolates.

Integron frequency in *E. coli* isolates

The presence of class 1 and 2 integrons were detected in 123 (41%) *E. coli* isolates, of which 65% (80) carried class 1 integron, while 26% (32) carried class 2 integron. Both integron classes were only found in 11 isolates (8.9%). Furthermore, two class 1 integrons approximately with amplicons of 1600 bp and 1000 bp were found in one strain. This isolate was resistant to seven antimicrobials including fluoroquinolones.

Phylogenetic grouping

The majority of the isolates belonged to D phylogenetic group A (50.7%), followed by groups A (28%), B1 (17%) and B2 (4.3%) (Figure).

DISCUSSION

Current studies show an increasing trend of antimicrobial resistance including different resistance rates and MDR prevalence in *E. coli* of broiler origin (20-22). In the present study, very high levels of resistance to several antimicrobials were detected. In addition, MDR trait was also widespread. In a European survey conducted by European Antimicrobial Susceptibility Surveillance in Animals (EASSA), antimicrobial susceptibility test results obtained from France, The Netherlands, The United Kingdom and Sweden were compared (23). On average, antimicrobial resistance rates of the present study were higher than the EASSA study, only comparable to streptomycin resistance rates obtained from France, The Netharlands and the UK. However, exceptionally, tetracycline resistance rate (85.4%) obtained from France was higher than our study. Also, in this multicentre study, resistance against third generation cephalosprins was not detected in the included countries. The prevalence rates of resistance observed in our study are also higher than a study carried out in Belgium (24). An exception for this study was higher prevalence rate of of third generation cephalosporin resistance (44%) than found in our study (3.7%). Morever, the authors reported over 58% of the isolates as MDR. Present study demonstrate that the isolates carried a high percentage (41.3%) of class 1 and 2 integrons in *E. coli* isolated from commercial broiler flocks in Turkey. In a recent study, Marchant *et al*., who investigated time-dependent change of integrons in *E. coli* isolates from chicken, reported a prevalence of 50% in Spain, and found no variations in the frequency of integron (7). Similarly, Kang *et al*. found prevalence

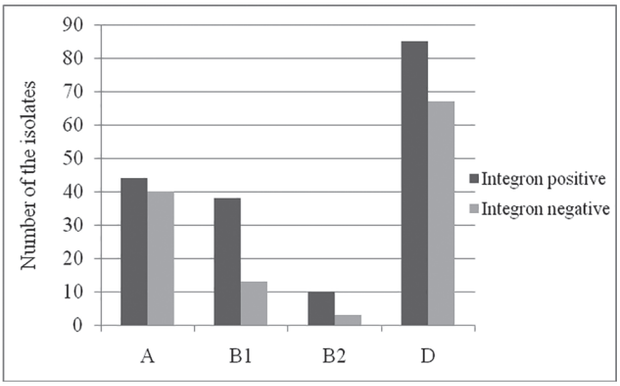

Figure: Distribution of integrons according to phylogentic *E. coli* groups.

of class 1 integron in *E. coli* isolates from poultry as 44.2% in Korea (25). However, higher prevalence rate (61.3%) of integron was reported by Zhang *et al*. in China (9).

Gene cassette arrays of class 1 and class 2 integrons have not been determined in this study. However, in previous studies performed in *E. coli* isolates carrying class 1 and 2 integrons from poultry, it has been shown that gene cassette arrays was mostly related with *aad*A, *dfr*A and *sat* genes conferring resistance to strepromycin/spectinomycin, trimethoprim and streptothricin, respectively (7, 9, 25). Phylogenetic groups A and B2 were reported as being more common than B1 and D groups in the gut of poultry (7, 26). In contrast, in the current study, phylogenetic group D that includes extraintestinal strains was found as the most common group followed by group A, B1 and B2. This result suggests that gut flora of poultry is a source of extraintestinal pathogenic *E. coli* (ExPEC) and may lead to contamination of chicken carcasses with ExPEC strains at slaughter (27, 28). In our study, we found statistically significant difference between phylogroups and integron presence (Figure) (P= 0.030). Similar results were also reported in a previous study by Cocchi *et al*. (29). However, Marchant *et al*. did not detect any association between the presence of integrons and phylogenetic group affiliation (7).

Leverstein-van Hall *et al*. highligted a significant correlation between MDR and presence of integron among Enterobactericeae members, since integrons are frequently located on transferable genetic elements such as plasmids, transposoons, and this makes the spread of resistance easier not only within the same species but also to other genera (30). Indeed, all integron carrying isolates showed MDR profile for four to eight classes of antimicrobial ($p<0.001$). As shown in Table 2, although higher resistance frequencies were usually observed in integron carrying isolates in comparison with integron negative isolates, only statistically significant differences were detected in trimethoprim, streptomycin, sulfamethoxazole, gentamicin, ciprofloxacin, nalidixic acid, kanamycin, chloramphenicol. Lower or no association was observed for ampicillin, tetracycline, amoxycillin-clavulanic acid, cefotaxime, ceftazidime and cefoxitin, probably due to other resistance mechanisms involved. On the other hand, the high frequency of integrons among *E. coli* strains did not indicate that integrons are the main contributers for the emergence of MDR phenotype in this study. Because the isolates without class 1 and 2 integrons also displayed MDR phenotype and carried *aad*A and *dfr*A1 resistance genes. Thus, it can be ascertained that integrons contributed partly to MDR of the isolates.

The reason for observing a high level of resistance to chloramphenicol (48.7%) detected in our study is ambiguous, since the use of this drug was banned in food producing animals in Turkey (Regulation No: 2002/68 of 19 December 2002). However, similar results were previously reported in other studies (7, 9, 25). This could be explained by the persistence of chloramphenicol resistant strains in the environment (24) or co-existence of chloramphenicol resistance genes with other resistance genes on the same mobile genetic elements (32).

The main resistance mechanism to fluoroquinolones is mutations in the quinolone resistance-determining regions (QRDR) of *gyr*A and *par*C genes (19). Development of resistance to fluoroquinolones in commensal, pathogenic and zoonotic bacteria is of great concern since these antimicrobials are considered as clinically important drugs for human medicine (32). Higher quinolone resistance (76%) observed in this study could be attributed to misuse and overuse of these antimicrobials in both humans and animals in Turkey for many years.

Third- and fourth-generation cephasporins are other clinically important antimicrobials (32). Thus, high prevelance of resistance to these drugs may lead to treatment difficulties in human medicine. Although, a low prevalence of ESBL producing *E. coli* was detected in present study, presence of these bacteria in food animals is considered to be a potential risk. However, this study was not designed to demonstrate the prevalence of ESBL-producing *E. coli*. To serve that purpose, selective isolation methods are needed to determine the true prevalence of these bacteria. For instance,

in a recent study, higher contamination of chicken meat with ESBL/pAmpC producing *E. coli* was reported by Pehlivanlar Önen *et al*. (28).

The present study is the first to characterize antimicrobial resistance and its underlying genetic mechanisms, as well as presence of integron and associated gene cassettes in commensal *E. coli* strains from commercial poultry flocks in Turkey. High rate of MDR found in this study indicate establishment of antimicrobial resistance surveillance program to evaluate the effects of antimicrobial usage in animals, to determine current status of antimicrobial resistance and to develop control strategies to prevent emergence and dissemination of antimicrobial resistance.

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