Investigation of Antimicrobial Resistance and Integron Profiles of Poultry Pathogenic *Escherichia coli*

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ABSTRACT

Integrons are mobile DNA elements which play an important role in the acquisition and transmission of antimicrobial resistance genes. The rapid spread of multidrug resistant bacteria threaten public health giving also causes economic problems worldwide. The aim of this study was to evaluate the presence of integron in Avian Pathogenic *Escherichia coli* (APEC) isolated from internal organs in broiler chickens with respiratory system conditions and to determine the contribution of integrons to antimicrobial resistance in these microorganisms. For this purpose, a total of 112 (82.9%) *E. coli* isolates isolated from the internal organs of 135 colibacillosis suspected broiler chickens constituted the material of this study. Antibiotic resistance of the isolates was determined by the disk diffusion method. The identification of bacteria at species level and the presence of integron gene (class 1, 2, 3) were investigated by polymerase chain reactions (PCR). It was determined that 70.5%, 68.8%, and 58.9% of isolates were resistant to ampicillin, tetracycline, and trimethoprim sulfamethoxazole, respectively. While 14.3% (16/112) of all isolates in the study was sensitive to all antibiotics used, 85.7% (96/112) of the isolates were resistant to only one antibiotic. Moreover, multiple antibiotic resistance (MDR) was found in 64.3% (72/112). The integron was detected among 52.7% (46.4% of them class 1; 1.8% of class 2; 4.5% class 1 and class 2) of the isolates while in 47.3% were without the integron. In this study, no integron was detected in any of the antibiotic susceptible isolates. However, 61.4% (59/96) of isolates with antibiotic resistance; 33.3% (8/24) of the non-MDR isolates; 70.8% (51/72) of the MDR isolates were detected carrying the integron. All isolates with antibiotic resistance were not carrying integron. However, the presence of an integron was significantly higher in isolates resistant to trimethoprim sulfamethoxazole, ampicillin, tetracycline, gentamicin, florfenicol and to ciprofloxacin resistant isolates. In these cases, the presence of integron may have played an important role in the resistance of APEC isolates to antimicrobial drugs. This study is important in that it is the first study to show the presence of integrons in *E. coli* isolates originating from broilers in western Turkey. The availability of strains of *E. coli* that are multi-resistant to antibiotics in poultry farms may also pose a risk to the public health. Studies focusing on the structure of integrons involved in the transfer of resistance genes may provide not only a better understanding of the mechanisms of resistance, but also provide useful information for the development of policies regarding the use antimicrobials.

Key words: Antibiogram; Broiler; Integron; Multi Drug Resistant Bacteria; *Escherichia coli.*

INTRODUCTION

Poultry colibacillosis is a systemic poultry disease caused by Avian Pathogenic *Escherichia coli* (APEC), which causes economic losses in the poultry industry (1). For the prevention and control of colibacillosis in poultry, the elimination of APEC's entry into the poul-

try coops, the protection of birds from infection related with predisposing factors, the immunization of birds and the treatment of birds with antimicrobial agents are fundamental processes (1,2). Due to the fact that the management and vaccination procedures are not always effective, antimicrobial therapy is considered the most common intervention method to reduce poultry colibacillosis. However, APEC strains are becoming increasingly resistant to a wide range of antimicrobial drugs (2), indicating that the treatment of colibacillosis in poultry may become challenging in the near future.

The distribution of antimicrobial drug resistance among bacterial populations through horizontal gene transfer poses a serious threat in terms of both veterinary and human medicine (3). Recently, it has been shown that integrons play an important role in the acquisition and dissemination of antimicrobial resistance genes among bacterial populations (4). Integrons form a special recombination system that encodes resistance to antimicrobials and disinfectants, which can capture and can be expressed in gene cassettes (5). According to the sequence homology of the integrase (*int*) genes, several different classes of integrons have been identified (5, 6). Class 1 integrons are the most frequently identified and characterized integron class among members of the family *Enterobacteriaceae*. Class 1 integron was detected on Tn21 whereas class 2 integron was detected on Tn7 transposons. Class 3 integron was recently been described in *Serratia marcescens* isolates in Japan (4, 7). Class 4 integron has been described only in *Vibrio cholera* and it is not known whether it is associated with antibiotic resistance (4, 7).

Studies have been performed concerning the important role of integrons in the horizontal transfer of antimicrobial resistance genes in commensal and pathogenic *E. coli* isolated from humans and animals, (5, 6, 7). Although there are a few studies related to the presence of integron in APECs (8), many studies have focused on monitoring of integron related to antibiotic resistance in *E coli* isolated from poultry feces (9) meat and meat products (10). However, according to the best knowledge of the authors; nothing is known concerning *E. coli* isolated from poultry internal organs in Turkey, the presence of integrons and effects on antimicrobial resistance.

The aim of this study was to investigate the presence of integron genes on *E. coli* isolated from internal organs of broiler chickens with respiratory diseases and the effects of the presence of integrons on antibiotic resistance.

MATERIAL AND METHOD

Study Material

In this study, a commercial poultry farm disease diagnostic laboratory brought to the attention of colibacillosis, in Ross 308 chickens aged between 16-41 days. Internal organs (heart, lung, liver) samples aseptically taken during necropsy of 135 broiler chickens which were used as material for this study. On post mortem of the birds pericarditis, peritonitis, perihepatitis, air sac inflammation and arthritis were found. In this study, material was obtained from birds that were not treated with antibiotics.

Isolation and identification

Isolation of *E. coli* was performed using standard bacteriological methods (11). The surface of the internal organ under examination was branded with a flame. An incision with a sterile scalpel was performed on the affected area. The specimen was taken from the incision site with a sterile loop. A loopful of the sample suspension was streaked onto Eosin Methylene Blue (EMB) Agar (Merck 1.01347, Darmstadt, Germany) and incubated for 24 h at 37°C aerobically. The following day, the colonies showing metallic green sheen on the EMB agar were characterized microscopically (11). Putative *E. coli* colonies were then transferred on to Nutrient Broth (Merck 1.05443, Darmstadt, Germany) for further identification using biochemical tests. The identification of the isolates was performed by Gram stain morphology, oxidase test, EMB agar growth characteristics and indole, methyl red, Voges Proskauer, citrate (IMVIC) tests (11). The isolates were stored in Brain Heart Infusion Broth (Oxoid CM 1135, Cheshire, UK) containing glycerol 20% at -20°C. *E. coli* ATCC 25922 was used as a reference organism (Manassas, VA 20108 USA).

Antibiotic susceptibility tests

Antimicrobial resistance of the isolates was investigated by standard disk diffusion method (12). The *E. coli* isolates were characterized for their resistance to 7 antibiotics [am-

Table 1: Primers used in this study, target genes, sequences, melting temperature (Tm), product lengths, use of sources and primers.

Primer	Target (Gene)	Sequence (5'-3')	Tm	Product Length (bp)	Reference
***usp*F**	Universal stress protein gene (*usp*)	CCTCCCGCACGATGATC	59.5	884	(16)
***usp*R**		TCCACGCATCGTCAGGC	62.5		
***int*1F**	Class 1, 2, 3 *int* genes	CCTCCCGCACGATGATC	57.6	280	(17)
***int*1R**		TCCACGCATCGTCAGGC	57.6		
***int*2F**		TTATTGCTGGGATTAGGC	51.4	233	(7)
***int*2R**		ACGGCTACCCTCTGTTATC	56.7		
***int*3F**		AGTGGGTGGCGAATGAGTG	58.8	600	(7)
***int*3R**		TGTTCTTGTATCGGCAGGTG	57.3		

picilline (AMP), tetracycline (TE), ciprofloxacin (CIP), trimethoprim/sulfamethoxazole (SXT), gentamicin (G), florfenicol (FFC), colistin sulphate (CT)] belonging to seven different antibiotic families (Table 1). Zone diameters of susceptibility testing results were categorized as sensitive (S), intermediate (I), or resistant (R) based on the Clinical and Laboratory Standards Institute (CLSI) (12) and Comite de l'Antibiogramme de la Societe Française de Microbiologie (CASFM) (13) standards. *E. coli* ATCC 25922 strains were used as quality control for the antibiotic susceptibility tests. The antibiotics tested were purchased from Oxoid, Hampshire UK. *E. coli* ATCC 25922 was used as a control strain. Multiple drug resistance (MDR) was defined as resistance to 3 or more unrelated antibiotics (14).

DNA extraction, purity and quantity controls

DNA extraction from *E. coli* was performed as recommended by the manufacturer using a commercial genomic DNA extraction kit (Fermentas, Massachusetts, USA). DNA purity and quantity controls were also performed. The OD260/OD280 ratio of 1.6-2.0 indicated DNA purity (15).

Polymerase chain reaction (PCR)

The universal stress protein gene (*usp*A) was used in PCR examination for the verification of the *E. coli* isolates (16). In the APECs isolated in the study, the presence of integron genes was determined by PCR amplification of integrase specific fragments of the *int* genes (7, 17). PCR, for each sample was carried out on a volume of 30 µl, final concentration was 10x Taq enzyme buffer solution 1x, 25 mM $MgCl_2$ 2 mM, 10 mM dNTP 0.2 mM, 100 ρmol primer (for each) 0.4 ρmol, 5 U Taq DNA polymerase 1.5 U (Fermentas, Massachusetts, USA), 2 µl of each DNA. The prepared tubes were loaded in the thermalcycler (Boeco, Hamburg, Germany).

The DNA was amplified using the following protocol: an initial denaturation at 95˚C for 5 min, followed by 30 cycles of denaturation (95˚C for 15 secs), annealing (56˚C for *usp*A, *int*1, *int*3 and 50˚C *int*2 for 15 secs) and extension (72˚C for 1 min), with a single final extension of 7 min at 72˚C. On electrophoresis, a 2% agarose gel stained with safe view (100 ml/6 µl) (ABM, Richmond, Canada) was used and the gel was exposed to 100 volts for 45 min. After electrophoresis, the gel was placed in the chamber of the transilluminator device connected to the computer and photographed under UV light (Vilbert Lourmat, Collegien, France). The primers used in the study are presented in Table 1 (7, 16, 17).

Statistical analysis

SPSS software version 13 was used for statistical analysis. Chi-Square ($\chi 2$) test was used to calculate association between antibiotic resistance and integron existence. The statistical significance level was defined as $P<0.05$ at the 95% confidence interval.

RESULTS

Isolation and identification

In this study, 112 (82.9%) *E. coli* isolates were obtained from internal organs of 135 broiler chickens suspected of colibacillosis. All isolates were determined to be Gram negative rod, oxidase -, indole +, MR +, VP -, citrate - and identified as *E. coli*.

Following PCR with the *usp*A specific primers, 884 bp long product was obtained in all 112 isolates. It was molecularly confirmed that all isolates were *E. coli* (Figure 1). After this verification, antibiotic resistance of 112 isolates was examined.

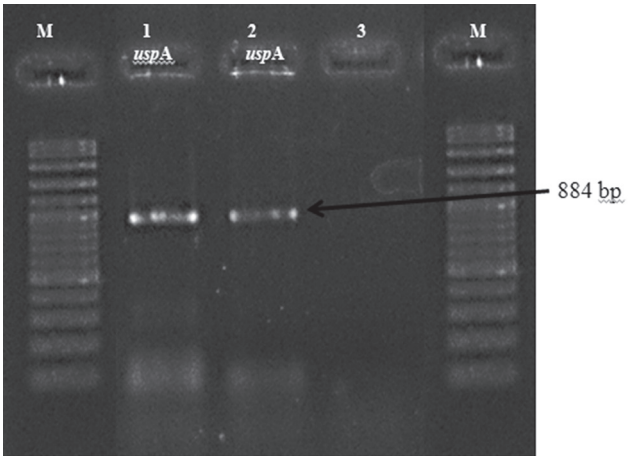

Figure 1: *usp*A PCR: gel electrophoresis images of *E. coli* isolates. 1. *E. coli* ATCC 25922 (Positive control) 2. *E. coli* field isolate 3. Negative Control (*E. faecalis* ATCC 25912 strain) M: Marker (100 bp DNA Ladder).

Antibiotic resistance

The resistance profiles to 7 antibiotics from seven different antimicrobial families were studied. Out of 112 *E. coli* strains, 79 (70.5%) were found as resistant to ampicillin, 77 (68.8%) to tetracycline, 66 (58.9%) to trimethoprim/sulphametaxazole, 61 (54.5%) to ciprofloxacin, 44 (39.3%) to florfenicol, 42 (37.5%) to gentamicin, 7 (10.4%) to ceftriaxone and cefoperazone, 3 (4.5%) to, 17 (15.2%) to colistin sulphate (Table 2, Figure 2).

In summary, 14.2% of the isolates were found as susceptible to all antibiotics used, while 64.3% of the isolates were multi-resistant. It was determined that 11.6%, 14.3%, 10.7%, 25.0%, and 2.7% of the isolates were resistant to 3, 4, 5, 6, and 7 antimicrobial families, respectively.

Determination of the presence of integrons

While 52.7% of the isolates were carrying integrons; 47.3% did not carry. 46.4% of one hundred and twelve *E. coli* isolates were only class 1; 1.8% only class 2; 4.5% of both class 1 and class 2 integrons were found to be carried together. No class 3 integron bearing isolate was found to be present (Table 3, Figure 3).

Comparison of antimicrobial resistance and integron profiles

A total of 33 antibiotic resistant phenotypes were found in 96 antimicrobial resistant isolates. The numbers of isolates belonging to each resistance phenotype and their integron classes are shown in Table 4. In this study, no integron was detected in any antibiotic susceptible isolates; 61.4% (59/96) of isolates with antibiotic resistance; 33.3% (8/24) of the non-multiple resistant isolates; 70.8% (51/72) of the multiple resistant isolates carried integrons.

It was determined that the integron positivity was significantly higher in SXT (P=0.001), AMP (P=0.001), TE (P=0.001), G (P=0.001), FFC (P=0.01) and CIP (P=0.01)

Table 2: Antibiotics used in this study, disc contents, groups, mechanism of actions, antibiogram evaluation criteria and isolates susceptibility and resistance to antibiotics status.

Antibiotic	Disc content (µg)	Group (Mechanism of Action)	<R ≥S (Reference)	*E. coli* (n=112)		
				S n (%)	I n (%)	R n (%)
AMP	10	Penicillin (Inhibits cell wall synthesis)	13-17 (12)	32 (28.6)	1 (0.9)	79 (70.5)
TE	30	Tetracycline (Protein Synthesis, 30 S inhibitor)	11-15 (12)	31 (27.7)	4 (3.6)	77 (68.8)
SXT	1.25/ 23.75	Folate path inhibitor (Inhibits folate synthesis)	10-16 (13)	46 (41.1)	0 (0.0)	66 (58.9)
CIP	5	Quinolone (Inhibits DNA gyrase enzyme)	15-21 (12)	45 (40.2)	6 (5.4)	61 (54.5)
FFC	30	Fenicol (Protein synthesis)	15-19 (*Pasteurella* spp.) (13)	65 (58.0)	3 (2.7)	44 (39.3)
G	10	Aminoglycosides (Protein synthesis)	12-15 (12)	62 (55.4)	8 (7.1)	42 (37.5)
CT	50	Polymyxin (Lipopeptide)	15-15 (13)	49 (43.8)	46 (41.0)	17 (15.2)

resistant isolates and was not significant in CT (P=0.61) resistant isolates (Table 5).

DISCUSSION

Colibacillosis caused by APEC strains is recognized as one of the major threats to the poultry industry (1, 2). In this study, 82.9% *E. coli* isolation was performed from internal organs of broiler chickens with colibacillosis in western Turkey. In other countries, the prevalence rates of colibacillosis range from 54.4% (18) to 86.7% (18, 19). This high isolation rate indicates that *E. coli* plays an important role in the epidemiology of broiler infections. *E. coli* was not isolated from 17.1% of broilers with respiratory system disease symptoms in the study. The possible reason for this maybe that broiler chickens may have been suffering from a viral or other bacterial respiratory system disease.

The use of antibiotics in poultry for treatment or preventive purposes may result in problems in the treatment of infections because of the selection of resistance among pathogenic bacteria (2, 20). However, the development of resistance especially in zoonotic bacteria can be seen as a public health problem which results in primary treatment failures (20). Antimicrobial resistance may vary according to the antibiotics used. In this study, the highest rate of resistance (70.5%) in APEC isolates was determined against ampicillin, an antibiotic of the penicillin group. Ampicillin resistance was also reported as 43.0% in Jamaica (21), 58.0% in Bangladesh (22) and 87.5% in Iran (23). Similarly, high resistance to tetracycline group antibiotics has been reported in other studies around the world. Salehi and Bonab (24) reported 94% tetracycline resistance in APECs in Iran. In another study performed in Iran, 91% tetracycline resistance

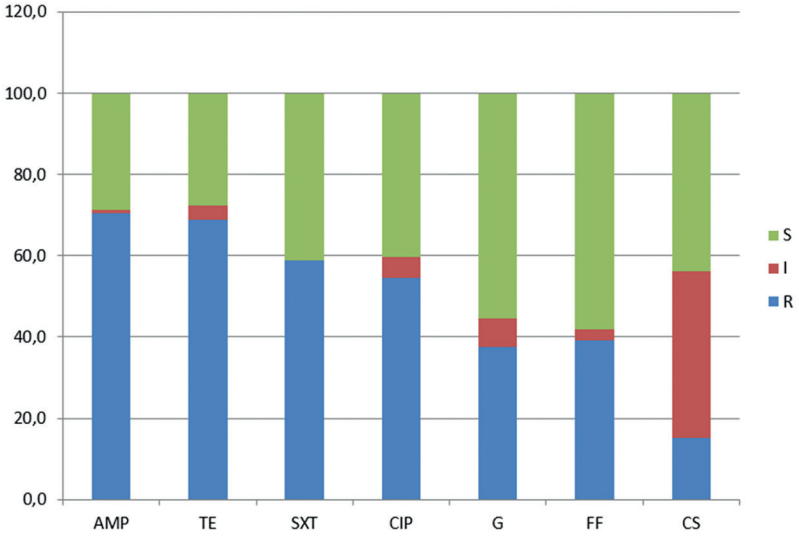

Figure 2: Antimicrobial susceptibility and resistance rates of APEC isolates.

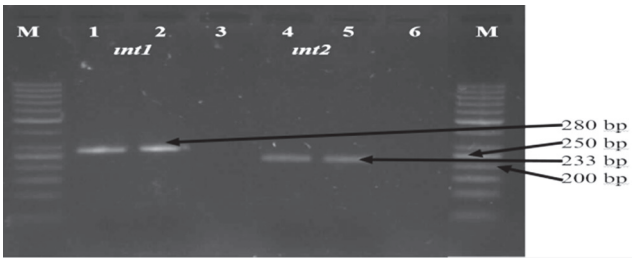

Figure 3: Image of integron classes of *E. coli* isolates in agarose gel. 1. Positive Control (*int*1 positive sequenced field isolate, 280 bp) 2. *int*1 positive field isolate 3. Positive Control (*int*2 positive sequenced field isolate, 233 bp) 4. *Int*2 positive field isolate M: Marker (100 bp DNA Ladder).

Table 3: Distribution of integron classes carried by *E. coli* isolate.

Integron classes	n (%)	n (%)
Class 1	52 (46.4)	59 (52.7)
Class 2	2 (1.8)	
Class 1+ Class 2	5 (4.5)	
Class 3	0 (0.0)	
Number of isolates without integron	53 (47.3)	53 (47.3)

n=112

was determined (25). 26.3% of APEC isolates in Thailand reported SXT resistance (26). In Iran, it reported that the trimethoprim resistance was 29% and sulfamethoxazole resistance was 45.6% in *E. coli* isolated from poultry (25). In a study conducted in Iran, ciprofloxacin resistance was reported as 67% (23). In Turkey, in another study, a similar rate of antibiotic resistance was reported (27). Beta-lactams, sulphonamides, tetracyclines, quinolones, aminoglycosides, fenicols are often used to treat diseases of poultry in Turkey (28). Therefore, it is natural to see resistance to drugs in this group. As a result, ampicillin, tetracycline, trimethoprim/sulfamethoxazole, ciprofloxacin, florfenicol and gentamicin antibiotics would not appear to be the right choice for treatment as resistance rates are high.

Integrons are found in the environment and/or in clinical *E. coli* isolates (4,5) and they may facilitate the spread of

Table 4: Antibiotic resistance phenotypes and integron classes of isolates.

	Resistance phenotypes	Number of isolates	Number of isolates carrying integron	Integron classes		
				Class 1	Class 2	Class1+Class2
1	TE	2	0	0	0	0
2	CT	3	1	1	0	0
3	AMP	5	3	2	0	1
4	CIP	3	0	0	0	0
5	FFC	1	0	0	0	0
6	TE,CT	1	0	0	0	0
7	SXT,CIP	1	0	0	0	0
8	CIP,CT	1	0	0	0	0
9	TE,CIP	1	0	0	0	0
10	AMP,SXT	2	2	2	0	0
11	TE,G	2	0	0	0	0
12	TE,AMP	2	2	1	0	1
13	TE,AMP,FFC	3	2	2	0	0
14	TE,AMP,SXT	6	4	4	0	0
15	TE, SXT,FFC	1	1	1	0	0
16	TE,AMP,CIP	2	0	0	0	0
17	TE, SXT,CT	1	1	1	0	0
18	TE,AMP, SXT,CIP	9	9	7	1	1
19	AMP, SXT,CIP,G	1	0	0	0	0
20	TE,AMP, SXT,FFC	3	2	2	0	0
21	TE,AMP,CIP, FFC	2	0	0	0	0
22	TE,AMP, SXT,CIP	1	1	1	0	0
23	AMP,SXT, CIP,G,CT	1	1	1	0	0
24	T,AMP,SXT, CIP, FFC	3	2	2	0	0
25	AMP,SXT, CIP,FFC,G	1	1	1	0	0
26	T,AMP,SXT,FFC,G	1	1	1	0	0
27	T,AMP,CIP,FFC,G	2	0	0	0	0
28	T,AMP,SXT, CIP, G	4	4	3	0	1
29	T,AMP,SXT,CIP, FFC,CT	1	0	0	0	0
30	T,AMP,SXT, CIP,FFC,G	22	16	15	0	1
31	T,AMP,SXT, CIP, CT,G	4	2	1	1	0
32	T,AMP,SXT,FFC,CT,G	1	1	1	0	0
33	T,AMP,SXT,CIP,FFC,CT,G	3	3	3	0	0
		96	59	52	2	5

resistance not only within the same species but also to other genera (4, 6). In the study, the integron positivity rates were consistent with previous studies. In this study, 52.7% (46.4% of them class 1, 1.8% only class 2, 4.5% of both class 1 and class 2) of the isolates carries an integron, while 47.3% of them did not have an integron. In general, class 1 integron, which is the most commonly detected integron class in *Enterobacteriaceae*, was the most frequently detected integron class in this study (4). In a study in Italy, the presence of class 1 and class 2 integrons isolated from 299 APEC strains in

Table 5: The relationship between the presence/absence of integron and antibiotic resistance.

		Integron positive	Integron negative	$\chi 2$	P	Result
AMP	Resistance positive	56	23	35.66	0.001	***Important
	Resistance negative	3	30			
TE	Resistance positive	51	26	18.16	0.001	***Important
	Resistance negative	8	27			
SXT	Resistance positive	52	14	43.96	0.001	***Important
	Resistance negative	7	39			
CIP	Resistance positive	39	22	6.81	0.01	**Important
	Resistance negative	20	31			
FFC	Resistance positive	30	14	6.99	0.01	**Important
	Resistance negative	29	39			
G	Resistance positive	31	11	12.04	0.001	***Important
	Resistance negative	28	42			
CS	Resistance positive	10	7	0.30	0.61	Not important
	Resistance negative	49	46			

poultry was investigated by using PCR and 55.9% of 167 isolates of APEC were reported as an integron-carriers. The Italian study reported 49.8% (149 isolates) of class 1 integron, 10.4% (31 isolate) of class 2 integron, 4.3% (13 isolates) of class 1 and class 2 integron together (8). The study in Egypt showed that 69.2% (9/13) of the APEC isolates were in the class 1 integron and 38.4% (5/13) were classified as class 3 integrons; however class 2 integron could not be detected (29). In another study in Egypt, in parallel with this study, integron class 1 was 29.3% and class 2 was 3.4%, but class 3 could not be detected (30). Goldstein *et al.* (2001) reported that in *E. coli* isolated from broilers, the coexistence of both integron classes was 11% (11/100) (7). Integrons are capable of carrying or integrating gene cassettes encoding resistance to antibiotics such as tetracycline, trimethoprim, aminoglycosides, β-lactams (4, 5). For this reason, in this study, it is important that while, none of the antibiotic susceptible isolates carried integrons, there was however a significant relationship between integron positivity and trimethoprim sulfamethoxazole, ampicillin, tetracycline, gentamicin resistance.

Infections caused by multiple antibiotic resistant bacteria are a challange to treat (3, 4). In this study, 64.3% of the isolates were multi-antibiotic resistant. Increased number of MDR strains in farm animals, especially in developing countries (23, 24), is disturbing and requires monitoring of the antimicrobial resistance of these pathogenic strains. The two main causes of antibiotic resistance in bacteria are thought to be incorrect antibiotic usage and horizontal transfer of resistance genes (2, 4). In this study, it was found that 33.3% (8/24) of the non-MDR isolates; 70.8% (51/72) of the MDR isolates were detected carrying the integron. Also, no integron was detected in any of the antibiotic susceptible isolates. Although it was known that integrons play a role in the resistance against antimicrobial drugs (4, 5), we observed that there was no integron in all APEC isolates resistant to antimicrobial agents. In this case, possibly other molecular resistance mechanisms such as plasmid or transposon may be considered, as other researchers have reported (17, 31).

In this study, we investigated the antimicrobial resistance, the prevalence of integrons and the role of antibiotic resistance in integron-mediated resistance in *E. coli*. This study is the most comprehensive study to date investigating integron gene carriage in *E. coli* isolates obtained from broiler internal organs in Turkey. It was seen in the study, that the number of antibiotic susceptible isolates was higher than the number of antibiotic resistant isolates. This situation indicates the alarming antibiotic resistance on farms in western Turkey. Based on this study and the detection of existing antibiotic resistance phenotypes in *E. coli* isolates should lead to the consideration of use of new antimicrobials in the region.

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