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Research Article

Detection of Extended-spectrum β-lactamase and AmpC β-lactamase Producing *Escherichia coli* Isolates from Chickens [1]

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Abstract

The aim of this study was to investigate the presence of Extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase producing *Escherichia coli* strains isolated from meat and faecal samples of chicken. Faecal samples (n: 384) were collected from broiler and egg-type healthy chickens in 43 flocks and 384 fresh chicken meat samples from local markets, supermarkets and slaughterhouses. *E. coli* isolates were tested phenotypically according to the guidelines of CLSI (2013) for ESBL production. Phenotypic detection of AmpC production was carried out by determination of resistance to cefoxitin and susceptibility to cefepime. Also, presence of genes encoding different types of β-lactamases (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and the mutation in the promoter of AmpC) in all phenotypically ESBL or AmpC producing *E. coli* was investigated by PCR assays. In this study, *bla*$_{CTX-M}$ (95.4%), *bla*$_{CTX-M-1}$ (81.8%), *bla*$_{CTX-M-8}$ (4.5%), *bla*$_{TEM}$ (45.4%) and *bla*$_{SHV}$ (2.2%) genes were detected from faecal samples and *bla*$_{CTX-M}$ (100%), *bla*$_{CTX-M-1}$ (28.5%) and *bla*$_{TEM}$ (7.1%) from meat samples. The only plasmidic AmpC β-lactamase found was the CIT type.

Keywords: *Escherichia coli, ESBL, AmpC, Chicken, PCR*

Tavuklarda Genişlemiş Spektrumlu β-laktamaz ve AmpC β-laktamaz Üreten *Escherichia coli* İzolatlarının Saptanması

Özet

Bu çalışmanın amacı, tavuk eti ve dışkı örneklerinden izole edilen Genişlemiş Spektrumlu β-laktamaz (GSBL) ve AmpC β-laktamaz üreten *Escherichia coli* izolatlarının varlığını ortaya koymaktır. Bu çalışmada, 43 kümesten sağlıklı tavuklara ait 384 dışkı örneği ve yerel marketlerden, süpermarketlerden ve kesimhanelerden 384 taze tavuk eti örneği toplandı. *E. coli* izolatları fenotipik olarak CLSI (2013) standartları kullanılarak GSBL üretimi yönünden incelendi. AmpC üretiminin fenotipik olarak saptanması sefoksitine direnç ve sefepime duyarlılık kriterleri ile belirlendi. Fenotipik olarak GSBL ya da AmpC üreten *E. coli* olarak saptanan izolatlarının hepsi PCR ile değişik tipteki β-laktamaz genlerinin (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, AmpC) varlığını ortaya koymak için incelendi. Bu çalışmada, dışkı örneklerinde *bla*$_{CTX-M}$ (%95.4), *bla*$_{CTX-M-1}$ (%81.8), *bla*$_{CTX-M-8}$ (%4.5), *bla*$_{TEM}$ (%45.4), *bla*$_{SHV}$ (%2.2) genleri ve tavuk eti örneklerinde ise *bla*$_{CTX-M}$ (%100), *bla*$_{CTX-M-1}$ (%28.5), *bla*$_{TEM}$ (%7.1) genleri saptandı. AmpC β-laktamaz grubunda sadece CIT geni belirlendi.

Anahtar sözcükler: *Escherichia coli, GSBL, AmpC, Tavuk, PCR*

INTRODUCTION

During the past decade, drug resistance has increased worldwide and also extended-spectrum β-lactamases (ESBL) is the most common mechanism of resistance to broad-spectrum cephalosporins in members of Enterobacteriaceae (mostly *Escherichia coli*) [1-3]. ESBL genes are located on plasmids those can easily harbour in bacterial species. Some ESBL genes are mutant derivatives of established plasmid-mediated β-lactamases (e.g., *bla*$_{TEM/SHV}$), and others are mobilized from environmental bacteria (e.g., *bla*$_{CTX-M}$). The epidemiology of ESBL genes is rather complex, changing quickly and shows marked geographic differences in distribution of genotypes of *bla*$_{CTX-M}$ β-lactamases [3]. Another large group of broad-spectrum β-lactamases are the AmpC enzymes, which are typically

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encoded on the chromosome of many Gram-negative bacteria. Also, AmpC-type β-lactamases may be carried on plasmids of bacterial species lacking the chromosomal AmpC gene [4].

The use of antimicrobial agents in poultry husbandry is a common problem. The authors emphasize that the antimicrobial-resistant poultry faecal *E. coli* strains can be transmitted to humans both directly and via the food chain [5]. The source of the colonization of ESBL/AmpC producing bacteria in humans is not completely understood, circumstantial evidence points also to a food-borne source [6]. Poultry meat can be contaminated with antimicrobial-resistant *E. coli* at slaughter and can also act as a reservoir of drug resistant bacteria. The use of cephalosporins in food-producing animals and in veterinary medicine could be a selective factor for the appearance of ESBL producing bacteria in animals [7].

Recent studies in Spain [8], France [9], Tunisia [10], Belgium [11], China [12], Great Britain [13], Netherlands [6], and England [14] show that CTX-M *E. coli* isolates are likely to be present in chickens globally. In Turkey, the prevalence has not been extensively investigated in poultry.

The present study was undertaken to determine the presence and prevalence of ESBL and AmpC β-lactamase producing *E. coli* strains in chicken in Marmara Region of Turkey.

MATERIAL and METHODS

The present study was approved by the Animal Care Committee of Istanbul University, Faculty of Veterinary Medicine, Approval no: 2012/17.

A total of 384 faecal samples (10 faeces pooled in a sterile faeces container and counted as one sample) were collected from healthy chickens in 43 flocks on 14 farms located in different areas of the Marmara Region in Turkey (Kırklareli, Edirne, Tekirdağ, Istanbul, Kocaeli, Yalova, Sakarya, Bursa, Balıkesir, Çanakkale), at intervals between November 2012 and October 2013. Additionally, a total of 384 retail chicken meat samples (breast, leg quarter) were collected from local markets, supermarkets and slaughterhouses located in the same region, between January-October 2013.

Five gram of the faecal and meat samples were inoculated into 15 ml of tryptic soy broth/TSB (Oxoid, USA) and were incubated for 16-18 h at 37°C. Subsequently, 10 µL of TSB was transferred onto MacConkey agar plate (Becton Dickinson, USA) supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, United Kingdom) and incubated aerobically overnight at 37°C. One presumptive *E. coli* colony was randomly selected on MacConkey agar and subcultured onto blood agar plate and identified by biochemical tests [15]. *E. coli* isolates were subjected for antibiotic susceptibility testing by using, cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefpodoxime (10 µg), aztreonam (30 µg), cefoxitin (30 µg), cefepime (30 µg) discs. Clinical Laboratory Standards Institute (CLSI) guidelines were followed for inoculum standardization, medium and incubation conditions, and internal quality control organisms *(E. coli* ATCC 25922) were used. The diameter of the zone of inhibition was measured and interpreted according to the guidelines of CLSI (2013). The isolates were tested for ESBL production by combination of disc diffusion test including cefotaxime and ceftazidime with and without clavulanic acid. An increase in the zone diameter of 5 mm or more, when either of the antimicrobial agents was combined with clavulanic acid, was considered evidence of ESBL production [16]. Phenotypic detection of AmpC production was carried out by determination of resistance to cefoxitin and susceptibility to cefepime [16,17].

DNA was extracted by Roche High Pure PCR Template Preparation Kit (Roche, France), according to the manufacturer's instructions. The presence of genes encoding different types of β-lactamases (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and the mutation in the promoter of *AmpC*) in all phenotypically ESBL or AmpC producing *E. coli* was studied.

For specific detection of the *bla*$_{CTX-M}$ genes (CTX-M-1, CTX-M-2, CTX-M-9, CTX-M-14, CTX-M-20 and CTX-M-21), consensus primers were chosen from regions with high levels of sequence homology to the *bla*$_{CTX-M}$ genes [18].

bla$_{CTX-M}$ genes groups 1, 2 and 9 were detected by multiplex PCR. Primer pairs and predicted amplicon sizes were: group 1, 5′-AAA AAT CAC TGC GCC AGT TC and 5′-AGC TTA TTC ATC GCC ACG TT (415 bp); group 2, 5′-CGA CGC TAC CCC TGC TAT T and 5′-CCA GCG TCA GAT TTT TCA GG (552 bp); group 9, 5′-CAA AGA GAG TGC AAC GGA TG and 5′-ATT GGA AAG CGT TCA TCA CC (205 bp). Amplification conditions were: initial denaturation at 94ºC for 5 min; 30 cycles of 94ºC for 25 s, 52ºC for 40 s and 72ºC for 50 s; and a final extension at 72ºC for 6 min [19].

The primer sequences, product sizes and cycling conditions used to amplify different β-lactamase genes by PCR are listed in *Table 1*.

Multiplex PCR for the purpose of identifying family-specific *AmpC* β-lactamase genes was performed as previously described [21]. The targets, *primers sequences used for PCR amplification and product sizes are summarized in Table 2*. The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 25 cycles of DNA denaturation at 94°C for 30s, primer annealing at 64°C for 30s, and primer extension at 72°C for 1 min. After the last cycle, a final extension step at 72°C for 7 min was added.

PCR product were analysed by gel electrophoresis with 2% agarose (Sigma-Aldrich, United Kingdom) and visualizations were performed.

Table 1. *Primer sequences, product sizes, cycling conditions and references*
Tablo 1. *Gen dizilimleri, amplikon büyüklüğü, amplifikasyon koşulları ve kaynaklar*

Target(s)	Primers (5' to 3', as synthesized)	Size (bp)	Cycling Conditions					References
bla_{CTX-M}	SCS ATG TGC AGY ACC AGT AA	550	94°C 3 min.	35 cycles			72°C 5 min.	[18]
	CCG CRA TAT GRT TGG TGG TG			95°C 30 s.	57°C 30 s.	72°C 45 s.		
CTX-M Group 8	TCG CGT TAA GCG GAT GAT GC	666	94°C 5 min.	25 cycles			72°C 6 min.	[19]
	AAC CCA CGA TGT GGG TAG C			94°C 25 s.	52°C 40 s.	72°C 50 s.		
CTX-M Group 25	GCA CGA TGA CAT TCG GG	327	94°C 5 min.	25 cycles			72°C 6 min.	[19]
	AAC CCA CGA TGT GGG TAG C			94°C 25 s.	52°C 40 s.	72°C 50 s.		
bla_{TEM}	ATG AGT ATT CAA CAT TTC CG	858	94°C 3 min.	35 cycles			72°C 5 min.	[20]
	CCA ATG CTT AAT CAG TGA GC			95°C 30 s.	55°C 30 s.	72°C 45 s.		
bla_{SHV}	CTT TAC TCG CTT TAT CG	475	95°C 5 min.	30 cycles			72°C 5 min.	[20]
	TCC CGC AGA TAA ATC ACC A			95°C15 s.	52°C 30 s.	72°C 90 s.		

Table 2. *Targets, primer sequences and product sizes*
Tablo 2. *Hedefler, gen dizilimleri ve amplikon büyüklükleri*

Target (s)	Primer	Primers (5' to 3', as synthesized)	Size (bp)
MOX-1, MOX-2, CMY-1, CMY-8-9-10-11	MOXM-F MOXM-R	GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG C	520
LAT-1-2-3-4, CMY-2-3-4-5-6-7, BIL-1	CITM-F CITM-R	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	462
DHA-1, DHA-2	DHAM-F DHAM-R	AAC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC	405
ACC	ACCM-F ACCM-R	AAC AGC CTC AGC AGC CGG TTA TTC GCC GCA ATC ATC CCT AGC	346
MIR-1T, ACT-1	EBCM-F EBCM-R	TCG GTA AAG CCG ATG TTG CGC CTT CCA CTG CGG CTG CCA GTT	302
FOX-1-2-3-4-5b	FOXM-F FOXM-R	AAC ATG GGG TAT CAG GGA GAT G CAA AGC GCG TAA CCG GAT TGG	190

RESULTS

Phenotypically Monitoring and Confirmation Tests

From faecal samples, 76 (19.7%) isolates on MacConkey agar (supplemented with cefotaxime) were positive for cefotaxime-resistant *E. coli*.

Distribution of β-lactam resistance phenotypes among cefotaxime-resistant faecal *E. coli* isolates from broilers on each farm is shown at *Table 3*.

From meat samples, 14 (3.6%) isolates on MacConkey agar (supplemented with cefotaxime) were positive for cefotaxime resistant *E. coli*. Out of those isolates only 2 (40%) isolates were ESBL producers, and 3 (60%) were AmpC β-lactamase producers.

Genetic Confirmation by PCR

In 44 phenotypically confirmed ESBL and/or AmpC β-lactamase producing *E. coli* isolates from faecal samples, 42 (95.4%) harboured at least one of the ESBL-gene: bla_{CTX-M} (95.4%), $bla_{CTX-M-1}$ (81.8%), $bla_{CTX-M-8}$ (4.5%), bla_{TEM} (45.4%) and for bla_{SHV} (2.2%). The only plasmid encoded AmpC β-lactamase found was the CIT type (n=29). $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, $bla_{CTX-M-25}$ or AmpC FOX, EBC, ACC, DHA and MOX groups were not detected.

Five phenotypically confirmed ESBL and AmpC β-lactamase producing *E. coli* isolates obtained from meat samples, all of them (%100) was harbouring at least one of ESBL-gene: bla_{CTX-M} (100%), $bla_{CTX-M-1}$ (28.5%) and/or bla_{TEM} (7.1%). The only plasmidic AmpC β-lactamase found was the CIT type (n=3). $bla_{CTX-M-2}$, $bla_{CTX-M-8}$, $bla_{CTX-M-9}$, $bla_{CTX-M-25}$, bla_{SHV} or AmpC FOX, EBC, ACC, DHA and MOX groups were not found. Distribution of β-lactamase genes is given in *Table 4*.

DISCUSSION

Antimicrobial resistance is recognized as one of the greatest threat and the most important global health challenge. In recent years, the prevalence of ESBL producing *E. coli* has been increasing in both human and veterinary medicine [3,22].

In this study, it has been demonstrated that ESBL producing *E. coli* strains are frequently present (39.4%) in faecal samples of broilers studied. The prevalence of ESBL producing *E. coli* has been reported between 6.7% and

Table 3. *Distribution of β-lactam resistance phenotypes among faecal E. coli isolates from broilers on each farm*
Tablo 3. *Fenotipik olarak β-laktam dirençli fekal E. coli izolatlarının broyler çiftliklerine göre dağılımı*

Farm Number	Number of Faecal Samples Analysed	Number of Cefotaxime -resistant *E. coli* isolates	Number of ESBL Producers	Number of AmpC Producers
1	31	3	1	-
2	38	8	-	5
3	33	9	4	-
4	22	11	2	4
5	35	7	3	1
6	19	6	3	-
7	28	7	2	1
8	25	-	-	-
9	18	-	-	-
10	25	14	8	3
11	28	-	-	-
12	32	10	7	-
13	32	-	-	-
14	18	1	-	-
TOTAL (%)	384	76 (19.7%)	30 (39.4%)	14 (18.4%)
			44 (57.8%)	

Table 4. *Distribution of β-lactamase genes*
Tablo 4. *β-laktamaz genlerinin dağılımı*

Samples	Genetic confirmation by PCR						
	bla_{CTX-M}	CIT Type	bla_{CTX-M} + CIT Type	$bla_{CTX-M-1}$	$bla_{CTX-M-8}$	bla_{TEM}	bla_{SHV}
Faecal (n=44)	42 (95.4%)	29 (65.9%)	27 (61.3%)	36 (81.8%)	2 (4.5%)	20 (45.4%)	1 (2.2%)
Meat (n=5)	5 (100%)	3 (60%)	3 (60%)	4 (80%)	-	1 (20%)	-

60.7%. Our results were relatively low according to some studies [9,13] while showed similarities to others [11,12].

ESBL producing *E. coli* was detected 40% of the isolates obtained from meat samples in this study. In European countries, the prevalence of ESBL genes in chicken meat have been detected in various studies; in the Netherlands, 79.8% [23], in Spain 67% [24] and in the United Kingdom 37% [25]. The different prevalence rates may be originated from detection methods, sampling procedures, regions, geographical conditions and the antibiotic policies.

In this study, 14 (18.4%) *E. coli* isolates from faecal samples were identified as AmpC (plasmidic or chromosomally) β-lactamase producers. The prevalence was lower in comparison to those detected by Smet et al.[11] 43% and Kolar et al.[20] 25.9%. On the other hand, Blanc et al.[23] reported the prevalence rate of *E. coli* isolates as AmpC β-lactamase producers was 6.7%. The comparison of our results with the mentioned studies shows that *E. coli* strains producing AmpC enzymes are mostly less prevalent.

According to our results, the CTX-M group β-lactamases were the predominant ESBL type. Similar results have been reported by Randall et al.[13], Overdevest et al.[3], Morris et al.[26], and Leverstein-van Hall et al.[27]. Also, the majority of those ESBL genes were identified as $bla_{CTX-M-1}$ (81.8%) in this study. Recent studies identified CTX-M-1 as the most prevalent ESBL type shared by human patients, healthy carriers, poultry, and retail chicken meat, suggesting recent cross-transmission between human and avian hosts [22]. In Turkey, Zarakolu et al.[28] and Gülamber et al.[29] reported that CTX-M-1 members were the most prevalent gene types in human isolates.

In this study, ESBL-producing *E. coli* isolates were detected in both faecal and food samples and $bla_{CTX-M-8}$ gene were detected from only two faecal isolates. On the contrary of our results, Jouini et al.[10] indicated that ESBL-producing *E. coli* isolates were detected in 10 (26%) of 38 food samples analyzed and in none of the tested animal faecal samples. Also, these authors reported that $bla_{CTX-M-8}$ gene was not detected from any of the tested animal faecal samples and pointed that the first time that the unusual CTX-M-8 β-lactamase has been detected in bacteria of non-human origin.

Phenotypically confirmed *E. coli* isolates obtained from faecal samples carried an ESBL-gene: bla_{TEM} (45.4%) and

from meat samples carried an ESBL-gene: bla_{TEM} (7.1%) Also, bla_{TEM} was always detected present in combination with other β-lactamase genes ($bla_{CTX-M-1}$, bla_{SHV}, bla_{CIT}). These findings were similar with previous studies reporting that TEM β-lactamases were the most frequent mechanism in *E. coli* isolates from food-producing animals [6,30].

bla_{SHV} was detected only in one isolate from faecal samples and was not detected in any of the isolates from meat samples. On the contrary to these results, Kolar et al.[20] described the most frequent ESBL types were SHV. In Spain, SHV-12-producing *E. coli* strains were then sporadically recovered in faecal samples from healthy chickens [24]. Carattoli [31] emphasize that this gene variant has been described in *E. coli* isolated from poultry in different countries; thus, it would be of particular interest to monitor its future global diffusion.

In the current study, of the plasmidic class C β-lactamases, only the bla_{CIT} type was identified. Various authors indicated that the most prevalent AmpC gene family was CIT including CMY-2, CMY-4, and two CMY-2 variants. Plasmid-encoded AmpC genes belonging to the CIT family have already been reported in food-producing animals and humans worldwide. These findings were consistent with previous studies as a major factor contributing to AmpC resistance [8,19,32].

Multiple β-lactamases within the same organism (e.g., multiple ESBLs or ESBL-AmpC combinations) can make phenotypic identification of the β-lactamases difficult. Unfortunately, for this reason, plasmid-mediated AmpC β-lactamase resistance goes undetected in most clinical laboratories [20]. Also, some researchers stated that two or more of β-lactamase resistance genes can be found in the frequently isolated Gram-negative bacteria [33]. In the current study, 27 faecal (61.3%) and 3 (60%) meat *E.coli* strains were observed as they possess the various ESBL and AmpC genes combinations.

Our results clearly show that ESBL and/or AmpC producing *E. coli* are present in most of the farms (71.4%) studied. The differences could be originated from antibiotic policies or increasing usage of cephalosporins in worldwide. Moreover, there are various methods for detection of ESBL and/or AmpC producing *E. coli*, making the results difficult to compare. Both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have revised and updated breakpoints for Enterobacteriaceae. The microbiology laboratories in Turkey, like many European laboratories, have implemented the new EUCAST guidelines in 2015. In the present study, the screening and confirmation tests of ESBL were applied and interpreted according to the guidelines of CLSI. In EUCAST guidelines, the formation of the zone of inhibition around cefpodoxime (10 mg) disk <21 mm in diameter (in CLSI this value is ≤17 mm) was accepted ESBL positive screening test. So that, if EUCAST standard had been used as a method, more isolates would be positive screening test, and confirmation test should be applied. Consequently, the prevalence is thought to be higher according to ESBL screening test breakpoints in guidelines issued by EUCAST [16,33].

To conclude, ESBL and/or AmpC producing *E. coli* strains are frequently present in this study and CTX-M type enzymes are the predominant ESBL type. CTX-M-1 is the most prevalent ESBL type and of the plasmidic class C β-lactamases, only the bla_{CIT} type is present. Further multi-disciplinary studies, parallel monitoring and surveillance programmes, and novel strategies in the spirit of 'One Health' are required since such data are currently missing.

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