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Characterization of extended-spectrum beta-lactamase-producing fecal *Escherichia coli* isolates in laying hens

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Summary: The purposes of the present study were to determine whether healthy laying hens in Burdur province of Turkey carried extended-spectrum beta-lactamase (ESBL)- producing *Escherichia coli* in their intestinal flora and to further characterize the *E. coli* isolates and ESBL genes. A total of 200 fecal samples from three laying hen farms were cultured in Brilliance *E. coli* / coliform Selective Agar supplemented with cefotaxime or ceftazidime. ESBL-producing *E. coli* isolates determined by phenotypic tests were screened by Polymerase Chain Reaction (PCR) for ESBL genes, and ESBL genes were sequenced. Phylogenetic analysis of the *E. coli* isolates were performed by PCR. ESBL-producing *E. coli* were isolated from 12 (6.0%) laying hen fecal samples by phenotypic tests. According to PCR screening and sequence analysis, *bla*$_{\text{CTX-M}}$ group 1 (*bla*$_{\text{CTX-M-3}}$ or *bla*$_{\text{CTX-M-15}}$) genes were detected in the *E. coli* isolates. Phylogenetic analysis of the *E. coli* isolates showed that group D was the predominant group (58.3%) and none of the isolates belonged to group B2. Consequently, present study reports the first characterisation of CTX-M-3 and CTX-M-15 type ESBL-producing *E. coli* isolates on laying hen production from Turkey.

Keywords: CTX-M-3, CTX-M-15, *Escherichia coli*, laying hens, phylogenetic analysis.

Yumurtacı tavuklarda genişlemiş spektrumlu beta laktamaz üreten dışkı kökenli *Escherichia coli* izolatlarının karakterizasyonu

Özet: Bu çalışmada, Burdur ilindeki yumurtacı tavukların bağırsak mikroflorasında genişlemiş spektrumlu beta laktamaz (GSBL) üreten *Escherichia coli* varlığının ortaya çıkarılması ve izole edilen *E. coli* izolatlarının ve taşıdıkları GSBL genlerinin karakterize edilmesi amaçlandı. Toplam üç tavuk çiftliğinden alınan 200 dışkı örneği, içerisine sefotaksim ve seftazidim ilave edilmiş Brilliance *E. coli* / koliform Selektif Agara ekildi. Fenotipik testlerle belirlenen GSBL üreten *E. coli* izolatları GSBL genleri yönünden Polimeraz Zincir Reaksiyonu (PZR) ile tarandı ve GSBL genlerinin sekans analizi yapıldı. İzolatların filogenetik analizi PZR ile gerçekleştirildi. Fenotipik testler ile GSBL üreten *E. coli* 12 (%6.0) adet dışkı örneğinde belirlendi. PZR ve sekans analizine göre, *E. coli* izolatlarında *bla*$_{\text{CTX-M}}$ grup 1 genleri (*bla*$_{\text{CTX-M-3}}$ ve *bla*$_{\text{CTX-M-15}}$) belirlendi. İzolatların filogenetik analizi grup D'nin en yaygın olduğunu (%58.3) ve izolatların hiç birinin B2 grubundan olmadığını gösterdi. Sonuç olarak, bu çalışma Türkiye'de yumurta tavuklarında CTX-M-3 and CTX-M-15 type GSBL üreten *E. coli* izolatlarını tanımlayan ilk rapordur.

Anahtar sözcükler: CTX-M-3, CTX-M-15, *Escherichia coli*, filogenetik analiz, yumurtacı tavuk.

Introduction

Beta-lactam antibiotics are among the most useful treatment options for bacterial infections of human and animals. The most common bacterial resistance to beta-lactam antibiotics can be achieved by the production of beta-lactamase enzymes by the resistant bacteria that hydrolyse the beta-lactam ring (amid bond) of the antibiotics (11). A group of beta-lactamases known as extended-spectrum beta-lactamases (ESBLs) provide resistance to Gram-negative bacteria against several antibiotics including penicillins, 1st - 4th generation cephalosporins and monobactams (e.g., aztreonam). ESBLs are not active against carbapenems (e.g., imipenem, meropenem and ertapenem) or cephamycins (e.g., cefoxitin). Beta-lactamase inhibitors such as clavulanic acid (CLA) and tazobactam, inhibit the ESBLs and this characteristic is used in their laboratory detection and confirmation (19). ESBLs are plasmid mediated and TEM, SHV and CTX-M groups are the most prevalent ESBLs in *Enterobacteriaceae* (19). ESBLs of TEM and SHV groups originate from TEM-1/TEM-2 and SHV-1 beta-lactamase genes (*bla*$_{\text{TEM-1}}$/*bla*$_{\text{TEM-2}}$ and *bla*$_{\text{SHV-1}}$), respectively, via mutations (19). On the other hand, CTX-M beta-lactamase genes (*bla*$_{\text{CTX-M}}$) have been captured from the chromosome of *Kluyvera* spp. by conjugation (20). Based on aminoacid sequence similarities CTX-M type ESBL variants are divided into 5 groups, called group 1, 2, 8, 9 and 25 (19).

The presence of ESBL-producing *Escherichia coli* isolates and ESBL types that they produced have been investigated in poultry productions from various parts of the world (4, 16, 17, 25). However, in Turkey, little is known about avian ESBL-producing *E. coli*. In the only study conducted with laying hen farms in Turkey (Marmara region), phenotypic detection of ESBL-producing *E. coli* isolates and PCR detection of ESBL genes were performed but ESBL variants were not revealed by sequencing in that study (3). Until now there is only one Turkish study conducted in which both the presence and types of ESBLs in healthy chickens were investigated in a small-scale local study in Turkey (23) but it covered only the broiler chickens. Such information for laying hen productions in Turkey is necessary to better understand the epidemiology of ESBLs in poultry and to develop control strategies. Therefore, the present study was carried out to show the emergence of ESBL-producing *E. coli* isolates in healthy laying hens in Burdur province of Turkey and to further characterize the *E. coli* isolates and ESBL genes.

Materials and Methods

Sample collection: The study population included three laying hen farms which constitute all chicken farms in Burdur province located in southwest of Turkey. Two hundreds fecal samples (50 samples from each of farm A and B, and 100 samples from farm C) were collected from cages by using sterile swabs provided that only one fecal sample from a cage. The fecal samples were placed in sterile screw-top vials, transported to the laboratory on ice and held at 4°C until processing within 24 h.

Selective isolation and phenotypic confirmation of ESBL-producing E. coli isolates: Firstly, a 10% suspension of a fecal sample in buffered peptone water (Lab M, UK) was prepared and incubated at 37°C for 24 h under aerobic conditions. Then, 50 µl was evenly spread onto Brilliance *E. coli*/coliform Selective Agar (Oxoid, UK) supplemented with cefotaxime (CTX, 2 µg/ml) (Sigma Aldrich, Germany) or ceftazidime (CAZ, 2 µg/ml) (Sigma Aldrich, Germany) and the plates were incubated at 37°C for 24 h under aerobic conditions.

One colony from each plate per positive sample was selected randomly and *E. coli* identification was performed based on conventional methods (Gram staining, acid and gas from glucose, catalase test, citrate utilization, decarboxylation of lysine, hydrogen sulphide production, indole production, methyl red-voges proskauer test, orthonitrophenyl-beta-D-galactopyranoside activity, oxidase test and urease production**)** (26). DNAs of *E. coli* isolates were extracted by using a genomic DNA purification kit (Thermo Fisher Scientific Inc.) and genetic confirmation was carried out by Polymerase Chain Reaction (PCR) amplification targeting *E. coli* 16S rRNA gene (24).

ESBL production by *E. coli* isolates was confirmed by the combined disc method as recommended by the CLSI (8).

Antibiotic susceptibility testing: Susceptibility of ESBL-producing *E. coli* isolates to 8 beta-lactam antibiotics were investigated by the agar disc diffusion test on MHA (Oxoid, UK) according to the CLSI protocols (8). The list of the antibiotic discs (Oxoid, UK) tested were as follows: ampicillin (AMP, 10 µg), aztreonam (ATM, 30 µg), cefepime (FEP, 30 µg), cefpodoxime (CPD, 10 µg), ceftriaxone (CRO, 30 µg), cefuroxime (CXM, 30 µg), cephalothin (CEF, 30 µg), and imipenem (IPM, 10 µg).

To explore if the isolates had co-resistance to other classes of antibiotics, they were additionally subjected to susceptibility testing against the following antibiotics (Oxoid, UK) by agar disc diffusion test on MHA (Oxoid, UK) as recommended by CLSI (8): chloramphenicol (CHL), ciprofloxacin (CIP, 5 µg), enrofloxacin (ENR, 5 µg), florfenicol (FFC, 30 µg), gentamicin (GEN, 10 µg), kanamycin (KAN, 30 µg), nalidixic acid (NAL, 30 µg), streptomycin (STR, 10 µg), sulfamethoxazole-trimethoprim (SXT, 25 µg) and tetracycline (TET, 30 µg).

Antibiotic susceptibility test results were evaluated according to inhibition zone diameters recommended by CLSI (6, 7, 8). The isolates were classified as resistant or susceptible. The *E. coli* isolates with the same antibiotic susceptibility phenotype from both media (supplemented with CTX or CAZ) per positive fecal sample were considered as the same isolate. In the present study, multidrug-resistance was described as resistance to more than 3 classes of antibiotics except beta-lactams.

PCR and sequencing: PCR screening of bla_{TEM} (1, 13), bla_{SHV} (13, 22) and $bla_{\text{CTX-M}}$ type (9, 13, 15, 21) genes of phenotypically confirmed ESBL-producing *E. coli* isolates was carried out as described previously with slight modifications in the cycling conditions. The cycling conditions for detection of bla_{TEM} gene were as follows: initial denaturation at 94ºC for 5 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 48ºC and 1 min at 72ºC, with a final extension step at 72ºC for 10 min. The cycling conditions for bla_{SHV} gene detection were initial denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 30 sec, 58ºC for 30 sec and 72 ºC for 1 min, and a final extension at 72ºC for 7 min. The cycling conditions for $bla_{\text{CTX-M}}$ gene (universal) consisted of initial denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 30 sec, 54ºC for 30 sec and 72ºC for 1 min, and a final extention step at 72ºC for 7 min. Finally, detection of $bla_{\text{CTX-M}}$ group 1, 2, 9 and 8/25 genes were performed with the following PCR cycling conditions: initial denaturation at 94 ºC for 5 min, 35 amplification cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min, and a final extension step at 72ºC for 7 min. *E. coli* ATCC 35218 ($bla_{\text{TEM-1}}$) and *Klebsiella pneumoniae* ATCC 700603 ($bla_{\text{SHV-18}}$) were used as

positive control strains for *bla*$_{TEM}$ and *bla*$_{SHV}$ genes, respectively. *E. coli* NCTC 13461, *E. coli* NCTC 13462, *E. coli* NCTC 13463, *Enterobacter cloacae* NCTC 13464 and *K. pneumoniae* NCTC 13465 were used as positive controls for detection of *bla*$_{CTX-M}$ group 1, group 2, group 8, group 9 and group 25 genes, respectively. *E. coli* ATCC 25922 was included in all PCRs as a negative control.

PCR products obtained from ESBL-producing *E. coli* isolates were subjected to DNA sequencing. DNA sequencing was carried out by the Refgen Genetical Research and Biotechnology Company (Gölbaşı-Ankara, Turkey). Sequencing was performed with both forward and reverse primers which were used in the PCR. To determine the variants of beta-lactamase genes, the sequences were compared with NCBI GenBank sequences using BLAST. Finally, nucleotid sequences of selected ESBL genes were submitted to NCBI GenBank.

Phylogenetic analysis: A triplex PCR protocol was used to reveal phylogenetic groups (A, B1, B2 and D) of all *E. coli* isolates in the present study, as described by Clermont et al. (5) with the modified PCR conditions (14). *E. coli* ATCC 25922 was used as positive control strain in the triplex PCR. For better discrimination of the strains, phylogenetic subgroups (A: A_0 and A_1; B2: $B2_2$ and $B2_3$; D: D_1 and D_2) were also investigated as described by Escobar-Páramo et al. (10).

Results

After the enrichment step and inoculation on Brilliance *E. coli*/coliform selective agar with CTX (2 µg/ml) or CAZ (2 µg/ml), *E coli* colonies were detected on both cultures of 27 fecal samples. Biochemical identification and PCR for 16S rRNA gene confirmed all of them as *E. coli* (Figure 1). Further characterization using the combined disc method confirmed 12 *E. coli* isolates (12/200, 6%) from two farms, farm B and C, as ESBL producers. Within farm isolation rate was 16% (8/50) for farm B and 4% (4/100) for farm C.

In antibiotic susceptibility testing against beta lactams, all ESBL producing *E. coli* isolates were found to be resistant to AMP, ATM, CTX, CPD, CAZ, CRO, CXM, CEF, susceptible to FOX and IPM. Five (41.7%) *E. coli* isolates were resistant to FEP. According to antibiotic susceptibility testing against other classes of antibiotics, all isolates were susceptible to GEN, FFC, and CHL. Resistance ratios for KAN, STR, CIP, ENR, NAL, TET, and SXT were 25%, 8.3%, 25%, 25%, 75%, 58.3% and 8.3%, respectively. One ESBL-producing *E. coli* isolate (CTX-M-15 producer) (1/12, 8.3%) from farm C was determined as multidrug-resistant (Table 1).

PCR screening for the *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes in phenotypically-confirmed ESBL-producing *E. coli* isolates indicated that all isolates (12/12, 100%) harbored *bla*$_{CTX-M}$ group 1 genes (ESBL) (Figures 2 and 3) and 2 of which (2/12, 16.7%) possessed also *bla*$_{TEM}$ gene (Figure 4). *bla*$_{SHV}$ gene was not detected in the isolates (Table 1).

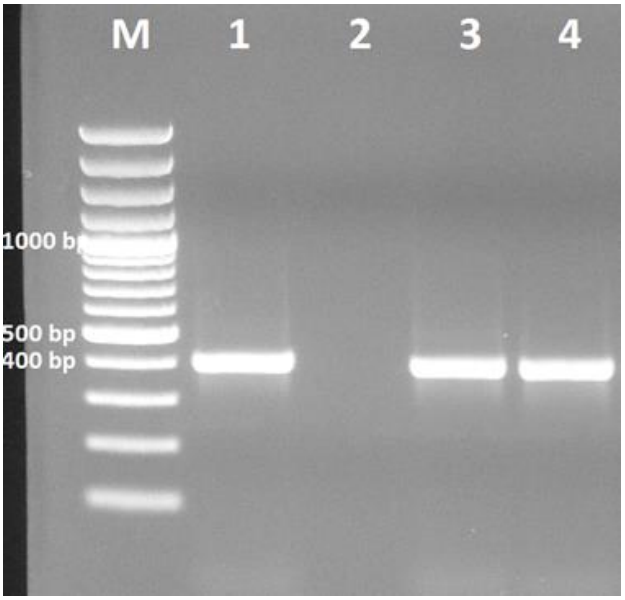

Figure 1. PCR performed with 16S rRNA gene specific primers (401 bp). M: Marker (100 bp), 1: Positive control (*E. coli* ATCC 25922), 2: Negative control (PCR mixture without template DNA), 3 and 4: *E. coli* isolated from laying hens (isolates: F73 and F92).

Şekil 1. 16S rRNA geni için spesifik primerlerle (401 bp) yapılan PZR. M: Marker (100 bp), 1: Pozitif kontrol (*E. coli* ATCC 25922), 2: Negatif kontrol (kalıp DNAsız PZR karışımı), 3 ve 4: Yumurta tavuklarından izole edilen *E. coli* (izolatlar: F73 ve F92).

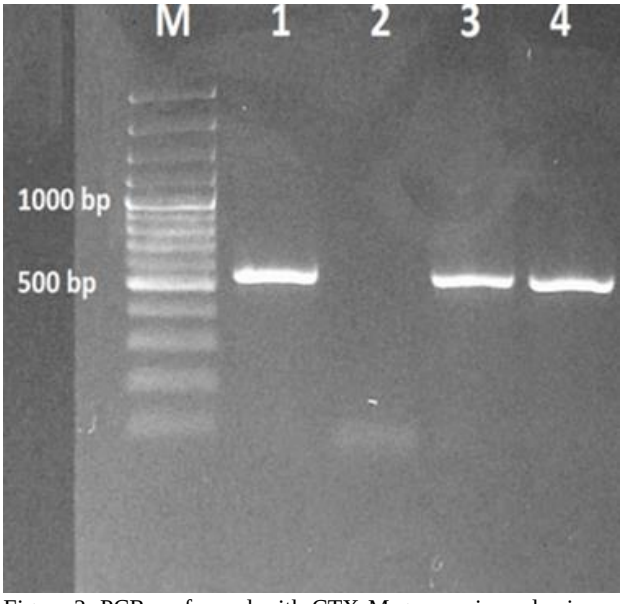

Figure 2. PCR performed with CTX-M gene universal primers (543 bp). M: Marker (100 bp), 1: Positive control (*E. coli* NCTC 13461), 2: Negative kontrol (PCR mixture without template DNA), 3 ve 4: CTX-M gene positive *E. coli* isolated from laying hens (isolates: F73 and F92).

Şekil 2. CTX-M geni universal primerleri (543 bp) ile yapılan PZR. M: Marker (100 bp), 1: Pozitif kontrol (*E. coli* NCTC 13461), 2: Negatif kontrol (kalıp DNAsız PZR karışımı), 3 ve 4: Yumurta tavuklarından izole edilen CTX-M geni taşıyan *E. coli* (izolatlar: F73 ve F92).

Table 1. Distribution of ESBL-producing *E. coli* isolates (n= 12) according to phylogenetic group and antibiotic resistance profiles.
Tablo 1. GSBL üreten *E. coli* izolatlarının (n=12) filogenetik grup ve antibiyotik direnç profillerine göre dağılımı.

Herd	Fecal sample (n)	*E. coli* isolated (n) (%)	Phylogenetic type	ESBL type	Antibiotic susceptibility profile
B	50	8 (16.0)	B1 (n= 2)	CTX-M-3	- (n= 2)
			D2 (n= 6)	CTX-M-15	TET, NAL (n= 5) TET (n= 1)
C	100	4 (4.0)	B1 (n= 3)	CTX-M-15	KAN, NAL, CIP, ENR (n= 3)
			D1 (n= 1)	CTX-M-15	[a]STR, SXT, NAL, TET (n= 1)

n: Number, [a]: Multidrug-resistant *E. coli* isolate.
n: Sayı, [a]: Çoklu antibiyotik direnci gösteren *E. coli* izolatı.

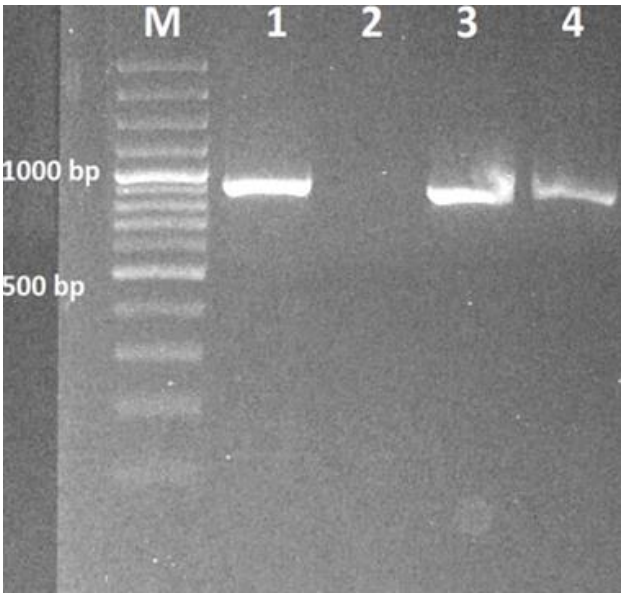

Figure 3. PCR performed with CTX-M gene group 1 primers (891 bp). M: Marker (100 bp), 1: Positive control (*E. coli* NCTC 13461), 2: Negative control (PCR mixture without template DNA), 3 ve 4: CTX-M gene (group 1) positive *E. coli* isolated from laying hens (isolates: F73 and F92).
Şekil 3. CTX-M geni grup 1 primerleri (891 bp) ile yapılan PZR. M: Marker (100 bp), 1: Pozitif kontrol (*E. coli* NCTC 13461), 2: Negatif kontrol (kalıp DNAsız PZR karışımı), 3 ve 4: Yumurta tavuklarından izole edilen CTX-M geni (grup 1) taşıyan *E. coli* (izolatlar: F73 ve F92).

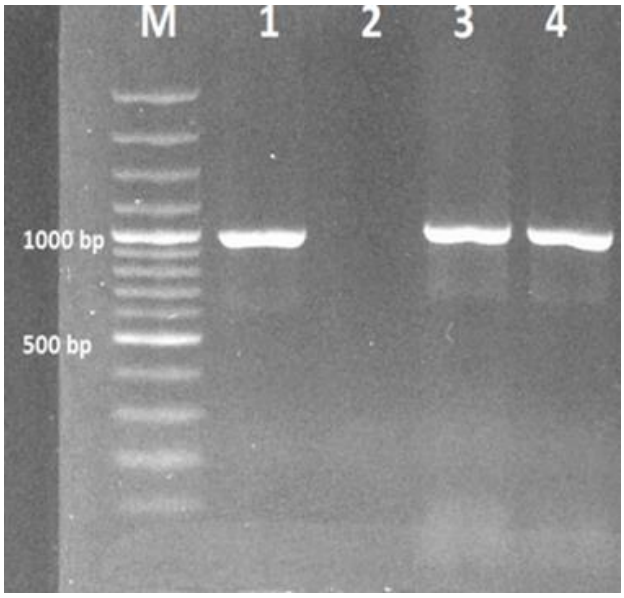

Figure 4. PCR performed with TEM gene specific primers (966 bp). M: Marker (100 bp), 1: Positive control (*E. coli* ATCC 35218), 2: Negative control (PCR mixture without template DNA), 3 ve 4: TEM gene positive *E. coli* isolated from laying hens (isolates: F151 and F196).
Şekil 4. TEM geni için spesifik primerlerle (966 bp) yapılan PZR. M: Marker (100 bp), 1: Pozitif kontrol (*E. coli* ATCC 35218), 2: Negatif kontrol (kalıp DNAsız PZR karışımı), 3 ve 4: Yumurta tavuklarından izole edilen TEM geni taşıyan *E. coli* (izolatlar: F151 ve F196).

DNA sequencing of $bla_{\text{CTX-M}}$ genes detected in PCR screening indicated CTX-M-15 as predominant ESBL (10/12, 83.3%) and CTX-M-3 as less frequent type (2/12, 16.7%). Additionally, DNA sequencing of 2 bla_{TEM} gene PCR products showed that they are TEM-1 beta-lactamase which is not an ESBL (Table 1). Assigned accession numbers for $bla_{\text{CTX-M-3}}$ (*E. coli* isolate: F73) and $bla_{\text{CTX-M-15}}$ gene (*E. coli* isolate: F92) nucleotid sequence data submitted in Genbank are KP303591 and KX274458, respectively.

According to phylogenetic typing of *E. coli* isolates, isolates from farm B belonged to B1 (n= 2) and D_2 (n= 6) phylogenetic groups and the isolates from farm C belonged to D_1 (n= 1) and B1 (n= 3) phylogenetic groups (Table 1).

Discussion and Conclusion

Avian *E. coli* isolates which are ESBL producers have been reported in poultry in various parts of the world (4, 16, 17, 25). However, to date, majority of the studies focused on broiler chickens and large-scale studies on the presence and extent of ESBL-producing *E. coli* isolates in laying hens are limited. Bortolaia et al. (4) reported *E. coli* isolates producing CTX-M group 1 (CTX-M-1/61), group 2 (CTX-M-2/20/44) and group 9 (CTX-M-14/17) beta-lactamases in layer flocks in Denmark. Wasyl et al. (25) isolated 3 CTX-M-1 and CTX-M-1+TEM-1b-producing *E. coli* from the laying chicken flocks in Poland. In the present study, isolation rate of ESBL-producing *E. coli* was determined as 6.0% in laying hens in Burdur province of Turkey and similarly CTX-M group-1 (CTX-M-3 and CTX-M-15 types) ESBLs were determined. Therefore,

our study is the first report showing the existence of CTX-M-3 and CTX-M-15 type ESBL-producing *E. coli* in laying hen farms from Turkey.

Plasmids bearing genes for ESBL production commonly carry other resistance determinants, such asaminoglycosides, phenicols, quinolones, sulfamethoxazole-trimethoprim and tetracycline, resulting in co-resistance to these antibiotics (18, 19). Similarly, in the present study we determined co-resistance to antibiotics mentioned above except phenicols (CHL and FFC), but the number of multidrug-resistant isolates were very low.

The increase in the prevalence of ESBL-producing *E. coli* is attributed to clonal spread of ESBL-producing strains and/or horizontal transfer of plasmids carrying ESBL genes (18). Studies showed that there was a dramatical increase in detection rates of CTX-M type ESBLs during the last decade globally (19). Similarly, $bla_{\text{CTX-M}}$ gene was the only ESBL type detected in *E. coli* isolates in the present study; SHV and TEM type ESBLs were not detected. Hence, the present study also supportsthe increasing trend of the emergence and dissemination of CTX-M type ESBLs in animal production globally.

In the present study, the predominant ESBL was CTX-M group 1 (CTX-M-3 and -15) which is similar to the reports of human isolates in Turkey (2, 12). It is known that CTX-M (particularly CTX-M-15)-producing *E. coli* isolates cause both nosocomial and community-onset infections, such as bacteriemia, gastroenteritis and urinary tract infections, in human (19). Therefore, existence of CTX-M-15 type ESBL-producing *E. coli* isolates indicates the risk for people working in these farms and consuming egg from these farms. Additionaly, the data obtained in the present study show that there is similarity between human and poultry in the epidemiology of ESBL genes.

Phylogenetic analysis of *E. coli* isolates indicates that most of the commensal *E. coli* isolates belong to groups A and B1 and, virulent extraintestinal isolates belong to group B2 and lesser extent to group D (5). Phylogenetic analysis of all *E. coli* isolates in the present study showed that group D (D_1 and D_2) was the predominant group and was followed by group B1; none of the isolates belonged to group B2. Although none of the isolates in the present study were from B2 phylogenetic group, the isolates from group D should be considered as a potential risk for severe infections both in animals and human.

Additionally, the results of phylogenetic analysis indicate that few parent *E. coli* isolates carrying ESBL genes exist in the laying hen farms in Burdur province of Turkey. Therefore, prevention of dissemination of the low number parent *E. coli* isolates will be critical to keep the prevelance of ESBL-producing *E. coli* in low levels in the laying hen production in the region.

Consequently, we report the emergence of CTX-M-3 and -15 type ESBL-producing *E. coli* isolates from 4 different phylogenetic groups in healthy laying hens in Burdur province of Turkey. Even if there is a low level of isolation in layers at present, it should be our major concern because of the possibility of transfer of ESBL genes between animal species as well as to humans by direct contact or by food chain. Thus, further researches including humans, food chain and environments of animals should be conducted to obtain information which will be helpful in order to develop control strategies in the field. Furthermore, investigations to reveal the relationship between antibiotic use and development of ESBL-producing *E. coli* isolates in poultry productions should be conducted.

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