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Existence of Plasmidic AmpC Beta-Lactamase-Producing *Escherichia coli* Isolates in Healthy Laying Hens

Faruk PEHLIVANOGLU

[1] *Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology, Burdur, Turkey*

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SUMMARY

In the present study, determination of prevalence of plasmidic AmpC (pAmpC) beta-lactamase-producing *Escherichia coli* in intestinal flora of laying hens in Burdur province of Turkey and characterization of pAmpC beta-lactamase-producing *E. coli* isolates were aimed. Two hundreds twenty five fecal samples from all laying hen farms (n=4) in Burdur province were collected and cultured in Brilliance *E. coli*/coliform selective agar supplemented with cefotaxime or ceftazidime. Presumptive AmpC beta-lactamase-producing *E. coli* isolates were determined by a phenotypic test and the isolates were screened by PCR for bla_{pAmpC} genes. Susceptibilities of the *E. coli* isolates to beta-lactams and other classes of antibiotics were investigated by agar disc diffusion test and finally phylogenetic analysis of the *E. coli* isolates was performed by multiplex PCR. pAmpC beta-lactamase-producing *E. coli* was isolated from 15 (6.7%) laying hen fecal samples. bla_{CITM} family gene was found in all *E. coli* isolates. The pAmpC beta-lactamase-producing isolates showed co-resistance to several classes of antibiotics (aminoglycosides, quinolones, sulfamethoxazole-trimethoprim and tetracycline). According to phylogenetic analysis, the *E. coli* isolates belonged to A_1, B1 and D_1 groups. Consequently, by the present study the first isolation of pAmpC beta-lactamase-producing *E. coli* isolates with multidrug-resistance phenotype on lying hen production from Turkey was reported.

Key Words: *Chicken, Escherichia coli, Multiple antibacterial drug resistance, AmpC beta-lactamases*

ÖZET

Sağlıklı Yumurta Tavuklarında Plasmid Kökenli AmpC Beta Laktamaz üreten *Escherichia coli* Varlığı

Bu çalışmada, Burdur ilinde yetiştirilen yumurtacı tavukların bağırsak mikroflorasında plazmid kökenli AmpC (pAmpC) beta laktamaz üreten *Escherichia coli* izolatlarının prevalansını belirlemek ve belirlenen pAmpC beta laktamaz üreten *E. coli* izolatlarının karakterizasyonunun yapılması amaçlandı. Burdur ilindeki tüm yumurtacı tavuk çiftliklerinden (n=4) 225 dışkı örneği toplandı ve içerisine sefotaksim veya seftazidim ilave edilerek hazırlanmış *E. coli*/koliform selektif agarda dışkı örneklerinin kültürleri yapıldı. AmpC beta laktamaz üreten *E. coli* izolatlarının ön tanısı fenotipik bir test ile yapıldı ve izolatlar bla_{pAmpC} beta laktamaz genleri için PZR ile tarandı. *E. coli* izolatlarının beta laktam ve diğer sınıflardan antibiyotiklere olan duyarlılıkları agar disk difüzyon testi ile araştırıldı ve son olarak *E. coli* izolatlarının filogenetik analizi multipleks PZR ile gerçekleştirildi. pAmpC beta laktamaz üreten *E. coli* 15 adet (%6.7) dışkı örneğinden izole edildi. CIT familyasına ait bla_{pAmpC} geni (bla_{CIT}) tüm *E. coli* izolatlarında bulundu. pAmpC beta laktamaz üreten izolatlar çeşitli sınıflardan antibiyotiklere (aminoglikozidler, kinolonlar, sulfametoksazol-trimethoprim ve tetrasiklinler) karşı dirençli bulundu. Filogenetik analiz sonuçlarına göre *E. coli* izolatları A_1, B1 ve D_1 gruplarına ait oldukları belirlendi. Sonuç olarak, Türkiye'de yumurtacı tavuk üretiminde pAmpC beta laktamaz üreten ve çoklu antibiyotik direnci gösteren *E. coli* izolatlarının ilk kez bu çalışmayla ortaya konulduğu görüldü.

Anahtar Kelimeler: *Tavuk, Escherichia coli, Çoklu antibakteryel ilaç dirençliliği, AmpC beta laktamazlar*

INTRODUCTION

Beta-lactams are among the most effective antibiotics used for treatment of bacterial infections in human and animals but high resistance rates are often observed in both commensal and pathogen bacteria. Bacterial resistance to beta-lactams occurs mostly by the production of beta-lactamase enzymes which inactivate the antibiotic by hydrolysing the beta-lactam ring of the antibiotic (Frere 1995). A group of beta-lactamases called AmpC beta-lactamases can confer resistance to penicillins, cephalosporins including oxyimino-cephalosporins (e.g.,

Corresponding author: Faruk PEHLIVANOGLU
Mehmet Akif Ersoy Univ., Veteriner Fak., Mikrobiyoloji AD, Burdur, Turkey e-mail: pehlivanoglu@mehmetakif.edu.tr

cefotaxime, ceftazidime and ceftriaxone), cephamycins (e.g., cefoxitin and cefotetan), and aztreonam (variably) (Jacoby 2009). AmpC beta-lactamases can be inhibited by cloxacillin and 3-aminophenylboronic acid, but it's activity is not affected by the clavulanic acid. In Gram-negative bacteria, they can be plasmid or chromosome mediated (Jacoby 2009). Plasmid mediated AmpC (pAmpC) beta-lactamases have arisen through the transfer of chromosomal genes and difference of pAmpCs from chromosomal AmpCs is being uninducible (Thompson 2001). pAmpC beta-lactamases are divided into 6 families called ACC (Ambler class C), CIT (origin, *Citrobacter freundii*), DHA (site of discovery, Dhahran hospital in Saudi Arabia), EBC (origin, *Enterobacter claocae*), FOX (resistance to cefoxitin) and MOX (resistance to moxalactam) according to differences in aminoacid sequences (Perez-Perez and Hanson 2002; Jacoby 2009). There is no current guideline recommended by Clinical and Laboratory Standards Institute (CLSI) and British Society of Antimicrobial Chemotherapy (BSAC) for the detection of AmpC beta-lactamases.

There are several investigations from various parts of the world to reveal the existence and extend of AmpC beta-lactamase-producing *E. coli* in poultry productions (Wasyl et al. 2012; Kameyama et al. 2013; Hille et al. 2014; Maamar et al. 2016). In Turkey, there are only two studies conducted in chicken farms but those studies covered only broiler chicken farms (Unal et al. 2014; Basaran Kahraman et al. 2016). On the other hand, a study from Laube et al. (2013) showed that carriage rate for AmpC beta-lactamase-producing *E. coli* increased with age of broilers. Since laying hens have longer life span than broilers, prevalence of AmpC beta-lactamase-producing *E. coli* can be higher in laying hen production than broilers. Thus, the present study was performed to show the prevalence of pAmpC beta-lactamase-producing *E. coli* isolates in healthy laying hens in Burdur province of Turkey and to further characterize the *E. coli* isolates.

MATERIALS and METHODS

Sampling

In the present study 4 laying hen farms, which constitute all chicken farms in Burdur province, were sampled. All fecal samples (n= 225) were collected from cages by using sterile swabs provided that only one fecal sample from one cage. Fifty samples were collected from each of farm A and B, 100 samples were collected from farm C, and 25 samples were collected from farm D. The fecal samples were put into sterile screw-top vials, transported to the laboratory on ice within 2 h and kept at 4 °C until processing within 24 h.

Selective isolation

Firstly, an enrichment protocol was performed to fecal samples before plating onto selective agar. Briefly, a 10% suspension of each fecal sample in buffered peptone water (Lab M, UK) was prepared and incubated at 37°C for 24 h under aerobic conditions. Fifty microliters from each suspension was 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 (one colony from the selective agar with CTX and one colony from the selective agar with CAZ) per positive fecal sample was selected randomly and *E. coli* identification was performed according to following identification tests: Gram staining, acid and gas from glucose, catalase test, citrate utilization, hydrogen sulphide production, indole production, methyl red-voges proskauer test, orthonitrophenyl-beta-D-galactopyranoside activity, oxidase test and urease production (Winn et al. 2006). Finally, genetic confirmation of *E. coli* was performed by PCR with a primer pair spesific to 16S rRNA gene (Wang et al. 2002) after extracting DNA from each *E. coli* isolate by using a commercial DNA purification kit (Thermo Fisher Scientific Inc.)(Table 1).

Presumptive determination of AmpC beta-lactamase-producing E. coli isolates

The AmpC beta-lactamase-producing *E. coli* isolates were determined by agar disc diffusion test on Mueller Hinton Agar (MHA) (Oxoid, UK) by using cefoxitin (FOX, 30 µg) disc (Oxoid, UK). According to CLSI zone diameters for *Enterobactericeae,* zone diameter equal and lower than 14 mm were accepted for evidence of FOX resistance (CLSI 2014).

Polymerase chain reaction and sequencing

PCR detection of bla_{pAmpC} genes in cefoxitin resistant *E. coli* isolates was performed by using the protocol described by Perez-Perez and Hanson (2002) with slight modification in protocol. Information about the primers were presented in Table 1. Two sets of triplex PCR (1: bla_{ACC}, bla_{CIT}, bla_{FOX} and 2: bla_{DHA}, bla_{EBC}, bla_{MOX}) were established for detection of bla_{pAmpC} gene families. *Taq* DNA polymerase enzyme, deoxyribonucleotide triphosphates and buffers used in PCR mixture were provided by Thermo Fisher Scientific Inc. Cycling conditions for both of triplex PCRs were 5 min at 94 ºC for initial denaturation, followed by 35 cycles of 45 sec at 94 ºC, 45 sec at 64 ºC and 1 min at 72 ºC, and a final elongation step of 7 min at 72 ºC.

Antibiotic susceptibility testing

Susceptibility of AmpC beta-lactamse-producing *E. coli* isolates to beta-lactam antibiotics and to other classes of antibiotics were investigated by the agar disc diffusion test on MHA (Oxoid, UK) according to the CLSI protocols (CLSI 2014). The beta-lactams antibiotic discs (Oxoid, UK) tested were 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). The antibiotics (Oxoid, UK) from other classes tested were 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).

The isolates were classified as resistant, intermediate or susceptible. Evaluation of the zone diameters was performed according to following criteria: CLSI zone diameter standards for *Enterobacteriaceae* (CLSI 2014) were used for AMP, ATM, CAZ, CEF, CHL, CIP, CPD, CRO, CTX, CXM, IPM, NAL and STR, CLSI document VET01-S2 (CLSI 2013) for FFC, GEN and SXT and, CLSI document M31-A3 (CLSI 2010) for ENR, KAN and TET.

The *E. coli* isolates of a single fecal sample cultured on two selective media (supplemented with CTX or CAZ) and with the same antibiotic susceptibility phenotype were accepted as the same isolate. In the present study, an *E. coli* isolate resistant to more than 3 classes of antibiotics excluding beta-lactams is accepted as multidrug-resistant.

Phylogenetic analysis

A triplex PCR protocol developed by Clermont et al. (2000) and modified by Higgins et al. (2007) was used for the phylogenetic analysis of the *E. coli* isolates. This method is based on presence and absence of *chuA* and *yjaA* genes, and DNA fragment TspE4.C2 of *E. coli*. Information about the primers were presented in Table 1. The phylogenetic groups of the *E. coli* isolates were assigned according to following criteria: the phylogenetic group A (*chuA*-, TspE4.C2-), B1 (*chuA*-, TspE4.C2+), B2 (*chuA*+, *yjaA*+), or D (*chuA*+, *yjaA*-). Additionally, phylogenetic subgroups (A: A_0 and A_1; B2: $B2_2$ and $B2_3$; D: D_1 and D_2) were investigated as described by Escobar-Páramo et al. (2006). *E. coli* ATCC 25922 was used as positive control strain (*chuA*+, *yjaA*+ and TspE4.C2+) in the triplex PCR.

Table 1. Primers used in the present study

Target	Primer	Product size (bp)
bla$_{MOX}$	5'-GCTGCTCAAGGAGCACAGGAT-3' 5'-CACATTGACATAGGTGTGGTGC-3'	520
bla$_{CIT}$	5'-TGGCCAGAACTGACAGGCAAA-3' 5'-TTTCTCCTGAACGTGGCTGGC-3'	462
bla$_{DHA}$	5'-AACTTTCACAGGTGTGCTGGGT-3' 5'-CCGTACGCATACTGGCTTTGC-3'	405
bla$_{ACC}$	5'-AACAGCCTCAGCAGCCGGTTA-3' 5'-TTCGCCGCAATCATCCCTAGC-3'	346
bla$_{EBC}$	5'-TCGGTAAAGCCGATGTTGCGG-3' 5'-CTTCCACTGCGGCTGCCAGTT-3'	302
bla$_{FOX}$	5'-AACATGGGGTATCAGGGAGATG-3' 5'-CAAAGCGCGTAACCGGATTGG-3'	190
chuA	5'-GACGAACCAACGGTCAGGAT-3' 5'-TGCCGCCAGTACCAAAGACA-3'	279
YjaA	5'-TGAAGTGTCAGGAGACGCTG-3' 5'-ATGGAGAATGCGTTCCTCAAC-3'	211
TspE4.C2	5'-GAGTAATGTCGGGGCATTCA-3' 5'-CGCGCCAACAAAGTATTACG-3'	152
16S rRNA	5'-CCCCCTGGACGAAGACTGAC-3' 5'-ACCGCTGGCAACAAAGGATA-3'	401

RESULTS

In the selective isolation, presumptive *E. coli* colonies were observed on both types of selective media in 19 of 200 fecal samples. The number of isolates observed on only medium containing CAZ was 7 but with only medium containing CTX, there was only 1 isolate. According to identification tests and PCR confirmation, all isolates were identified as *E. coli*. Eight *E. coli* isolates from farm A and 7 *E. coli* isolates from farm B were determined as presumptive AmpC beta-lactamase producers by agar disc diffusion test with FOX. No presumptive AmpC beta-lactamase-producing *E. coli* was isolated from the animals in farm C and D.

PCR screening of the presumptive AmpC beta-lactamase-producing *E. coli* isolates for the *bla*$_{pAmpC}$ gene families (*bla*$_{ACCM}$, *bla*$_{CIT}$, *bla*$_{DHA}$, *bla*$_{EBC}$, *bla*$_{FOX}$ and *bla*$_{MOX}$) showed that all of them (n=15) harbored *bla*$_{CIT}$ family genes and 7 isolates which were from the farm A were additionally harbored *bla*$_{MOX}$ family genes. Therefore, farm-level prevalence was found to be 50% (2/4) and individual animal prevalence was found to be 6.7% (15 /225) for the pAmpC beta-lactamase-producing *E. coli* in Burdur province.

Table 2. Antibiotic susceptibilities of pAmpC beta-lactamase-producing *E. coli* isolates

Beta lactams	pAmpC producing *E. coli* (n=15)	
	R (n)	I (n)
AMP	15	0
ATM	1	7
FEP	0	0
CTX	15	0
FOX	15	0
CPD	15	0
CAZ	15	0
CRO	15	0
CXM	5	10
CEF	15	0
IPM	0	0
Other antibiotics		
GEN	0	0
KAN	7	0
STR	7	6
CIP	1	8
ENR	5	10
NAL	9	5
TET	14	0
SXT	13	0
FFC	0	3
CHL	0	0

Table 3. Distribution of pAmpC beta-lactamase-producing *E. coli* isolates (n=15) according to phylogenetic groups, *bla*$_{pAmpC}$ genes and antibiotic resistance profiles

Farm	Phylogenetic group	pAmpC family	n	Antibiotic susceptibility profile
A	A_1 (n= 1)	CIT	1	NAL, ENR, TET
	A_1 (n= 7)	CIT + MOX	4	STR, KAN, SXT, NAL, TET *
			3	STR, KAN, SXT, NAL, ENR, TET*
B	B1 (n= 6)	CIT	5	SXT, TET
			1	SXT, NAL, ENR, TET
	D_1 (n= 1)	CIT	1	NAL, ENR, CIP

*Multidrug-resistant isolates

In the antibiotic susceptibility testing against beta-lactams, all of the pAmpC beta-lactamase-producing *E. coli* isolates (15/15, 100%) were found resistant to AMP, CTX, CPD, CAZ, CRO and CEF and susceptible to FEP and IPM. For aztreonam, 7 isolates (7/15, 46.7%) were susceptible and one isolate (1/15, 6.7%) was resistant (Table 2). The antibiotic susceptibility testing against other classes of antibiotics, the highest resistance was detected against TET (14/15, 93.3%). All AmpC beta-lactamase-producing *E. coli* isolates (15/15, 100%) were found to be susceptible to CHL and GEN. Resistance ratios of the AmpC beta-lactamase-producing *E. coli* isolates to KAN, STR, CIP, ENR, NAL and SXT were 46.7%, 46.7%, 6.7%, 33.3%, 60.0% and 86.7%, respectively (Table 2). In the present study, 7 of 15 (46.7%) pAmpC beta-lactamase-producing *E. coli* isolates were determined as multidrug-resistant (Table 3). On the other hand, farm A had 8 isolates with 3 different antibiotic susceptibility profiles and farm B had 7 isolates with 3 different antibiotic susceptibility profiles (Table 3).

According to phylogenetic analysis, 8 (8/15, 53.3%) *E. coli* isolates belonged to group A (subgroup A_1), 6 (6/15, 40%) isolates to group B1 and the remaining 1 (1/15, 6.7%) isolate to group D (subgroup D_1). Distribution of phylogenetic groups of the pAmpC beta-lactamase-producing *E. coli* isolates according to farm is presented in Table 3.

DISCUSSION

Avian pathogenic and commensal *E. coli* isolates which are AmpC beta-lactamase producer have been reported in poultry in various parts of the world even though majority of the studies focused on only broiler chickens (Wasyl et al. 2012; Kameyama et al. 2013; Hille et al. 2014; Maamar et al. 2016). In Turkey, no information exists on prevalence of AmpC beta-lactamse-producing *E. coli* in healthy laying hens. Thus, our study is the first report showing the existence and extend of pAmpC beta-lactamase (CIT and MOX family)-producing *E. coli* in laying hen farms from Turkey. In the present study, the herd-level and individual animal prevalence of pAmpC beta-lactamase-producing *E. coli* were determined as 50% and 6.7%, respectively. These ratios indicate the emerging problem for animals in Burdur province of Turkey.

It is known that the plasmids carrying bla_{AmpC} genes often posses the resistance genes for aminoglycosides, phenicols, quinolones, sulfamethoxazole-trimethoprim and tetracycline (Thompson 2001; Jacoby 2009). Therefore, AmpC beta-lactamase-producing *E. coli* isolates are frequently multidrug-resistant. Likewise, we determined resistance to the aminoglycosides, quinolones, sulfamethoxazole-trimethoprim and tetracycline in pAmpC beta-lactamase-producing *E. coli* isolates. In total, 46.7% (7/15) of pAmpC beta-lactamase-producing *E. coli* isolates showed multidrug-resistance. These multidrug resistant isolates are probably the same strain because all of them were isolated from farm A, and they belonged to the same phylogenetic group (A_1) and possessed the same bla_{pAmpC} genes (bla_{CIT} and bla_{MOX})(Table 2).

Phylogenetic analysis of all *E. coli* isolates in the present study (n= 15) showed that group A (subgroup A_1) is the predominant group (8/15), followed by group B1 (6/15) and group D (subgroup D_1) (1/15); none of the isolates belonged to group B2. 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 (Clermont et al. 2000). On the other hand, Campos et al. (2008) showed that commensal *E. coli* strains of poultry origin from A and B1 phylogenetic groups carried various virulence genes, such as *sit*A (the protein involved in the iron transport), *irp*-2 (iron repressible protein), *fyu*A (ferric yersiniabactin), *iuc*A (aerobactin synthetase) and *iha* and $lpfA_{\text{OI157/O154}}$ (adhesion related genes described for STEC and EHEC). Therefore, multidrug resistant pAmpC beta-lactamase-producing *E. coli* isolates from phylogenetic groups A and B1 in the present study should not be ignored.

From the phylogenetic analysis, we can state that low number parent *E. coli* isolates carrying bla_{pAmpC} genes present in the laying hen farms in Burdur province of Turkey. In farm A the isolates (n=8) were from only one phylogenetic group (A_1) and they carried only bla_{CIT} or both bla_{CIT} and bla_{MOX} genes. In farm B, the isolates (n=7) were from only two phylogenetic groups (B1 or D_1) and all of them carried the same bla_{pAmpC} gene (bla_{CITM}).

AmpC beta-lactamase-producing Gram negative bacteria are responsible for various infections in human, such as meningitis, urinary tract infection and nosocomial infections (Jacoby 2009). It has been suggested that food producing animals can be a potential reservoir for AmpC-beta-lactamase-producing *E. coli* isolates and these isolates can be transmitted to humans via the food chain or by direct contact. Voets et al. (2013) showed that AmpC beta-lactamase-producing *E. coli* isolates from poultry meat and human clinical cases carry the same bla_{AmpC} gene on the same plasmid. Therefore, continuous surveillance of both pathogenic and commensal *E. coli*, which are AmpC beta-lactamase producer, in poultry production in Burdur region will be essential for the timely detection and prevention of dissemination of such isolates to humans.

CONCLUSION

In conclusion, the present study is the first report revealing the existence of AmpC beta-lactamase-producing *E. coli* isolates in healthy laying hens in Turkey. This study indicates that control programs are necessary to prevent the transfer of multidrug resistant pAmpC beta-lactamase-producing *E. coli* isolates to other animal species and to humans by direct contact or by food chain, even if low number parent pAmpC beta-lactamase-producing *E. coli* isolates exist in the farms in Burdur province of Turkey. Furthermore, additional studies should be conducted in laying hen flocks in different locations of Turkey to better understand the epidemiology of AmpC beta-lactamase-producing *E. coli* isolates in laying hen production in Turkey.

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