



Prevalence and molecular characterization of extended-spectrum β -lactamase (ESBL) and plasmidic AmpC β -lactamase (pAmpC) producing *Escherichia coli* in dogs

Özkan ASLANTAŞ^{1)*} and Ebru Şebnem YILMAZ²⁾

¹⁾Department of Microbiology, Faculty of Veterinary Medicine, Mustafa Kemal University, TR-31030 Hatay, Turkey

²⁾Department of Biology, Faculty of Art and Science, Mustafa Kemal University, TR-31030 Hatay, Turkey

ABSTRACT. This study aimed to determine the prevalence of fecal carriage of extended spectrum β -lactamase (ESBL) and/or plasmidic AmpC β -lactamase (pAmpC) producing *Escherichia coli* among dogs (n=428) in Turkey. Polymerase chain reaction (PCR) and sequencing were used to characterize genes encoding β -lactamase and plasmid mediated quinolone resistance (PMQR). Antimicrobial susceptibility testing and PCRs for virulence genes and phylogenetic groups were also performed. Cefotaxime resistant *E. coli* isolates were detected in 95 (22.2%) of the swab samples. Sequencing analysis results showed occurrence of various β -lactamase genes: *bla*_{CTX-M-15} (62), *bla*_{TEM-1b} (42), *bla*_{CMY-2} (22), *bla*_{CTX-M-3} (16), *bla*_{CTX-M-1} (15), *bla*_{OXA-1} (9) and *bla*_{SHV-12} (3) alone or in combination. The most frequently encountered phylogenetic group was group A₁ (35.8%), followed by group D₂ (22.1%), B₁ (15.8%), D₁ (9.5%), A₀ (7.4%), B₂ (5.3%) and B₃ (4.2%), respectively. PMQR genes, *aac(6)-Ib-cr*, *qnrS1* and *qnrB10* were detected in 25.3, 10.5 and 1.1% of the isolates, respectively. While all isolates were susceptible to imipenem and amikacin, resistance rates to non- β -lactam antibiotics ranged from 20.0% for tobramycin to 56.8% for tetracycline. The virulence genes were only detected in 34 (36.2%) of the isolates and this isolates carried single or various combination of virulence genes of *iucD*, *papC*, *papE*, *f17a-A* and *eaeA*. Four isolates were identified as human virulent pandemic CTX-M-15 producing *E. coli* clone O25b:ST131/B2. To the best of our knowledge, this is the first study to show fecal carriage of ESBL/pAmpC type β -lactamase producing *E. coli* isolates among dogs in Turkey.

KEY WORDS: dog, *Escherichia coli*, ESBL, molecular characterization, pAmpC

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The extended spectrum cephalosporins (ESCs) are critically important antimicrobials for the treatment of bacterial infections in both human and veterinary medicine [50]. Increased use of these drugs led emergence of ESC resistant Gram negative bacteria in humans and animals [47]. Main resistance mechanisms to ESCs are frequently related to plasmid mediated production of enzymes, extended spectrum β -lactamases (ESBLs) and AmpC β -lactamases (pAmpC) [25, 36]. Carriage of ESBL and AmpC genes on plasmids enhances transfer of these genes to other bacteria by conjugation. Furthermore, these plasmids carry genes conferring resistance to various classes of antimicrobials, such as fluoroquinolones, aminoglycosides, sulphonamides, trimethoprim. This makes treatment options for infections caused by ESBL and/or AmpC producing bacteria very limited [39].

CTX-M type ESBLs was first detected in a laboratory dog in Japan in 1986 [33]. This type of ESBLs showed a significant international dissemination since 2000, becoming the most prevalent type of ESBL throughout the world and, outnumbering other ESBL types such as TEM and SHV. Currently, there are different 172 CTX-M β -lactamases types (<http://www.lahey.org/studies/>), clustered into five phylogroups (CTX-M-1, -2, -8, -9 and -25). CTX-M-1 phylogroup, which includes CTX-M-15, is the most widely distributed all over the world [8].

The genes encoding AmpC type β -lactamases are chromosomal- or plasmid-mediated and most pAmpC genes are derived from the chromosomal *ampC* genes of several members of the family *Enterobacteriaceae* [38]. CMY family is the most prevalent pAmpC β -lactamase [25] and 136 different types of this family have been described to date (<http://www.lahey.org/studies/>). Among the CMY family, CMY-2 type is most frequently encountered around the world. In Turkey, CMY-2 producing *E. coli* has been reported in humans, cattle and retail meats [3, 37, 52].

*Correspondence to: Aslantaş, Ö., Department of Microbiology, Faculty of Veterinary Medicine, Mustafa Kemal University, 31030, Hatay, Turkey.
e-mail: ozkanaasantas@yahoo.com

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Due to their close contact with humans, companion animals may play a role as a reservoir of transmitting antimicrobial resistant bacteria [18]. Recently, Ljungquist *et al.* [32] showed household transfer of ESBL-/pAmpC producing *E. coli* between humans and dogs in Sweden. Another worrying development is the recent emergence of highly virulent human pandemic B2-O25b-ST131 clone of CTX-M-15 producing *E. coli* strains from companion animals of different species in various European countries [17, 40, 46]. This clone, isolated in 2008, was implicated in human urinary tract and bloodstream infections, and proved difficult to treat due to extensive multidrug resistance [42]. The risk associated with the transfer of these multiresistant bacteria from humans to companion animals and vice versa is a great concern for both canine welfare and public health.

Recently, presence of ESBLs/pAmpC β -lactamase producing fecal *E. coli* isolates from cattle and chicken has been reported in Turkey [3, 4]. However, data on ESBL/pAmpC β -lactamase producing *E. coli* in dogs is not available. Therefore, the objectives of this study is to determine the prevalence of ESBL and/or pAmpC producing *E. coli* from dogs in three cities and to characterize positive isolates with molecular methods.

MATERIALS AND METHODS

Sample collection, isolation and identification of E. coli

Between January 2014 and June 2014, a total of 428 non-duplicate rectal swabs from companion dogs in Hatay (n=148), Mersin (n=142) and Adana (n=138) cities which are located in southern Turkey. Age of dogs was ranging from one month to 16 year (median age 3 years), including 47 breeds. Among the dogs sampled, 45.1% (n=193) were females and 54.9% (n=235) were males. Dogs had no clinical symptoms and admitted to veterinary clinics for routine physical examination, parasite screening or vaccination procedures.

Selective isolation of ESBL producing E. coli

The swabs were inoculated onto 2 μ g/ml cefotaxime containing Brilliance *E. coli*/Coliform Agar Selective Agar (Oxoid, U.K.) for selective isolation and incubated at 35°C for 24 hr. Screening concentration of cefotaxime indicating ESBL/pAmpC production was determined in accordance with Clinical Laboratories Standards Institute (CLSI) criteria [12]. One typical colony was selected and passaged onto Blood Agar to obtain pure cultures. Identification was done using classical methods (Gram stain, catalase, oxidase, indole, MR-VP, citrate and urease), and using species-specific PCR [49].

Antimicrobial susceptibility testing

Antimicrobial susceptibility of ESBL/pAmpC producing *E. coli* isolates tested by disk diffusion method and interpreted according to Clinical Laboratories Standards Institute (CLSI) criteria [12]. The antimicrobials tested were ampicillin (10 μ g), amoxicillin-clavulanic acid (10/20 μ g), nalidixic acid (30 μ g), ciprofloxacin (5 μ g), cefotaxime (10 μ g), cefpodoxime (10 μ g), cefepime (30 μ g), ceftazidime (30 μ g), ceftiofur (30 μ g), chloramphenicol (30 μ g), gentamicin (10 μ g), tobramycin (10 μ g), amikacin (10 μ g), kanamycin (30 μ g), tetracycline (30 μ g) and sulfamethoxazole-trimethoprim (1.25/23.75 μ g).

Production of ESBL phenotype was evaluated by disk combination method using ceftazidime, cefotaxime, cefpodoxime disks alone or in combination with clavulanic acid and double disk synergy test (DDST) using amoxicillin/clavulanic acid, cefotaxime and ceftazidime disks. Those cefotaxime-resistant *E. coli* isolates not only showing a negative ESBL-phenotype but also showing resistance to amoxicillin/clavulanic acid and ceftiofur were accepted as pAmpC phenotype. *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative controls, respectively. The isolates that were resistant to three or more antimicrobial from different classes were defined as multidrug resistant (MDR).

DNA extraction

DNA extraction was done using boiling method, as previously reported by Ahmed *et al* [1].

Determination of ESBL and PMQR genes

The ESBL genes *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{OXA} were investigated by PCR as described by Ahmed *et al.* [1]. The pAmpC genes were detected with the same method as described by Pérez-Pérez and Hanson [38]. Screening of PMQR genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib* and *qepA* genes were searched as suggested by Cavaco *et al.* [9], Kim *et al.* [28] and Park *et al.* [35]. All ESBL/pAmpC β -lactamase and PMQR positive amplicons were sequenced from both ends and sequencing results were compared to reported sequences available in GenBank.

Phylogenetic grouping

The phylogenetic analysis of the ESBL/pAmpC β -lactamase producing *E. coli* isolates were determined by the triplex PCR as previously reported by Clermont *et al.* [10]. Phylogenetic groups and subgroups (A₀, A₁, B₁, B₂, B₃, D₁ and D₂) were determined according to Escobar-Páramo *et al.* [16].

Determination of pandemic B2 O25b-ST131 clone

Pandemic B2 O25b-ST131 *E. coli* CTX-M-15 clone was investigated using allele specific PCR developed by Clermont *et al.* [11].

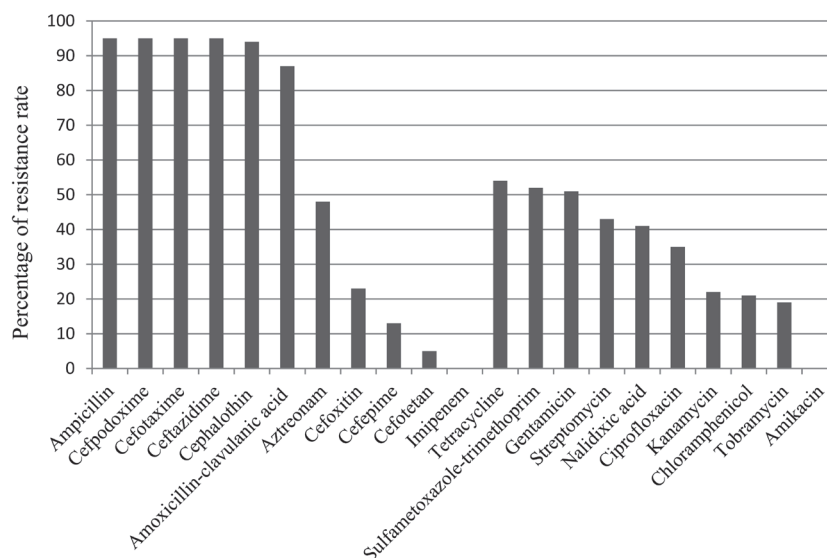


Fig. 1. Antimicrobial susceptibility of ESBL/pAmpC producing *E. coli* isolates.

Determination of virulence genes

The presence of 18 virulence genes [*iucD*, *hlyA*, *cnf1*, *papC*, *papE-F*, *sfa/focDE*, *fl17A*, *fl17a-A*, *fl17b-A*, *fl17c-A*, *17d-A*, *afa D-8*, *afa E-8*, *clpG*, *cnf2*, *stx1*, *stx2*, *eaeA*] were examined by PCR [6, 7, 27, 29, 49, 53].

Pulsed field gel electrophoresis (PFGE) analysis

Clonality of *E. coli* B2-O25-ST131 CTX-M-15 isolates was determined by pulsed-field gel electrophoresis (PFGE) technique with *XbaI* restriction of DNA in Public Health Institution of Turkey (Ankara) as described previously [15].

Statistical analysis

Differences in frequencies of ESBL/pAmpC producing *E. coli* isolates according to provinces, different age groups and genders were evaluated using Pearson's or likelihood ratio chi-square tests. IBM SPSS Statistics package Version 23 (IBM Corp., Armonk, NY, U.S.A.) was used for the statistical analysis. *P* value <0.05 was considered as statistically significant.

Ethics

The study was approved by the Animal Ethical Committee of Mustafa Kemal University (2012/08/15).

RESULTS

Isolation results

Cefotaxime resistant isolates were recovered in 95 (22%) of 428 dog. Out of 95 cefotaxime resistant *E. coli* isolates, 72 (75.8%) isolates exhibited ESBL phenotype, while the remaining 23 (24.2%) isolates showed a pAmpC phenotype, characterized by resistance to amoxicillin-clavulanic acid and cefoxitin. Prevalence of ESBL/pAmpC positive *E. coli* was 18.3% (26/142) in Mersin, 31.8% (47/148) in Hatay and 22% (22/138) in Adana. Significant differences between fecal carriage and provinces were observed (*P*=0.004). However, no significant differences between genders were observed (*P*=0.595). Furthermore, there were also no significant differences between different age groups (*P*=0.089).

Antimicrobial susceptibility

The results of antimicrobial susceptibility of ESBL/pAmpC producing *E. coli* isolates are given in Fig. 1. All isolates were resistant to ampicillin, cefotaxime, ceftazidime, cefpodoxime, but susceptible to imipenem. Resistance rates for the rest of other β -lactams were: 98.9, 91.6, 50.5, 24.2, 13.7 and 5.3% for cephalothin, amoxicillin-clavulanic acid, aztreonam, cefoxitin, cefepime and cefotetan, respectively. As for the non- β lactam antimicrobials, while all isolates were susceptible to amikacin, various rates of resistance were observed for tetracycline (56.8%), gentamicin (53.7%), sulfamethoxazole-trimethoprim (45.3%), streptomycin (45.3%), nalidixic acid (43.2%), ciprofloxacin (36.8%), kanamycin (22.2%), chloramphenicol (22.1%) and tobramycin (20%). The most of the isolates (67.4%) showed MDR phenotype. MDR to six, five, four and three antimicrobial classes were observed in 14 (%), 23 (%), 10 (%), 17 (%) isolates, respectively. Resistance to one and two classes of antimicrobials were detected in 17 and 14 isolates, respectively.

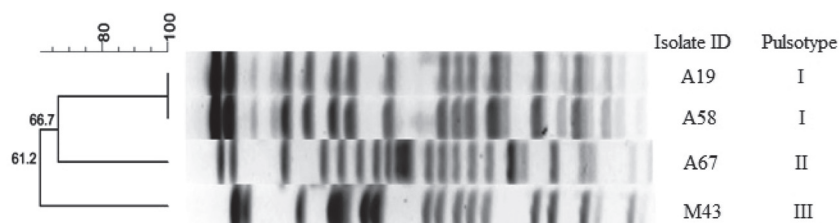


Fig. 2. Dendrogram showing the relationship of four B2-O25b-ST131 CTX-M-15 positive *E. coli* isolates based on *Xba*I-generated PFGE profiles.

Detection of ESBL/pAmpC β -lactamase genes

ESBL/pAmpC β -lactamase genes identified in *E. coli* isolates were: CTX-M-15 (n=46), CTX-M-1 (n=13), CTX-M-3 (n=11), CTX-M-15+CMY-2 (n=14), CTX-M-3+CMY-2 (n=5), CTX-M-1+CMY-2 (n=2), CTX-M-3+SHV-12 (n=1), CTX-M-15+SHV-12 (n=2) and CMY-2 (n=1).

Determination of PMQR genes

Among 35 ciprofloxacin resistant isolates, PMQR genes were found in 22 (62.9%) isolates including 17 *aac(6')-Ib-cr*, four *qnrS1*, one *aac(6')-Ib-cr + qnrS1*. In addition, PMQR genes were also detected in nine ciprofloxacin susceptible isolates, of which four had *aac(6')-Ib-cr*, four carried *qnrS1* and one harbored *qnrB10*.

Phylogenetic grouping

Most of ESBL/AmpC producing *E. coli* isolates belonged to phylogenetic group A₁ (34/95; 35.8%), followed by group D₂ (21/95; 22.1%), B₁ (15/95; 15.8%), D₁ (9/95; 9.5%), A₀ (7/95; 7.4%), B₂ (5/95; 5.3%) and B₃ (4/95; 4.2%), respectively.

Determination of pandemic O25:ST131 clone

Pandemic O25:ST131 clone was detected in four (4.3%) isolates producing CTX-M-15, which belongs to B₂ phylogenetic group. This clone was detected in Adana (n=3) and Mersin (n=1) isolates but not in Hatay.

PFGE analysis and detection of virulence genes

PFGE results revealed presence of three pulsotype among *E. coli* B2-O25-ST131 CTX-M-15 isolates (Fig. 2). Virulence genes were detected in 36.2% (n=34) of 95 ESBL/pAmpC β -lactamase producing *E. coli* isolates and these isolates were only found as positive for *iucD*, *f17a-A*, *papC*, *papE* and *eaeA* genes. Other virulence genes were not detected. Single or various combination of virulence genes were observed among the isolates. The most common gene encountered was *iucD* gene, which was found in 31 isolates. Also, *f17a-A* fimbrial adhesin in two (2.1%) isolates and *papE-papC*, *eaeA* and *papE* genes in one (1.1%) isolate were detected.

DISCUSSION

This is the first study on the prevalence of ESBL/pAmpC producing *E. coli* from dogs conducted in Turkey. Our study showed that prevalence of fecal carriage of ESBL/pAmpC producing *E. coli* was 22.2% (95/428). Our results are higher than those reported in France (18.5%), in Mexico (17%), in Algeria (14.7%) [20, 41, 54], but comparable to those reported in China (24.5%) [45]. In addition, significant differences between fecal carriage and provinces were observed ($P=0.004$). Although past treatment histories were not recorded during sampling, it was speculated that the significant differences observed between fecal carriage and provinces could be related to variations in overall antibiotic use among these populations but not to individual risk factors.

According to studies carried out in different countries, it seems that types and subtypes of ESBL/pAmpC enzymes in *E. coli* strains isolated among dogs vary geographically. CTX-M-15 was the most common enzyme determined in this study, produced by 21 isolates alone and 41 isolates combined with one or more β -lactamase genes. In addition to *bla*_{CTX-M-15}, *bla*_{SHV-12} was observed in three isolates combined with other β -lactamase genes. CTX-M-15 were reported in healthy dogs by several studies [17, 24, 34, 41, 54]. CTX-M-15 is also the most commonly reported enzyme type in *E. coli* isolates from human clinical samples [2, 13, 19]. Recent studies in Turkey shows presence of this enzyme among ESBL producing *E. coli* isolates in cattle and retail meat [3, 37]. To a lesser extent, CTX-M-3 and CTX-M-1, were detected in nine and three isolates alone, also eight and 12 isolates in combination with other β -lactamase genes, respectively.

In this study, *aac(6')-Ib-cr* was the most common PMQR gene, detected in 24 (25.3%) of ESBL/pAmpC producing *E. coli* isolates. Moreover, *aac(6')-Ib-cr* was more frequently found in CTX-M-15 producing isolates (14/24, 58.3%), and to lesser extent, with other CTX-Ms including CTX-M-1 (4) and CTX-M-3 (6). Recently, Liu *et al.* [31] reported the frequent combination of *bla*_{CTX-M-15} and *aac(6')-Ib-cr* in CTX-M producing *E. coli* from dogs and cats in United States. In this study, PMQR genes were also detected in nine ciprofloxacin susceptible isolates. Similar finding was also reported by Zhao *et al.* [55], who found that 21 of

39 *qnr* positive isolates were ciprofloxacin susceptible. Liao *et al.* [30] recently reported that the PMQR genes were not associated with selective pressure caused by quinolones, the presence of PMQR genes were more linked to cephalosporin use than quinolone use, suggesting that other resistance genes might have more effect on PMQR prevalence.

Emergence of virulent human pandemic O25b-ST131/B2 CTX-M-15 producing *E. coli* clone draws significant international attention as a major cause of human infections [42]. Recently, emergence of B2-O25b-ST131 clone producing CTX-M-15 and CTX-M-27 enzymes have been reported in canine clinical samples in Japan [21], in Portugal [40] and in U.K. [46]. Similarly, Ewers *et al.* [17] reported presence of CXT-M-15 producing *E. coli* from dogs with urinary tract infections from different European countries (Netherlands, Germany, Spain, Denmark and France) belonged to this clone. This is the first report of the pandemic O25b-ST131/B2 *E. coli* CTX-M-15 clone in commensal dog *E. coli* isolates in Turkey. PFGE analysis showed that the isolates had different profiles (Fig. 2), suggesting genomic diversity within this clone. This result is not adequate to ascertain the source and direction of possible transmission. For that reason, to elucidate possible transmission route of this clone from human to companion animals or vice versa, genetic relatedness of human and animal isolates should be evaluated using more isolate in Turkey. However, considering the current findings, it is possible to say that dogs might play a role in spreading of this pandemic clone to community.

Trott [47] reported that ceftiofur use in veterinary clinical settings increased CMY-2 type AmpC β -lactamase selection both in *Salmonella* spp. and *E. coli* isolates. In this study, 23 (24.5%) isolates were found as pAmpC β -lactamase producer and all carried the *bla*_{CMY-2} gene, but the *bla*_{CMY-2} gene was almost found in combination with other β -lactamase genes, except an isolate. A similar result has been reported in the Republic of Korea by So *et al.* [44], who found the *bla*_{CMY-2} gene in 12 isolates in combination with the *bla*_{CTX-M} genes and alone in three isolates. Although presence of CMY-2 has been previously reported in *E. coli* isolates in Turkey from cattle [3] and broilers (unpublished data), this is the first study that report on occurrence of *bla*_{CMY-2} carrying *E. coli* among dogs in Turkey.

ESBL and/or pAmpC producing bacteria have also been reported resistant to other classes of antibiotics rather than beta-lactams and treatment options for infections caused by these bacteria are very limited [22, 23, 39]. In this study, the isolates were also resistant to non- β -lactams in various rates. This could be explained by co-existence of ESBL and/or pAmpC genes with other resistance determinants on same plasmids [14, 51].

It is considered that virulent extra-intestinal *E. coli* (ExPEC) strains usually belonged to phylogenetic groups B2 and D in comparison with other phylogenetic groups. In contrast, commensal strains frequently belonged to phylogenetic groups A and B1. Therefore, phylogenetic grouping is used to determine potential pathogenic strains for screening purposes and to establish a link between virulence and phylogenetic groups [11]. In this study, the isolates carrying virulence genes belonged mainly to phylogenetic group A (17 isolates) and D (10 isolates), but to a lesser extent, B2 (six isolates) and B1 (one isolate). Similar results were also reported by Ben Sallem *et al.* [5] and Wagner *et al.* [48]. Johnson *et al.* [26] reported that ExPEC strains may take part in intestinal microbiota of healthy dogs without showing any clinical symptoms and may pose a risk for the transfer of these bacteria to humans. Wagner *et al.* [48] indicated that multidrug resistant (MDR) *E. coli* isolates from canine urinary tract infections have a reduced virulence genotype compared to susceptible ones.

Regarding with the distribution of ESBL/pAmpC strains according to phylogenetic groups, all phylogenetic groups were determined among isolates with various rates. However, most of the isolates belonged mainly to phylogenetic group A (41/95) and D (30/95), but to a lesser extent, B1 (15/95) and B2 (9/95). Our results are consistent with studies indicating that majority of ESBL/pAmpC producing isolates belonged to commensal phylogenetic groups A and B1 [43, 54].

In conclusion, this is the first study on the presence of ESBL and/or pAmpC producing *E. coli* strains among dogs in Turkey and distribution of genes encoding β -lactamase genes. High prevalence of *bla*_{CTX-M} and *bla*_{CMY-2} genes emphasize the possibility of transfer of these resistant bacteria to humans. Companion animals due to their close contact with their owners should be monitored regularly to prevent the dissemination of resistant bacteria. Detailed molecular analyses are needed to reveal the genetic relationship of ESBL/pAmpC producing strains isolated from different sources including dogs and humans.

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