Detection of extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* in companion dogs

B Deepthi, M Srivani, RN Ramani Pushpa and Chaitanya Y

**DOI:** https://doi.org/10.22271/tpi.2020.v9.i9Sd.5257

**Abstract**

Faecal samples collected from 70 companion dogs were cultured for isolation of *E. coli* and confirmed by PCR targeting *E16S* gene. A total of 51 *E. coli* isolates were recovered and screened for ESBL resistance by phenotypic and genotypic methods. For selective isolation of ESBL producing *E. coli*, samples were inoculated onto cefotaxime containing Mc conkey agar, and this process resulted in recovery of 38 isolates. These suspect ESBL producers were further confirmed by combination disc method and double disc synergy test which revealed an overall incidence of 56.8% (29/51). Multiplex PCR assays revealed presence of *blaTEM*, *blaSHV*, *blaOXA* and *blaCTX-M* group1, 2 and 9 genes in 12, 5, 2, 5, 8 and 3 isolates respectively. Two isolates have combination of *blaCTX-M* group genes. Indiscriminate use of antibiotics in companion animals was described as a risk factor for faecal colonization of ESBL producing *E. coli* and transmission to humans. Hence, data on antibiotic resistance patterns of companion animals is quintessential.

**Keywords:** *E. coli*, companion dogs, ESBL, antibiotic resistance, multiplex PCR

**1. Introduction**

Antimicrobial resistance is a large and increasing problem in both human (Coque *et al.*, 2008) [12] and veterinary medicine (Wieler *et al.*, 2011) [25]. The use and misuse of antibiotics has played a role in the emergence and spread of resistant bacteria in animal and human communities. The Infectious Diseases Society of America has listed *Escherichia coli* and *Klebsiella* spp as two out of six pathogens for which new drugs are urgently needed in order to combat resistance development (Talbot *et al.*, 2006) [22]. Emerging resistance to the β-lactam antimicrobials is troubling; this diverse antimicrobial class includes the penicillins, cephalosporins, cefamycins, carbapenems, and monobactams. Resistance to beta-lactam antibiotics is mediated by bacterial enzymes called beta-lactamasases that are encoded by beta-lactamase (*bla*) genes like *blaTEM* (Temoniera β-lactamase), *blaSHV* (sulphhydryl variable), *blaOXA* (oxacillinase), *blaCTX-M* (Cefotaximase-Munich), *blaAmpC* (Bush and Jacoby, 2010) [9]. Extended-spectrum beta-lactamases (ESBLs) are variants of beta-lactamasases that confer resistance to third generation cephalosporins as well as monobactams and are inhibited by beta-lactamase inhibitors (Bush and Jacoby, 2010) [9].

The gastrointestinal tract is an important reservoir for antimicrobial-resistant (AMR) Gram-negative organisms (Wellington *et al.*, 2013) [24]. MDR (resistance to three or more antimicrobial classes) (Magiorakos *et al.*, 2012) [16]. Extended-spectrum beta lactamasates (ESBL) and AmpC-producing faecal *Escherichia coli* carried by dogs are of particular concern. They may act as a reservoir for self-infection, which may lead to further transmission of resistance genes, as well as pathogens and resistance genes potentially being transferred into other hosts, including people, other pets and the environment (Ahmed *et al.*, 2015) [1]. ESBL and AmpC enzyme production results in co-resistance to the majority of the commonly used beta-lactam antimicrobials, including third generation cephalosporins (Pitout, 2010) [20]. ESBL is frequently plasmid mediated and AmpC beta lactamase activity is chromosomal (cAmpC) or plasmid-mediated (pAmpC) (Peter-Getzlaff *et al.*, 2011) [19]. Location of these genes in plasmids indicates that they can be transferred via horizontal gene transfer between bacteria within and between bacterial species (Broland *et al.*, 2013) [18].

Studies related to the detection of beta-lactam resistance in *Escherichia coli* of canine origin have been relatively less explored in India. The aim of the present study was to determine the prevalence and distribution of ESBL encoding genes in *E. coli* isolated from the faeces of...
healthy and diarrhoeic dogs presented to Teaching Veterinary Clinical Complex, Gannavaram.

2. Materials and Methods
2.1 Sample collection
A total of 70 rectal swabs were collected from healthy (30) and diarrhoeic (40) companion dogs presented to TVCC, Gannavaram with various clinical ailments. Samples from healthy dogs were collected based on defecation history i.e. no history of diarrhoea for at least 4 weeks. Among the dogs sampled, 41.4% (n=29) were females and 58.6% (n=41) were males.

2.2 Selective isolation and confirmation of ESBL producing E. coli by PCR
The swabs were inoculated onto 2 µg/ml cefotaxime containing Mac conkey Agar (Himedia) for selective isolation and incubated at 37 °C for 24 hr. A typical isolated colony was selected and passaged onto blood agar to obtain pure culture. Identification was done using conventional culture methods and biochemical tests (Gram stain, catalase, oxidase, indole, MR-VP, citrate and urease).

*E. coli* thus isolated was further confirmed by species-specific PCR. DNA extraction was done using boiling and snap chilling method. About 2 mL of overnight grown *E. coli* culture was taken in microfuge tube and centrifuged at 10,000 rpm for 5 minutes (min) (Eppendorf). The pellet was suspended in 400 µL of nuclease free water and heated for 10 min in a boiling water bath. The microfuge tube was transferred immediately on to ice. After five min, the tube was centrifuged at 8000 rpm for 5 min at 4 °C and the supernatant was used as template for PCR reactions. Quantification of DNA was done by nanodrop, and DNA samples were diluted was used as template for PCR. Quantification of DNA was done by nanodrop, and DNA samples were diluted.

Identification was done using conventional culture methods and biochemical tests (Gram stain, catalase, oxidase, indole, MR-VP, citrate and urease).

*E. coli* thus isolated was further confirmed by species-specific PCR. DNA extraction was done using boiling and snap chilling method. About 2 mL of overnight grown *E. coli* culture was taken in microfuge tube and centrifuged at 10,000 rpm for 5 minutes (min) (Eppendorf). The pellet was suspended in 400 µL of nuclease free water and heated for 10 min in a boiling water bath. The microfuge tube was transferred immediately on to ice. After five min, the tube was centrifuged at 8000 rpm for 5 min at 4 °C and the supernatant was used as template for PCR reactions. Quantification of DNA was done by nanodrop, and DNA samples were diluted was used as template for PCR. Quantification of DNA was done by nanodrop, and DNA samples were diluted.

Identification was done using conventional culture methods and biochemical tests (Gram stain, catalase, oxidase, indole, MR-VP, citrate and urease).

*E. coli* thus isolated was further confirmed by species-specific PCR. DNA extraction was done using boiling and snap chilling method. About 2 mL of overnight grown *E. coli* culture was taken in microfuge tube and centrifuged at 10,000 rpm for 5 minutes (min) (Eppendorf). The pellet was suspended in 400 µL of nuclease free water and heated for 10 min in a boiling water bath. The microfuge tube was transferred immediately on to ice. After five min, the tube was centrifuged at 8000 rpm for 5 min at 4 °C and the supernatant was used as template for PCR reactions. Quantification of DNA was done by nanodrop, and DNA samples were diluted was used as template for PCR. Quantification of DNA was done by nanodrop, and DNA samples were diluted.

Identification was done using conventional culture methods and biochemical tests (Gram stain, catalase, oxidase, indole, MR-VP, citrate and urease).

2.3 Phenotypic methods of ESBL detection
*E. coli* isolated in the present study were screened for ESBL production by testing their susceptibility to various antimicrobials by disk diffusion method and interpreted according to Clinical Laboratories Standards Institute (CLSI) criteria (CLSI 2018) [11]. The antimicrobials tested were cefotaxime (10 µg), ceftazidime (10 µg), ceftriaxone (30 µg), aztreonam (30 µg) and Amoxycclavulanate (10 µg). Resistance to at least one of the five antibiotics used was considered as positive phenotype screening test (PST) for possible ESBL production (Drieux et al., 2008) [13].

All samples found to be positive in PST were subjected to phenotype confirmatory test (PCT) by combination disk method (CDM) and Double disk synergy test (DDST). In CDM, ESBL producing isolates were subcultured in nutrient broth and incubated at 37 °C and later streaked on Mueller Hinton Agar. Two pairs of disks (i.e., with and without clavulanic acid) were placed: ceftazidime (CAZ, 30 µg), ceftazidime plus clavulanic acid (CAC, 30/10 µg) and cefotaxime (CTX, 30 µg), cefotaxime plus clavulanic acid (CEC, 30/10 µg). An isolate was confirmed for ESBL production for the inhibition zone diameter around combination disc was ≥5 mm (synergy effect) when compared to discs containing respective cephalosporin alone (Drieux et al. 2008) [13].

In DDST, ESBL producing isolates were sub cultured in nutrient broth and incubated at 37 °C and later streaked on Mueller Hinton agar. Cefotaxime, ceftriaxone and aztreonam discs were placed around amoxicillin/clavulanic acid disc at a distance of 15mm. A clearly visible extension of edges of inhibition zone towards amoxicillin/clavulanic acid disc was regarded as positive CPT. This enhanced zone of inhibition towards clavulanate disc was called keyhole phenomenon which indicates a positive synergy test.

2.4 Genotypic methods of ESBL detection
All the *E. coli* isolates were subjected to detection of ESBL genes by PCR. Two multiplex PCR assays were standardized. One assay for simultaneous detection of *bla*TEM, *bla*SHV and *bla*OXA genes and the other for detection of *bla*CTX-M groups (Group-1, 2 and 9). The primer sequences and product sizes used to amplify ESBL genes are listed in Table 1.

| Antimicrobial family | Genetic marker | Sequence (5’---- 3’) | Amplicon n size (bp) |
|----------------------|----------------|----------------------|----------------------|
| **Beta-lactams**     |                |                      |                      |
| TEM-F                |                | CATTTCGTTGCGCCCTTTAAC CGTTCATCCATAAGTGGCGTGAC | 800bp |
| TEM-R                |                |                      |                      |
| SHV-F                |                | AGGCGGTTAGACCAATAAAC AATCCCCAGATAAATACACCAC | 713 bp |
| SHV-R                |                |                      |                      |
| OXA-1like F          |                | GCCACCCAGATTCAACTTCAAG GACCCCAAGTTTCCTGTAAAGT | 564 bp |
| OXA-1like R          |                |                      |                      |
| **Extended spectrum beta lactams** | | | |
| *CTX-M* group 1      |                | TTAGGAAATGTTGCGCCGTGA CGATATCGTTGGTGATCAACCA | 688 bp |
| *CTX-M* group 2      |                | CGTAAACGCCAGATGAC CGATATCGTTGGTGATCACCAT | 404 bp |
| *CTX-M* group 9      |                | TCAAGCCTTGCGAGCTGTG TGATTCCTGCGCCTGAG | 561 bp |

3. Results and Discussion
Of the rectal swabs obtained from 70 dogs; *E. coli* was isolated from 51 (Healthy dogs, n=19; diarrhoeic dogs, n=32) samples. All the *E. coli* isolates were biochemically characterized and amplified 231 bp product in the PCR targeting *16S* gene of *E. coli* (Fig.1).
The PCR targeting **E16S** gene was successfully used earlier by other workers for the molecular confirmation of *E. coli* isolated from diverse sources (Ahmadi *et al.*, 2015)\(^2\). Among the 51 *E. coli* isolates subjected to the ESBL screening test, 14 isolates from healthy dogs and 24 isolates from diarrhoeic dogs were positive (Fig.2).

Confirmatory test results showed that 9 isolates from healthy dogs and 20 isolates from diarrhoeic dogs produced ESBL phenotypically (Fig 3 & 4).

**Table 2:** Distribution of ESBL producing *E. coli* isolates according to the phenotypic evaluation

| Group             | Healthy dogs | Diarrhoeic dogs | Total dogs |
|-------------------|--------------|-----------------|------------|
| *E. coli* isolated from | 19           | 32              | 51         |
| PST positive      | 14           | 24              | 38         |
| PCT positive      | 9            | 20              | 29         |

Knowledge on prevalence and distribution of resistance genes is important for understanding the mechanism of spread of antimicrobial resistance (AMR) genes since bacteria of animal origin can serve as a reservoir of such genes (Aslam *et al.*, 2009)\(^3\). In many genera of gram-negative bacteria, the most predominant β-lactamases are TEM, SHV, OXA and CTX type enzymes (Bradford, 2001)\(^7\). All the *E. coli* isolates obtained in the present study were included in the molecular assays. Of the 19 isolates obtained from healthy dogs, four isolates (21%) harboured *blaTEM*, one isolate (5.2%) harboured *blaSHV*, one isolate (5.2%) harboured *blaOXA*, two isolates (10.5%) harboured *blaCTX-M* Group 1, three isolates (15.7%) harboured *blaCTX-M* Group 2 and one isolate (5.2%) harboured *blaCTX-M* Group 9 genes. Of the 32 isolates obtained from diarrhoeic dogs, nine isolates...
(28.1%) harboured \textit{blaTEM}, four isolates (12.5%) harboured \textit{blaSHV}, one isolate (3.1%) harboured \textit{blaOXA}, three isolates (9.7%) harboured \textit{blaCTX-M} Group 1, five isolates (15.6%) harboured \textit{blaCTX-M} Group 2 and two isolates (6.2%) harboured \textit{blaCTX-M} Group 9 genes. One isolate from diarrhoeic dog harboured all the three \textit{blaCTX-M} Groups 1, 2 and 9 (Fig. 5 and 6).

The first ESBL producing \textit{E. coli} to be identified in dogs was recovered from a faecal sample from a laboratory dog in Japan (Matsumoto et al., 1988) (17), followed by dogs with urinary tract infections in Spain (Teshager et al., 2000) (23). Subsequently, ESBL producing \textit{E. coli} have been recovered from healthy and sick dogs in many parts of the world. The occurrence of beta lactamase production in \textit{E. coli} isolates obtained from healthy companion animals varies between 1-25.4% in various countries (Belas et al., 2014) (5). However, in this study higher prevalence rate was observed which might be overestimated due to smaller sample size. Similar percentages of ESBL producing \textit{E. coli} have been found in the studies of healthy and diarrhoeic dogs (Aslantas and Yilmaz, 2017) (4).

ESBL genes were even detected in PCT negative isolates also. It has been reported that isolates containing both ESBL and AmpC genes could be able to mask the effects of clavulanic acid used in the ESBL-confirming test due to AmpC enzymes. Thus, in this situation, it is possible that the presence of ESBLs might be underestimated (O’keefe et al., 2010) (18) in the phenotypic tests. In this study, eight isolates negative by phenotypic tests are found to be positive by genotypic tests. These results demonstrated the importance of molecular methods for the detection of ESBL. AmpC gene detection unfortunately couldn’t be undertaken in this study.

Antimicrobial treatment administered three months prior to the investigation was identified as a risk factor for the carriage of ESBL producing \textit{E. coli} in healthy dogs (40%) and diarrheic dogs (78.1%). Genotypic and phenotypic results were depicted in Table 3.

In the animals included in this study, there is a high level of intestinal carriage of ESBL producing \textit{E. coli} in healthy dogs (40%) and diarrheic dogs (78.1%). Genotypic and phenotypic results were depicted in Table 3.

The first ESBL producing \textit{E. coli} to be identified in dogs was recovered from a faecal sample from a laboratory dog in Japan (Matsumoto et al., 1988) (17), followed by dogs with urinary tract infections in Spain (Teshager et al., 2000) (23). Subsequently, ESBL producing \textit{E. coli} have been recovered from healthy and sick dogs in many parts of the world. The occurrence of beta lactamase production in \textit{E. coli} isolates obtained from healthy companion animals varies between 1-25.4% in various countries (Belas et al., 2014) (5). However, in this study higher prevalence rate was observed which might be overestimated due to smaller sample size. Similar percentages of ESBL producing \textit{E. coli} have been found in the studies of healthy and diarrhoeic dogs (Aslantas and Yilmaz, 2017) (4).

ESBL genes were even detected in PCT negative isolates also. It has been reported that isolates containing both ESBL and AmpC genes could be able to mask the effects of clavulanic acid used in the ESBL-confirming test due to AmpC enzymes. Thus, in this situation, it is possible that the presence of ESBLs might be underestimated (O’keefe et al., 2010) (18) in the phenotypic tests. In this study, eight isolates negative by phenotypic tests are found to be positive by genotypic tests. These results demonstrated the importance of molecular methods for the detection of ESBL. AmpC gene detection unfortunately couldn’t be undertaken in this study.

Antimicrobial treatment administered three months prior to the investigation was identified as a risk factor for the carriage of ESBL producing \textit{E. coli} in healthy dogs (40%) and diarrheic dogs (78.1%). Genotypic and phenotypic results were depicted in Table 3.

In the animals included in this study, there is a high level of intestinal carriage of ESBL producing \textit{E. coli} in healthy dogs (40%) and diarrheic dogs (78.1%). Genotypic and phenotypic results were depicted in Table 3.
occurrence of isolates having more than one type of ESBL/AmpC gene. Oxa-1 is the predominant gene detected followed by CTX-M group 2 variants. Penetration and the later global spread of CTX-M producing organisms have been produced with the participation of the so-called “epidemic resistance plasmids” often carried in multi-drug resistant and virulent high-risk clones. All these facts but also the incorporation and co-selection of emerging resistance determinants within CTX-M producing bacteria, such as those encoding carbapenemases, depict the currently complex pandemic scenario of multi-drug resistant isolates (Canton et al., 2012). Resistance conferred by ESBL is often associated with resistance to other classes of antibiotics, such as fluoroquinolones, aminoglycosides and trimethoprim-sulphamethoxazole, which can be explained in part because plasmids harbouring CTX-M frequently carry genes conferring resistance to other families of antibiotics (Ben Sallem et al., 2014). Present study reveals high incidence of ESBL producing E. coli in dogs which shows the alarming situation. These animals may act as a reservoir for antibiotic resistant genes and transmit the same to their owners. Whether pets are a source for humans cannot be determined by this observational study, but this reservoir should certainly be included in attribution studies for human infections. In view of this, pet owners shall be encouraged to dispose dog faeces safely and to practice hygienic measures.

4. References
1. Ahmed LN, Price LB, Graham JP. An exploratory study of dog park visits as a risk factor for exposure to drug-resistant extra-intestinal pathogenic E. coli (ExPEC). BMC Res Notes. 2015; (8):137.
2. Ahmadi MR, Haghkhah M, Derakhshandeh A, Aghamiri SM, Mirzaei A, Nazifi S et al. Identification of bacterial and fungal agents of clinical endometritis in dairy heifers and treatment by metronidazole or cephariprin. Theriogenology Insight: An Int. J of Repro. in all Anim. 2015; 5(2):99.
3. Aslam M, Diarra MS, Service C, Rempel H. Antimicrobial resistance genes in Escherichia coli isolates recovered from a commercial beef processing plant. J of Food Protoc. 2009; 72(5):1089-1093.
4. Aslantas O, Yilmaz ES. Prevalence and molecular characterization of extended-spectrum β-lactamase (ESBL) and plasmidic Amp C β-lactamase (pAmp C) producing Escherichia coli in dogs. The J of Vete. Medi. Sci. 2017; 79(6):1024-1030.
5. Belas A, Salazar AS, Gama LTD, Couto N, Pomba C. Risk factors for faecal colonisation with Escherichia coli producing extended-spectrum and plasmid-mediated Amp C β-lactamases in dogs. Vete. Record. 2014; 175:202.
6. Ben Sallem R, Ben Slama K, Saenz Y, Rojo-Bezares B, Estepa V, Jouini A et al. Prevalence and characterization of extended-spectrum β-lactamase (ESBL) and CMY-2 producing Escherichia coli isolates from healthy food-producing animals in Tunisia. Foodborne Pathogens and Disease. 2012; 9:1137-1142.
7. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clinical Microbiol. Reviews. 2001; 14(4):933-951.
8. Brolund A, Franze NO, Melefors O, Tegmark-Wisell K, Sandgren L. Plasmidome-analysis of ESBL-producing Escherichia coli using conventional typing and high-throughput sequencing. PLoS ONE, 2013, 8(6). doi:10.1371/journal.pone.0065793.
9. Bush K, Jacoby GA. Updated functional classification of β-lactamases. Antimicrobial Agents and Chemotherapy. 2010; 54(3):969-976.
10. Canton R, González-Alba JM, Galán JC. CTX-M Enzymes: Origin and Diffusion. Frontiers in Microbiology. 2012; 3:110.
11. Clinical and Laboratory Standard Institute. Performance standards for antimicrobial susceptibility testing; twenty-eighth informational supplement; M100. Wayne, 2018.
12. Coque TM, Baquero F, Canton R. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. Euro Surveillance. 2008; 13:1-11.
13. Driex L, Brossier F, Sougakoff W, Jarlier V. Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide. Clinical Microbiol. and Inf. 2008; 14:90-103.
14. Gandolfi-Decristophoris P, Petritin O, Ruggeri-Bernardi N, Schelling E. Extended-spectrum beta-lactamase-producing Enterobacteriaceae in healthy companion animals living in nursing homes and in the community. American J. of Inf. Cont. 2013; 41(9):831-835.
15. Hordijk J, Schoormans A, Kwakernaak M, Duin B, Broens E, Dierikx C et al. High prevalence of fecal carriage of extended-spectrum β-lactamase/AmpC-producing Enterobacteriaceae in cats and dogs. Frontiers in Microbiol. 2013; 4(242):1-5.
16. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiol. and Inf. 2012; 18(3):268-81.
17. Matsumoto Y, Ikeda F, Kamimura T, Yokota Y, Mine Y. Novel plasmid mediated β-lactamase from Escherichia coli that inactivates oxyiminocephalosporins. Antimicrobial Agents and Chemotherapy. 1988; 32:1243-1246.
18. O’Keefe A, Hutton TA, Schifferli DM, Rankin SC. First detection of CTX-M and SHV extended-spectrum β-lactamases in Escherichia coli urinary tract isolates from dogs and cats in the United States. Antimicrobial Agents and Chemotherapy. 2010; 54(8):3489-3492.
19. Peter-Getzlaff S, Polsfluss S, Poledica M, Hombach M, Giger J, Böttger EC et al. Detection of AmpC beta-lactamase in Escherichia coli: comparison of three phenotypic confirmation assays and genetic analysis. J. of Clinical Microbiol. 2011; 49(8):2924-2932.
20. Pitout JD. Infections with Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae: Changing Epidemiology and Drug Treatment Choices. Drugs. 2010; 70(3):313-33.
21. Sundong-boRui WU, Xian-jing HE, Shuang W, Yun-cheng L, Xu HAN, Yue-qiang W et al. Isolation of an SHV-12 β-lactamase-producing Escherichia coli strain from a dog with recurrent urinary tract infections. Antimicrobial Agents and Chemotherapy. 2000; 44:3483-3484.
22. Talbot GH, John Bradley, John E. Edwards, David Gilbert, Michael Scheld, John G. Bartlett, Bad Bugs
Need Drugs: An Update on the Development Pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America, Clinical Infectious Diseases. 2006; 42(5):657-668.

23. Teshager T, Dominguez L, Moreno MA, Saenz Y, Torres C, Cardenosa S. Isolation of an SHV-12 β-lactamase-producing Escherichia coli strain from a dog with recurrent urinary tract infections. Antimicrobial Agents and Chemotherapy. 2000; 44:3483-3484.

24. Wellington EMH, Alistair B, Boxall, Paul Cross, Edward J Feil, William H Gaze et al. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. Lancet Infect Dis. 2013; 13:155-65.

25. Wieler LH, Ewers C, Guenther S, Walther B, Becker AL. Methicillin-resistant staphylococci (MRS) and extended-spectrum beta-lactamases (ESBL) -producing Enterobacteriaceae in companion animals: Nosocomial infections as one reason for the rising prevalence of these potential zoonotic pathogens in clinical samples. International J of Medi. Microbiol. 2011; 301(8):635-641.