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

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DOI: https://doi.org/10.22271/tpi.2020.v9i9Sd.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) 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 β-lactam antibiotics is mediated by bacterial enzymes called beta-lactamases that are encoded by β-lactamase (bla) genes like blaTEM (Temoniera β-lactamase), blaSHV (sulfhydryl variable), blaOXA (oxacillinase), blaCTX-M (Cefotaximase-Munich), blaAmpCetc (Bush and Jacoby, 2010) (9). Extended-spectrum beta-lactamases (ESBLs) are variants of beta-lactamases that confer resistance to third generation cephalosporins as well as monobactams and are inhibited by β-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 lactamases (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) (15). ESBL and AmpC enzyme production results in co-resistance to the majority of the commonly used β-lactam antimicrobials, including third generation cephalosporins (Picout, 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 (Brolund et al., 2013) (16).

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 5minutes (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 to a final concentration of 30ng/ μL and 3-5 μL of this preparation was used as template for PCR. E. coli 16S rRNA gene specific primers quoted by Sun Dong et al. (2011) [21] (E16S-F:ATCAACCGGATTTCCCCAGTG E16S-R: CACTATCGGTCACTCAAGGAG) were used in this study. PCR reactions were carried out in an Eppendorf thermal cycler (AG5341). The amplification conditions were 5 min of denaturation at 95 °C, followed by 35 cycles of 95 °C for 1 min, 50 °C for 50 seconds(s), and 72 °C for 1 min, and a final extension step of 72 °C for 10 min.

Details of the primers used for the detection of ESBL genes are listed in Table 1.

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 Amoxyclovulanate (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 when 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. Cefazidime, cefotaxime, ceftiraxone 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 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 blaTEM, blaSHV and blaOXA genes and the other for detection of blaCTX-M groups (Group-1, 2 and 9). The primer sequences and product sizes used to amplify ESBL genes are listed in Table 1.

Table 1: Details of the primers used for the detection of ESBL genes

<table>
<thead>
<tr>
<th>Antimicrobial family</th>
<th>Genetic marker</th>
<th>Sequence (5’---- 3’)</th>
<th>Amplicon n size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-lactams</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-F</td>
<td>CATTTCGCGTGGCCCTTTATTC</td>
<td>800bp</td>
<td></td>
</tr>
<tr>
<td>TEM-R</td>
<td>CGTTCATCCATGTTGCGTTCGAC</td>
<td>713 bp</td>
<td></td>
</tr>
<tr>
<td>SHV-F</td>
<td>AGGCGGTCGAGCAAAATTAAAC</td>
<td>564 bp</td>
<td></td>
</tr>
<tr>
<td>SHV-R</td>
<td>ATCCCCGAGATATAATCACCAC</td>
<td>713 bp</td>
<td></td>
</tr>
<tr>
<td>OXA-1like F</td>
<td>GGCACCAAGATTCAACTTCAAG</td>
<td>862 bp</td>
<td></td>
</tr>
<tr>
<td>OXA-1like R</td>
<td>GACCCCAAGTTTCTCGTAAGTG</td>
<td>688 bp</td>
<td></td>
</tr>
<tr>
<td>Extended spectrum beta lactams</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M group 1</td>
<td>TTAGGGAATATGTGCCGCTGTA</td>
<td>404 bp</td>
<td></td>
</tr>
<tr>
<td>CTX-M group 2</td>
<td>CGATATCGTTGGTTGACCATCA</td>
<td>561 bp</td>
<td></td>
</tr>
<tr>
<td>CTX-M group 9</td>
<td>TCAAGCCTGCCAGCTGGT</td>
<td>688 bp</td>
<td></td>
</tr>
</tbody>
</table>

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 rRNA gene of E. coli (Fig.1).

--- 190 ---
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). 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).

ESBL production was confirmed in a total of 29 isolates. The distribution of ESBL producing E. coli isolates according to the phenotypic evaluation is shown in Table 2.

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). In many genera of gram-negative bacteria, the most predominant β-lactamases are TEM, SHV, OXA and CTX type enzymes (Bradford, 2001). 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 blaTEM, four isolates (12.5%) harboured blaSHV, one isolate (3.1%) harboured blaOXA, three isolates (9.7%) harboured blaCTX-M Group 1, five isolates (15.6%) harboured blaCTX-M Group 2 and two isolates (6.2%) harboured blaCTX-M Group 9 genes. One isolate from diarrhoeic dog harboured all the three blaCTX-M Groups 1, 2 and 9 (Fig. 5 and 6).

The first ESBL producing 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 E. coli have been recovered from healthy and sick dogs in many parts of the world. The occurrence of beta lactamase production in 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 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 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 E. coli in healthy dogs (40%) and diarrheic dogs (78.1%). Genotypic and phenotypic results were depicted in Table 3.

Table 3: Number of animals carrying ESBL genes in healthy and diarrhoeic dogs

<table>
<thead>
<tr>
<th>ESBL genes</th>
<th>Healthy dogs (n=19)</th>
<th>Diarrhoeic dogs (n=32)</th>
<th>Total (n=51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaTEM</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>blaSHV</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>blaOXA</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>blaCTX-M group 1</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>blaCTX-M group 2</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>blaCTX-M group 9</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>blaTEM+ blaOXA</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>blaCTX-M group 1+ group 9</td>
<td>-</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>blaCTX-M group 1+ group 2+ group 9</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

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occurrence of isolates having more than one type of ESBL/AmpC gene. blaTEM 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)[10]. 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)[6]. 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
22. Talbot GH, John Bradley, John E. Edwards, David Gilbert, Michael Scheld, John G. Bartlett, Bad Bugs...

