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

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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 *bla*TEM, *bla*SHV, *bla*OXA and *bla*CTX-M group1, 2 and 9 genes in 12, 5, 2, 5, 8 and 3 isolates respectively. Two isolates have combination of *bla*CTX-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-lactamases that are encoded by beta-lactamase (*bla*) genes like *bla*TEM (Temoniera β-lactamase), *bla*SHV (sulfhydryl variable), *bla*OXA (oxacillinase), *bla*CTX-M (Cefotaximase-Munich), *bla*AmpCetc (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 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 lactamases (ESBL) andAmpC-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 (Brolund *et al.,* 2013) [8].

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 ºC 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-bo *et al.* (2011) [21] (E16S-F:ATCAACCGAGATTCCCCCAGT E16S-R: CACTATCGGTCAGTCAGGAG) 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 ^{0}C, followed by 35 cycles of 95 °C for 1 min, 50 ºC for 50 seconds(s), and 72 ºC for 1 min, and a final extension step of 72 ºC for 10 min.

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 Amoxyclavulanate (10 µg). Resistance to atleast 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 ^{0}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 ^{0}C and later streaked on muellerhinton agar. Ceftazidime, 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 PCT. 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.

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

Antimicrobial family	Genetic marker F(5'-3') R(3'-5')	Sequence (5'---- 3')	Amplico n size (bp)
Beta-lactams	TEM-F TEM-R	CATTTCCGTGTCGCCCTTATTC CGTTCATCCATAGTTGCCTGAC	800bp
	SHV-F SHV-R	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	713 bp
	OXA-1like F OXA-1like R	GGCACCAGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	564 bp
Extended spectrum beta lactams	*CTX-M* group 1	TTAGGAAATGTGCCGCTGTA CGATATCGTTGGTGGTACCA	688 bp
	CTX-M group 2	CGTTAACGGCACGATGAC CGATATCGTTGGTGGTACCAT	404 bp
	CTX-M group 9	TCAAGCCTGCCGATCTGGT TGATTCTCGCCGCTGAAG	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 *E16S* gene of *E. coli* (Fig.1).

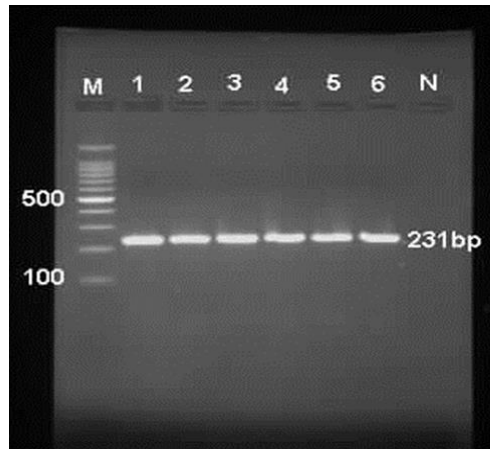

Fig 1: Molecular characterization by E16s rRNA species specific PCR for *E. coli*

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).

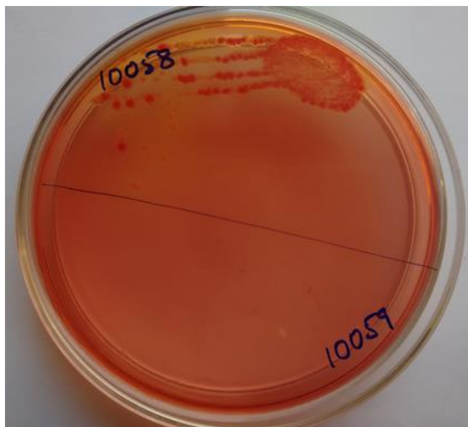

Fig 2: Phenotypic screening test:Multi Drug Resistant isolates cultured on Mc Conkey agar (with 2µg/ml cefotaxime)

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

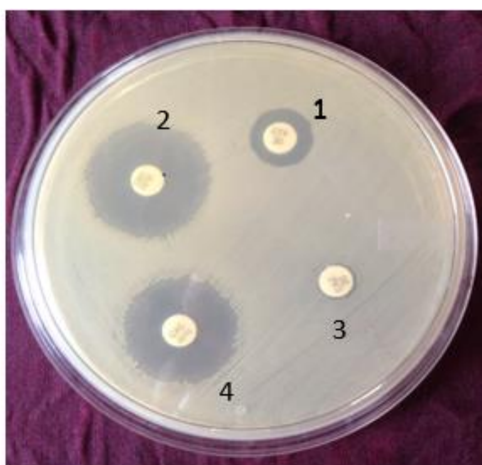

Fig 3: Phenotypic confirmatory test by Combination Disc Method. 1. Cefotaxime (CTX); 2. Cefotaxime + Clavulanic acid (CEC); 3. Ceftazidime (CAZ); 4. Ceftazidime + Clavulanic acid (CAC)

Fig 4: Double Disc Synergy Test- Enhanced zone towards clavulanate disc – “Key hole” phenomenon. 1. Ceftriaxone (CTR); 2. Ceftazidime (CAZ); 3. Aztreonam (AT); 4. Cefotaxime (CTX); 5. Ceftazidime + Clavulanic acid (CAC)

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.

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

Group	Healthy dogs	Diarrhoeic dogs	Total dogs
n screened	30	40	70
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 *bla*TEM, one isolate (5.2%) harboured *bla*SHV, one isolate (5.2%) harboured *bla*OXA, two isolates (10.5%) harboured *bla*CTX-M Group 1, three isolates (15.7%) harboured *bla*CTX-M Group 2 and one isolate (5.2%) harboured *bla*CTX-M Group 9 genes. Of the 32 isolates obtained from diarrhoeic dogs, nine isolates

(28.1%) harboured *bla*TEM, four isolates (12.5%) harboured *bla*SHV, one isolate (3.1%) harboured *bla*OXA, three isolates (9.7%) harboured *bla*CTX-M Group 1, five isolates (15.6%) harboured *bla*CTX-M Group 2 and two isolates (6.2%) harboured *bla*CTX-M Group 9 genes. One isolate from diarrhoeic dog harboured all the three *bla*CTX-M Groups 1,2 and 9 (Fig. 5 and 6).

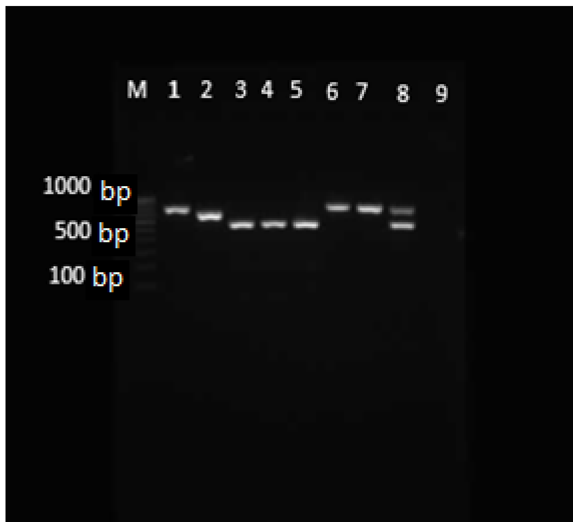

Fig 5: Multiplex PCR targeting *bla*TEM, *bla*SHV and *bla*OXA genes in ESBL producing *E. coli.* M- 100 bp DNA Ladder; 1- Standard *E. coli* carrying blaTEM (800 bp); 2-Standard *E. coli* carrying blaSHV (713 bp); 3-Standard Klebsiella carrying blaOXA (564 bp); 4 & 5- *E. coli* isolate carrying blaOXA (564 bp); 6 & 7 - *E. coli* isolate carrying blaTEM (800 bp); 8- E. coli isolate carrying blaSHV and blaOXA; 9- Negative control

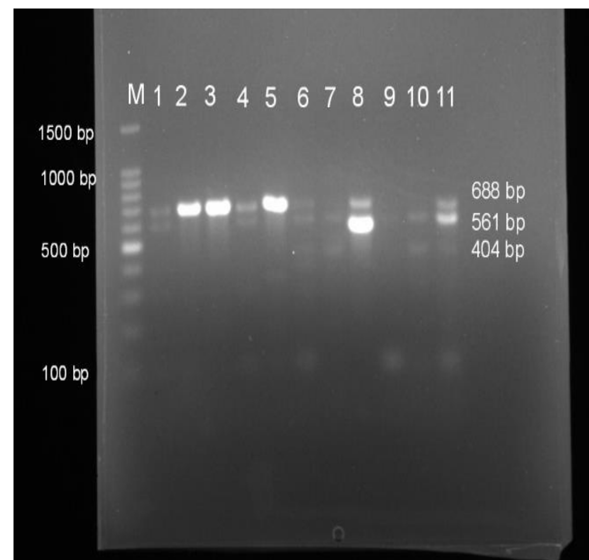

Fig 6: Multiplex PCR targeting *bla*CTX-M Group 1, Group 2 and Group 9 genes in ESBL resistant E. coli isolates. M- 100 bp DNA Ladder; 1,4,5,6 - *E. coli* isolate with group1 (688 bp) and group9 (561 bp); 2 &3 - *E. coli* isolate with group1 (688 bp); 7 &10 - *E. coli* isolate with group2 (404 bp) and group 9 (561 bp); 8 - *E. coli* isolate with group 1 (688 bp) and group 9 (561 bp); 11 - *E. coli* isolate with all the variants; 9- Negative control

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

	ESBL genes	Healthy dogs (*n*=19)	Diarrhoeic dogs (*n*=32)	Total (*n*=51)
No. of animals carrying	*bla*TEM	4	8	12
	*bla*SHV	1	4	5
	*bla*OXA	1	1	2
	*bla*CTX-M group 1	2	3	5
	*bla*CTX-M group 2	3	5	8
	*bla*CTX-M group 9	1	2	3
	*bla*TEM+ *bla*OXA	-	1	1
	*bla*CTX-M group 1+ group 9	-	1	1
	*bla*CTX-M group 1+ group 2+ group9	-	1	-

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 Enterobacteriaceae (Gandolfi-Decristophoris *et al.,* 2013) [14]. This treatment resulted in selection for the presence of ESBL producing *E. coli* in the feces. The treatment history of the animals included in our study is not known. Therefore, any possible contribution of previous antibiotic usage as described above could not be established.

Molecular analysis revealed a high variety of ESBL genes present, especially within diarrheic dogs (Table 2). In this group seven out of twenty animals harbored more than one ESBL gene. Few isolates carried a combination of both *bla*SHV and *bla*OXA and few isolates harboured different groups of *bla*CTX-M. Hordijk *et al*. (2013) [15] also reported

occurrence of isolates having more than one type of ESBL/AmpCgene. *bla*TEM 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.

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