RESEARCH ARTICLE

Isolation and Molecular Characterization of Extended Spectrum Beta-lactamase (ESBL) Producing *Escherichia Coli* from Dogs

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Abstract

The present study was aimed to isolate and characterize the Extended Spectrum Beta-lactamase (ESBL) producing *Escherichia coli* from faecal samples of dogs in Puducherry, India. In this study, 100 *E. coli* isolates were obtained from 50 healthy dogs and 50 diarrheic dogs, out of which 33 (33%) isolates were confirmed as ESBL producers by the combination disc method. All the *E. coli* isolates were genotypically confirmed for the presence of genes responsible for ESBL production. Out of 100 *E. coli* isolates, fourteen (14%), seven (7%) and three (3%) isolates were found to be positive for *bla*TEM, SHV and CTX-M genes, respectively. The present study highlighted ESBL-producing *E. coli* in dogs in Puducherry region.

Keywords: Antimicrobial resistance, *E. coli*, ESBL, Dogs, Genotypic confirmation Public health.

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Introduction

Antibiotics are the 'wonder drugs' to combat various infectious diseases caused by microbes. For decades, multiple varieties of antibiotics have not only been used for therapeutic purposes but practiced prophylactically across industries like agriculture and animal husbandry. Uncertainty has arisen, as microbes have become resistant to common antibiotics while the host remains unaware that antibiotic resistance has emerged (Zaman *et al.,* 2017).

The resistance mechanism developed by several bacteria, especially Enterobacteriaceae, in *E. coli* is the production of extended spectrum beta-lactamase (ESBLs) enzyme, which could hydrolyse beta-lactam group of antibiotics such as penicillin, cephalosporin, cefotaxime, ceftazidime, aztreonam and related oxyimino beta lactam (Bush *et al.,* 1995). The production of CTX-M mediates this resistance, TEM and SHV β-lactamases encoded by blaCTX-M, blaSHV, and blaTEM genes, respectively. These β-lactamase (bla) gene can be plasmid-mediated or expressed chromosomally (Bush and Jacoby 2010). ESBL-producing Enterobacteriaceae in dogs was first reported in Western Europe in the mid-1980s. (Knothe *et al.,* 1983) Since then, their incidence has been increasing steadily and has become a worldwide problem (Giraud *et al.,* 2003).

Reduced bacterial susceptibility to advanced antimicrobials may further enhance the uncontrolled spread of these resistant pathogens at the animal–environment–human interface. (Prestinaci *et al.,* 2015) One of the driving forces behind increased beta lactam resistance among *E. coli* is the use of third and fourth-generation cephalosporins.

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Furthermore, the conjugative nature of *E. coli* facilitates the transfer of beta-lactamase genes (blaCTX-M, blaSHV, and blaTEM genes) from resistant donors to susceptible bacteria (Eiamphungporn *et al.,* 2018). The ESBL-producing *E. coli* isolates from companion animals is increasing and leading to alarming that the cross species spread of these

resistant bacteria could be of great public health significance (Albrechtova *et al.,* 2012).

Although there are reports of phenotypic and genotypic detection of ESBL-producing *E. coli* in farm animals (Tewari *et al.,* 2019) and poultry (Samanta *et al.,* 2015) from India, the study on prevalence of ESBL producing *E. coli* in dogs is scanty in India. Considering which, the present study was undertaken with the objective of isolation and molecular characterization of ESBL producing *E. coli* from faecal samples of healthy dogs as well as diarrheic dogs samples.

Materials and Methods

Collection and Processing of Samples

A total of number 100 faecal samples (50 healthy and 50 diarrheic) were collected at the Veterinary Clinical Complex, Rajiv Gandhi Institute of Veterinary Education and Research (RIVER), Puducherry. The samples were collected in sterile cotton swab individually packed in polypropylene tube (M/s Hi-Media, Mumbai) and were transported to Department of Veterinary Microbiology Laboratory, RIVER, Puducherry within 2 hours and immediately processed for culture and isolation.

Isolation and Identification of *E. coli*

Each fecal sample was inoculated into Luria broth individually in sterile test tubes and incubated for 18 h at 37°C. A loopful of the enriched culture was streaked onto Mac Conkey's agar and incubated at 37°C for 24 h. Upon incubation, the pink lactose fermenting colonies were subjected to Gram's staining. The isolated gram-negative bacteria were identified upto species level as *E. coli* based on the morphology, cultural characters and biochemical reactions described in Bergey's Manual of Systematic Bacteriology (1984).

The *E. coli* isolates were further subjected to a polymerase chain reaction (PCR) using the primers targeting alr gene for genotypic confirmation (Yokoigawa *et al.,* 1999). The PCR amplification was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany) and the conditions for PCR reactions followed as per Yokoigawa *et al.,* 1999. The PCR products were analyzed by agarose gel electrophoresis. All the isolates confirmed as *E. coli* by PCR were taken up for further ESBL detection by phenotypic and genotypic method.

Phenotypic Identification of ESBL Production

All the *E. coli* isolates were screened for resistance against five indicator antimicrobial agents: Cefotaxime (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg) and aztreonam (30 µg). Resistance to at least one indicator antibiotic was considered a "positive" screening test for ESBL production. All the isolates positive by phenotypic screening were confirmed by double-disc method by using Ceftazidime (30 µg), ceftazidime plus clavulanic acid (30/10 µg) or cefotaxime (30 µg), cefotaxime plus clavulanic acid (30/10 µg) discs (Hi- Media) on Mueller–Hinton agar plates by Kirby-Bauer disc diffusion method following CLSI guidelines (CLSI, 2019). The zone of inhibition was determined after 16–18 h of incubation at 37°C.

An isolate was confirmed as ESBL producer when the inhibition zone diameter around the combination disc was ≥ 5 mm (synergy effect) when compared to disc containing cephalosporin alone (CLSI, 2019).

Genotypic Detection of ESBL Production

All the isolates identified as *E. coli* were further subjected to PCR with primers targeting *bla*TEM, SHV and CTX-M genes by uniplex PCR. Preparation of template DNA from *E. coli* strains was carried out as described by Zhang *et al.* (2015). The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master cycler, Germany) with primers targeting blaTEM and blaSHV according to the following programme, Initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 5 min as per Bhattacharjee et al., 2007. The PCR amplification of blaCTX-M was carried out using the following programmes-

- Initial denaturation at 95°C for 5 min followed by 5 cycles of denaturation at 95°C for 30 s,
- annealing at 65°C for 30 S, extension at 72°C for 30 s, 10 cycles of denaturation at 95°C for 30 s,

Table 1: Primers details used for PCR and its amplicon size

S. No	Primer name	Sequences	Amplicon size (bp)	References
1	alr gene	F5'CTGGAAGAGGCTAGCCTGGACGAG3' R5' AAAATCGGCACCGGTGGAGCGATC3'	366	(Yokoigawa et al., 1999)
2	TEM	F5' ATGAGTATTCAACATTTCCG 3' R5' CTGACAGTTACCAATGCTTA 3'	867	(Bhattacharjee et al., 2007)
3	SHV	F5' AGGATTGACTGCCTTTTTG 3' R5' ATTTGCTGATTTCGCTCG 3'	393	(Bhattacharjee et al., 2007)
4	CTX-M	F5' CAATGTGCAGCACCAAGTAA 3' R5' CGCGATATCGTTGGTGGTG 3'	540	(Dutta et al., 2013)

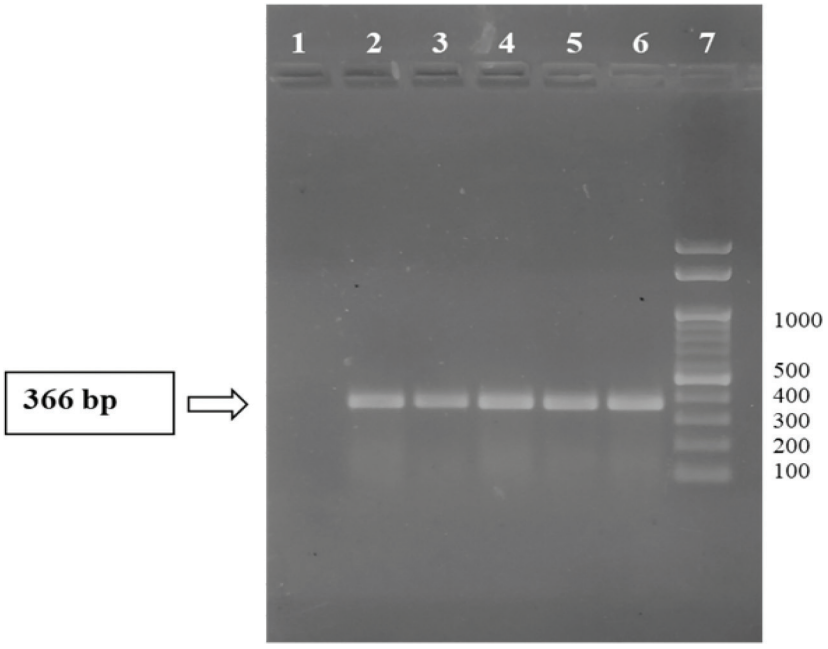

Fig 1: Agarose gel electrophoresis showing the results of polymerase chain reaction amplified product of 366 bp for the alr gene of *E. coli* isolates. Lane 1: Negative control; Lane 2,3,4,5&6: Field isolates and positive control for alr gene of *E. coli* respectively; Lane 7: 100 bp ladder.

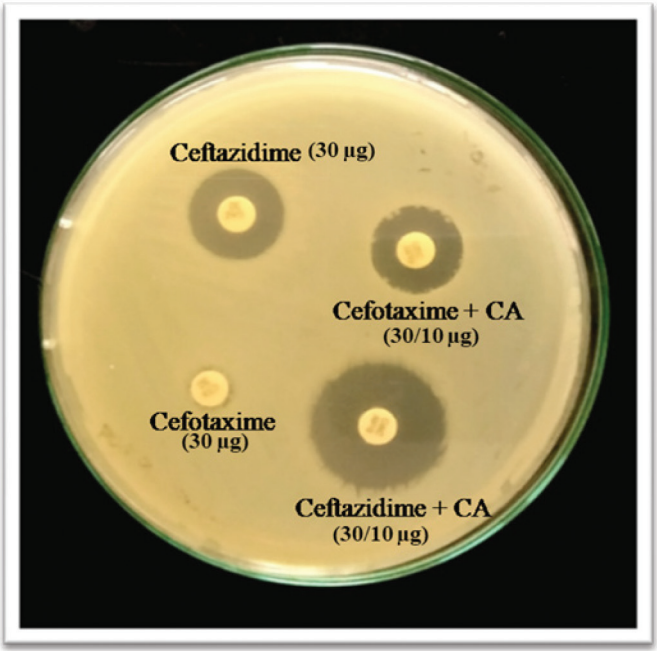

Fig 2: Phenotypic confirmation of ESBL production of *E. coli* isolate

- annealing at 62°C for 30 s, extension at 72°C for 30 s, 15 cycles of denaturation at 95°C for 30 s,
- annealing at 60°C for 30 s, extension at 72°C for 30 s, 15 cycles of denaturation at 95°C for 30 s,
- annealing at 58°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 7 min as per Dutta et al., 2013 and the primers details were described in Table 1. The PCR products were analyzed by agarose gel electrophoresis.

Results and Discussion

In the present study, among 100 canine faecal swabs (50 healthy and 50 diarrheic) examined, a total of 100 *E. coli* isolates were obtained, in using cultural and biochemical characterization followed by genotypic confirmation by PCR using alr gene specific for *E. coli* (366 bp) (Fig. 1).

In this study, a total of 33 (33%) *E. coli* isolates were confirmed as ESBL producers by combination disc method (Fig. 2). Of the 33 isolates, 16% (8/50) of the isolates were from the healthy dogs and 50% (25/50) isolates from the diarrheic dogs.

A similar frequency of occurrence of ESBL producing *E. coli* in dogs ranging from 1 to 33.3%, has been reported previously from various countries such as United States (O'Keefe *et al.,* 2010), Europe (Ewers *et al.,* 2010), Korea (So *et al.,* 2012), Netherland (Hordijk *et al.,* 2013), Switzerland (Huber et al., 2013), Denmark (Dierikx *et al.,* 2013), Germany (Schmiedel *et al.,* 2014) Similarly, Tamang *et al.* (2012), Mandakini *et al.* (2015) and Gundran *et al.* (2019) have also reported ESBL production in *E. coli* isolates from dogs ranging from 15.75 to 44.23 % from Korea, India and the Philippines.

The genotypic method helps confirm genes' presence in ESBL production in *E. coli* isolates. The isolates were screened for the presence of TEM, SHV, CTX-M or other bla genes by PCR amplification (Falagas and Karageorgopoulos, 2009). By Polymerase chain reaction, out of 100 *E. coli* isolates, fourteen (14%), seven (7%) and three (3%) isolates were found positive for bla TEM, SHV and CTX-M genes, respectively, by PCR (Fig. 3 to Fig. 5). Of the 14 TEM positive isolates, 16% (8/50) were from the healthy dogs and 12% (6/50) from the dogs with diarrhea.

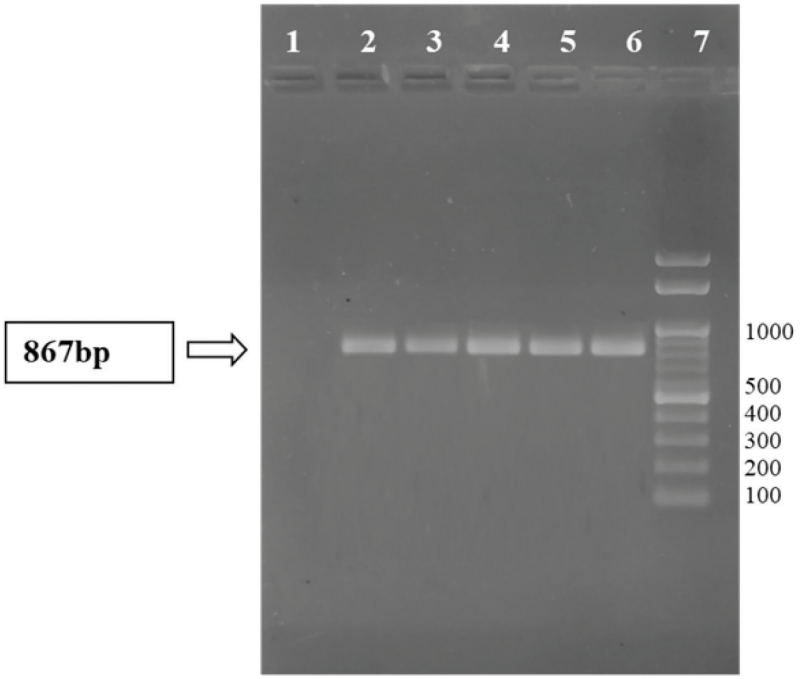

Fig 3: Agarose gel electrophoresis showing the results of polymerase chain reaction amplified product of 867 bp for the TEM gene in *E. coli* isolates. Lane 1: Negative control; Lane 2&3,4,5,6: Positive control and Field isolates for TEM gene in *E. coli*, respectively; Lane 7: 100 bp ladder.

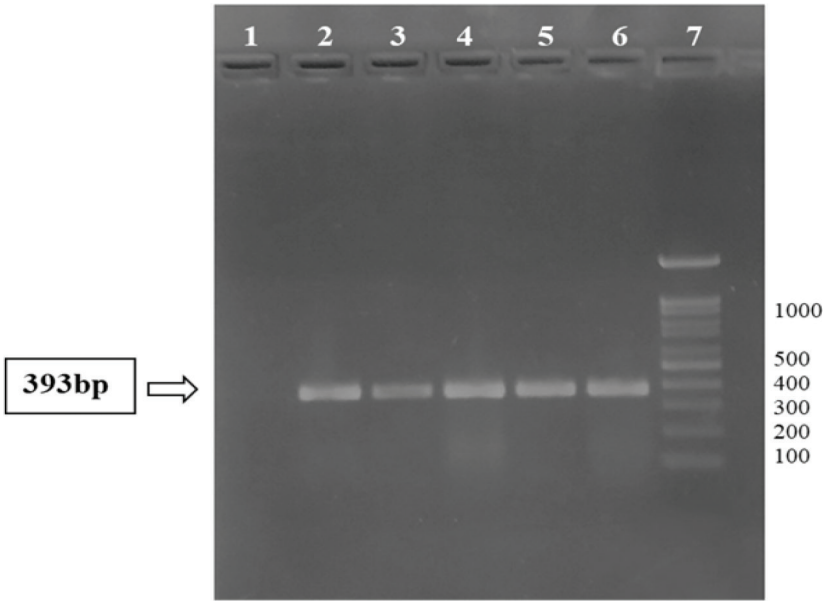

Fig 4: Agarose gel electrophoresis showing the results of polymerase chain reaction amplified product of 393 bp for the SHV gene in *E. coli* isolates. Lane 1: Negative control; Lane 2&3,4,5,6: Positive control and Field isolates for SHV gene in *E. coli* respectively; Lane 7: 100 bp ladder.

Similarly, of the 3 CTX-M positive isolates, 2% (1/50) were from healthy dogs and 4% (2/50) from dogs with diarrhea and all the 7 (14%) SHV positive isolates were from healthy dogs. In this study, the most common ESBL genotype among *E. coli* isolates was found to be SHV and TEM

Whereas the presence of TEM, SHV and CTX-M genes in ESBL-positive *E. coli* isolates at different ranges has been reported by several studies from different countries such as O'Keefe *et al.* (2010) detected one (9.09%) and 10 (90.09%) of 11 ESBL positive isolates harbored SHV and CTX-M respectively in isolates from dogs and cats associated with urinary tract infection in the United States. Similarly, Mandakini *et al.* (2015) detected TEM (6.47%) and CTX-M (2.94%) genes in isolates from faecal samples of diarrhoeic piglets in India.

Tamang *et al.* (2012) reported 1.91% (12/628) of the isolates confirmed phenotypically as ESBL producer harbored CTX-M gene in stray dogs in South Korea. Das *et al.* (2017) reported 36% and 12%isolates harbored blaCTX-M and blaTEM genes, respectively and none of the isolates carried blaSHV genes from subclinical mastitis in cattle in West Bengal. However,

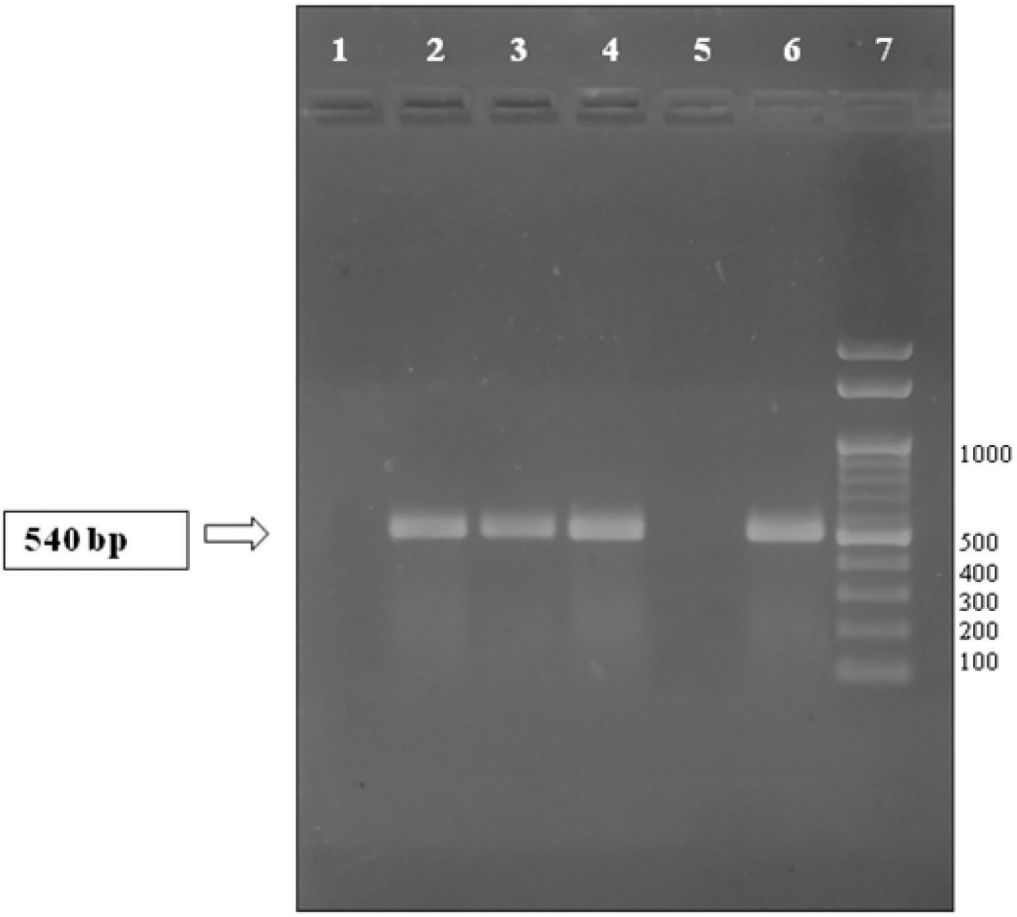

Fig 3: Agarose gel electrophoresis showing the results of polymerase chain reaction amplified product of 867bp for the TEM gene in *E. coli* isolates. Lane 1: Negative control; Lane 2&3,4,5,6: Positive control and Field isolates for TEM gene in *E. coli*, respectively; Lane 7: 100 bp ladder.

Rawat *et al*. (2018) reported a much higher prevalence of the TEM and SHV of 20% and 31.6% of the *E. coli* isolates from faecal samples of dairy cattle from Chhattisgarh, India.

In the present study, higher rates of positivity of ESBL production in *E. coli* confirmed by phenotypic methods in comparison with PCR might be because other resistance genes of ESBL production were not targeted in the present study. IMP, VIM, OXA, NDM, KPC, CMY, etc.

The difference in prevalence and occurrence of different genes between different studies can have several explanations. These include geographic differences, selection of animals, methods used for sampling, antibiotic usage and bacterial isolation (such as enrichment procedures and the media used).

This is the first study to describe the carriage of ESBL-producing *E. coli* in fecal samples of dogs in Puducherry. Hence such dogs harboring ESBL-producing *E. coli* may pose a threat to humans.

CONCLUSIONS

It can be concluded that a high prevalence 33% of ESBL-producing *E. coli* isolates in faecal samples of dogs in Puducherry. The most common ESBL genotype among *E. coli* isolates was SHV and TEM. The incidence of beta-lactamase resistance might probably result from indiscriminate use of third generation cephalosporins in canine practice, which may lead to therapeutic failures in treating the infections caused by *E. coli*.

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