Original Article

Prevalence and molecular characterization of extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* isolated from dogs suffering from diarrhea in and around Kolkata

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Abstract

Background: Dogs are the favorite companion animals among humans. The close interaction between dogs and people increases the risk of antibiotic resistance spreading. Surveillance for antimicrobial resistance and the identification of ESBL-producing *Escherichia coli* as an indicator bacterium is an important tool for managing antimicrobial drug therapy. 

Aims: The present study targeted to identify and characterize ESBL-producing *E. coli* among dogs suffering from diarrhea in and around Kolkata. 

Methods: Isolation and identification of *E. coli* from dogs suffering from diarrhea (n=70) along with screening for the production of both ESBL and AmpC. The isolates were further characterized through antimicrobial resistance profiling, resistance genes (*blaCTX-M, blaTEM, and blasuv*) screening, and phylogenetic group study. 

Results: Among the 70 isolates, 21 (30%) were confirmed ESBL producers. An antibiogram typing of ESBL-producing *E. coli* revealed that the majority of them were resistant to norfloxacin (85.7%) followed by tetracycline (61.90%), doxycycline (57.14%), piperacillin/tazobactam (52.38%), cotrimoxazole (47.62%), amikacin (46.22%), amikacin (23.81%), and chloramphenicol (19.05%). Major resistance genes included *blaCTX-M* (100%), *blatEM* (28.57%), and *blasuv* (9.50%). The predominant phylogenetic groups were phylogroup A (76%) followed by phylogroup D (24%). 

Conclusion: The current investigation reported a high prevalence of both ESBL and AmpC β-lactamase (AmpC) producing *E. coli*, co-resistance to a distinct group of antibiotics, and co-existence of different ESBL genes in dogs. Our findings highlight the importance of diagnostic antimicrobial susceptibility testing for proper antimicrobial therapy and to prevent antimicrobial resistance from spreading to humans from dogs in Kolkata and the surrounding area.

Key words: Antibiotics, Dog, *E. coli*, ESBL, PCR

Introduction

The emergence of extended-spectrum beta-lactamase (ESBL) and AmpC type beta-lactamase (AmpC) producing bacteria are of considerable concern in veterinary and human medicine (Laxminarayan et al., 2013; Ventola, 2015). The presence of infection due to ESBL/pAmpC-producing bacteria limits the treatment options with existing antibiotics in animals as well as humans (Goldstein et al., 2012). Antibiotic usage that is indiscriminate in humans and animals hastens the emergence of antibiotic resistance in both Gram-positive and Gram-negative bacteria. However, resistance in Gram-negative bacteria is the most serious threat to human and animal health (Laxminarayan et al., 2013; Kuenzli, 2016).

ESBLs and AmpC increase beta-lactam antibiotic resistance and are principally responsible for antibiotic resistance in *Enterobacteriaceae* (Pitout, 2012). The ESBL-producing *Enterobacteriaceae* are resistant to a different class of beta-lactams drugs such as penicillin, amoxicillin, extended-spectrum cephalosporins (ESCs) (Dahms et al., 2015; Kuenzli, 2016). Approximately 65% of total antibiotic comes under the beta-lactam family (Thakuria and Lahon, 2013), which consists of penicillin and its derivative, cephalosporins, carbapenems, cephamycins, and monobactams (Holten and Onusko, 2000). Beta-lactam antibiotics inhibit
bacterial cell wall formation, whereas beta-lactamases hydrolyse the beta-lactam ring of antibiotics, rendering them inactive (Džidić et al., 2008). ESBL mediated resistance can be prevented by beta-lactamase inhibitors for instance clavulanic acid, tazobactam, or sulbactam (Chong et al., 2011). Gene encoding beta-lactamase enzyme can be found either chromosomally or on the mobile genetic element which includes plasmid, gene cassettes, or transposons (Babić et al., 2006).

AmpC is mainly found in Gram-negative bacilli which are resistant to broad-spectrum antimicrobials that include penicillin, cephalexin, monobactams, and cephamycins. AmpC is easily impacted by cloxacillin, oxacillin, and aztreonam, but other enzyme inhibitors, such as clavulanic acid, sulbactam, and tazobactam have a limited effect on them (Jacoby, 2009). Organisms over-expressing AmpC are unaffected by beta-lactam drugs except for ceftazidime, ceftazidime, and carabapenem (Perez-Perez and Hanson, 2002). AmpC-producing Enterobacteriaceae has been largely reported in animal and human patients (Chakraborty et al., 2014).

*E. coli* belongs to the Enterobacteriaceae family and is a Gram-negative, rod-shaped, facultatively anaerobic coliform bacterium ( Clermont et al., 2000). The pathogenic variants of *E. coli* may cause either severe intestinal or extra-intestinal diseases (Dobrindt, 2005). The ESBL/AmpC-producing *E. coli* is also found in the gastrointestinal tracts of animals as commensal bacteria. Due to their close contact with humans, dogs may be able to transfer ESBL/AmpC-producing *E. coli* to humans. Previous studies reported pathogenic strains from humans in dogs (Ovejero et al., 2017), and humans and dogs within the same household were found to have identical ESBL/AmpC strains (Ljunquist et al., 2016).

Companion animals such as dogs are treated with several antibiotics for treatment similar to those used in humans on a regular basis. The most often related genes with animal resistance encoded various CTX-M beta-lactamases followed by TEM and SHV beta-lactamases (Hasman et al., 2005; Smet et al., 2010; Ewers et al., 2011; Ewers et al., 2012). The presence of ESBL and AmpC genes in Enterobacteriaceae has been documented in the feces of healthy dogs in Europe in recent years (Haenni et al., 2014; Damborg et al., 2016; Ljunquist et al., 2016). However, the plasmids harboring these ESBL and AmpC genes are significantly less well-known (Haenni et al., 2014; Damborg et al., 2016). In the recent decade, antimicrobial drug use has been linked to an increase in antimicrobial resistance in canine *E. coli* isolates. The *E. coli* strains causing canine diarrhea were the first pathogens that were detected by PCR which is considered the most reliable method to identify pathogens from normal intestinal bacteria (Normand et al., 2000).

The current research was carried out to investigate the prevalence of ESBL/AmpC-producing *E. coli* in dogs suffering from canine diarrhea in Kolkata, India. Because of the potential for ESBL/AmpC-producing *E. coli* isolates to be transmitted from dogs to their owners, we performed their further characterization through drug resistance profiling, identification of ESBL and AmpC resistance genes, and phylogenetic grouping.

### Materials and Methods

#### Bacterial isolates

The purpose of this study was to determine the prevalence and pattern of antibiotic resistance of ESBL and AmpC-producing *E. coli* isolates from dogs with canine diarrhea Supplementary Table 1 (ST1) from the Veterinary Clinical Complex, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences along with different private clinics of Kolkata, India. A total of 70 fecal samples were collected from different dogs suffering from canine diarrhea using sterile HiCulture™ Collecting Swab (HiMedia, India), and were transferred in an ice pack to the laboratory for processing within 2-4 h of collection. All the samples were collected by taking full consent from the owner of the dog.

#### Isolation of *E. coli* from canine fecal samples

Each of the 70 fresh samples was inoculated into 5 ml of Difco™ Nutrient Broth (BD, BBL, Difco, USA) for bacterial growth at 37°C for 24 h. The growth in nutrient broth was then transferred to Difco™ MacConkey Agar (BD, BBL, Difco, USA), and *E. coli* was isolated precisely by incubating at 37°C for another 24 h.

*E. coli* identification was confirmed once again by inoculation on HiCrome™ Eosin Methylene Blue (EMB) Agar plate (HiMedia, India) and incubation at 37°C for 24 h. Out of 70 (N) fecal samples processed, 2 colonies from each sample (70*2=140 isolates) were obtained based on colony morphology. Metallic green colored suspected colonies were picked up and further cultured in Difco™ Nutrient Broth (BD, BBL, Difco, USA), and simultaneously streaked into Difco™ Mueller Hinton Agar (MHA) (BD, BBL, Difco, USA) plates for additional study. For future usage, all pure cultures were stored at -70°C (15% LB Glycerol stock).

#### Morphological and biochemical confirmation of *E. coli* isolates

Suspected colonies were subjected to Gram staining with smeared slide by a standard protocol for morphological characterization of *E. coli*. Biochemical identification of the isolates was performed based on a standard biochemical test of *E. coli* described previously (Quinn et al., 2011; Vashist et al., 2013).

#### Phenotypic detection of ESBL production

Screening of ESBL production for *E. coli* isolates was performed through the Double Disc Diffusion test. Briefly, isolates were inoculated at 37°C overnight on a Difco™ Mueller Hinton Agar plate containing ceftazidime (30 µg), cefotaxime (30 µg), ceftazidime-clavulanic acid (30/10 µg), and cefotaxime-clavulanic acid (30/10 µg), respectively (Andrews, 2012; Kar et al., 2015). The zone of inhibition for the antibiotic discs of
ceftazidime (30 µg) and cefotaxime (30 µg) was compared with the corresponding discs of ceftazidime-
clavulanic acid (30/10 µg) and cefotaxime-clavulanic acid (30/10 µg), respectively. An increase of 50% or ≥5
mm in the zone of inhibition towards the disc of antibiotic-clavulanic acid compared to a disc of antibiotic
alone was considered positive for ESBL production.

Phenotypic detection of AmpC production
For detection of AmpC production, all confirmed as
ESBL E. coli strains (21) were subjected to the cefoxitin-
cloxacillin Double Disc Synergy Test (CC-DDST) test
(HiMedia, India) following the procedure mentioned
previously (Polsfuss et al., 2011). Cloxacillin’s inhibitory effect on AmpC enzymes is the basis for this
Test. When compared to cefoxitin alone, an increase of 4
mm in the zone of inhibition in the presence of
cloxacillin-cefoxitin combination was considered AmpC producing E. coli (Polsfuss et al., 2011).

Antimicrobial susceptibility testing
Antibiotic susceptibility pattern of phenotypically confirmed E. coli strains was determined by the disk
diffusion method against 10 different antibiotic discs
(HiMedia, India). Antimicrobial susceptibilities of the following antibiotics were investigated: doxycycline (30
µg), fosfomycin (200 µg), cotrimoxazole (25 µg),
chloramphenicol (30 µg), gentamicin (10 µg), imipenem
(10 µg), amikacin (30 µg), piperacillin-tazobactam
(100/10 µg), tetracycline (30 µg), and norfloxacin (10
µg). As per the Clinical and Laboratory Standard
Institute (CLSI), the isolates were classed as sensitive,
intermediately resistant, or resistant (CLSI, 2014).

DNA extraction and PCR based detection of uidA gene
The conventional heat lysis protocol was used to extract DNA from all of the phenotypically confirmed E.
coli isolates (140). DNAs were analyzed by PCR for the presence of uidA gene (Matloko et al., 2021), an
enzymatic marker for detection and validation of E. coli
in suspected isolates. The Veriti 96-well thermal cycler
(Applied Biosystems, USA) was used to run the PCR
reactions by the method described previously (Molina et
al., 2015). The amplified PCR products were analyzed by
1.5% (w/v) agarose gel containing ethidium bromide (0.5
µg/ml, Sigma, USA) and subsequently visualized by
gel documentation system. 162 bp PCR amplicons of
uidA gene were detected in 70 E. coli isolates (50%) out
of the 140 isolates (Fig. 1). The strains recovery rate is
50%.

PCR based detection of antimicrobial resistance
genes
All the confirmed ESBL-producing E. coli isolates
were screened by several PCR assays for the presence of
ESBL and AmpC genes using primers illustrated in
Supplementary Table 2 (ST2). PCR reaction was
performed in a total volume of 25 µL containing 0.5 µL
of each DNA template (50 ng/µL), 2.5 µL of 10X PCR
buffer, 2.0 µL of 25 mM MgCl₂, 0.5 µL of dNTPs mixture (10 mM) and 1 U of Taq DNA polymerase (5
U/µL). 0.5 µL (10 µM) of each primer was used in a
single PCR reaction and the final volume (25 µL) was
adjusted with nuclease-free water. All the isolates were
screened for the presence of major ESBLs genes blatem,
blashv, blactx-m (Bhattacharjee et al., 2007), and blaampc
(Shahid et al., 2012), minor ESBLs genes blaveb, blases,
and blaper (Dallenne et al., 2010), plasmid-mediated
fluoroquinolone resistance genes aac(6’)-Ib-cr, qnrA,
qnrB, and qnrS (Park et al., 2006; Robicsek et al., 2006;
Ciesielczuk et al., 2013), tetracycline resistance genes
tet(A), tet(B), tet(C), tet(D), and tet(E) (Ng et al., 2001),
mobile genetic elements, ISEcpl (Saladin et al., 2002),
tetrahydrofolate reductase genes, intgene1, and intgen2
(Machado et al., 2005), and pAmC gene groups,
blaACC, blaFOX, blasmox, blaIMAM, blactx, and blambr
(Pérez-Pérez and Hanson, 2002). PCR reactions were executed in Veriti 96-well thermal cycler
(Applied Biosystems, USA). The PCR products were
analyzed in 1.5% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml, Sigma, USA) and gel
documentation system was used to visualize the results.

Fig. 1: A 2% (w/v) agarose gel ofUidA gene fragments amplified from E. coli isolated from dogs. Lanes 1-4: UidA
gene specific PCR amplicons, Lane 5: 100 bp DNA ladder, and Lane 6: No DNA template control

Phylogenetic grouping by triplex PCR assays
A triplex PCR was carried out to resolve the phylogenetic group of E. coli based on PCR
amplification of two gene fragments (chuA and yjaA) and an
unknown DNA fragment TspE4.C2 as defined
formerly (Clermont et al., 2000). Each PCR reaction was
carried out separately in a 25 µL PCR mix containing 0.5
µL of each DNA template (50 ng/µL), 2.5 µL of 10X
PCR buffer, 2.0 µL of 25 mM MgCl₂, 0.5 µL of dNTPs
mixture (10 mM) and 1 U of Taq DNA polymerase (5
U/µL). 0.5 µL (10 µM) of each primer was used in a
single PCR reaction and the final volume (25 µL) was
adjusted with nuclease-free water. After initial
denaturation at 94°C for 4 min, samples were subjected
to one amplification regime comprising 35 cycles of
94°C for 45 s, 53°C for 45 s, and 72°C for 1 min,
followed by a final extension of 7 min at 72°C. The
amplified PCR products were electrophoretically examined by running them at 100 V for 45 min on a
1.5% (w/v) agarose gel containing ethidium bromide (0.5
g/ml) in Tris-borate-EDTA (TBE) buffer and visualizing
them under UV transillumination.
Results

Isolation and molecular documentation of *E. coli*

Out of 70 (N) faecal samples processed, 2 colonies from each sample \(70 \times 2 = 140\) isolates were obtained based on colony morphology. All the 140 phenotypically confirmed *E. coli* isolates were further characterized using PCR amplification of the *uidA* gene for molecular documentation of *E. coli*. *uidA* gene encodes β-glucoronidase enzyme which is considered a signpost for the *E. coli* isolates. 162 bp PCR amplicons of *uidA* gene were detected from all the 70 *E. coli* isolates (50%) (Fig. 1).

These, 70 isolates also exhibited rose pink dotted colonies in MacConkey agar (Fig. 2A) and metallic green colonies in EMB agar (Fig. 2B). Gram staining of suspected *E. coli* isolates showed Gram-negative (-ve) rod-shaped bacteria. In addition, all the 70 isolates followed typical biochemical properties of *E. coli* like catalase (+ve), oxidase (-ve), Indole (+ve), Methyl red (+ve), Voges Proskauer (-ve), Citrate (-ve), and urease (-ve).

Phenotypic characterization for ESBL and AmpC production

Out of 70 *E. coli* isolates, 21 (30%) of them were ESBL-producing *E. coli* isolates based on the zone of inhibition in the double disc diffusion test. The test was carried out for all *E. coli* isolates with the cefotaxime (CTX-30) antibiotic disc with and without clavulanic acid in an MHA plate for overnight incubation at 37°C and finally, a zone of inhibition ≥5 mm between 2 discs was documented as ESBL-producing *E. coli* (Fig. 3A).

Nineteen isolates (90.5%) were identified to be positive for AmpC production among 21 ESBL-producing *E. coli* strains by cefoxitin-cloxacillin Double Disc Synergy test (CC-DDST) (Fig. 3B). So, co-production of both ESBL and AmpC was reported in our study.

Antibiotic susceptibility pattern of ESBL-producing *E. coli* isolates

Disc diffusion test was carried out to detect antibiotic susceptibility/resistance patterns of *E. coli* isolates against different antibiotics. Antibiotic susceptibility pattern was classified as sensitive, intermediate, and resistant to each antibiotic depending on the respective zone of inhibition. None of the isolates tested positive for phosphomycin and imipenem resistance, although 10 isolates (48%) were identified as resistant intermediates to imipenem. The rate of antibiotic resistance in *E. coli* was 85.7% for norfloxacin, 61.90% for tetracycline, 57.14% for doxycycline, 52.38% for piperacillin/tazobactam, 47.62% for cotrimoxazole, 42.62% for gentamicin, 23.81% for amikacin, and 19.05% for chloramphenicol, respectively. Out of 21 ESBL positive isolates, 16 (76.19%) were multidrug-resistant. The overall antimicrobial resistance in ESBL-producing *E. coli* isolates is presented in Fig. 4 with MDR isolates highlighted and underlined in Table 1. **Fig. 2:** Colonial morphology of *E. coli* isolated from dogs. (A) *E. coli* isolates displayed rose pink dotted colonies in MacConkey agar, and (B) *E. coli* isolates demonstrated metallic green colonies in EMB agar. **Fig. 3:** Phenotypic characterization of *E. coli* isolated from dogs. (A) Detection of ESBL production based on zone of inhibition in Double Disc Diffusion test, and (B) Detection of AmpC production based on zone of inhibition in cefoxitin-cloxacillin Double Disc Synergy test (CC-DDST). **Fig. 4:** Antimicrobial resistance pattern of ESBL-producing *E. coli* isolates from dogs suffering from diarrhea. COT: Cotrimoxazole, IPM: Imipenem, FO: Fosfomycin, NX: Norfloxacin, TE: Tetracyclin, GEN: Gentamicin, AK: Amikacin, DO: Doxyccycin, C: Chloramphenicol, and PIT: Piperacilin+Tazobactam
Table 1: Antibiotic resistance genes profile, antibiogram, and phylogenetic grouping of ESBL/AMPC-producing E. coli isolated from dogs

| Isolates   | Antibiotic resistance determinants | Antibiotic resistance profile | Phylo-group |
|------------|-----------------------------------|-------------------------------|-------------|
| Sec-5      | blaCTXM, blaTEM, tet(A), aac(6’)-ib-cr, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-2g      | blaCTXM, tet(A), qnrS, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-2h      | blaCTXM, blaTEM, tet(A), tet(B), qnrS, qnrA, aac(6’)-ib-cr, blaSHV, integ 2, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-3a      | blaCTXM, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-5a      | blaCTXM | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-15b     | blaCTXM, tet(B), blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-17a     | blaCTXM, blaTEM, blaSHV, tet(A), qnrS, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-17b     | blaCTXM, blaTEM, tet(A), qnrS, qnrA, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-20a     | blaCTXM, blaTEM, tet(A), tet(B), qnrS, qnrA, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-20b     | blaCTXM, blaTEM, tet(A), tet(B), qnrS, qnrA, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-22a     | blaCTXM, blaTEM, tet(A), tet(B), qnrS, qnrA, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-23a     | blaCTXM, blaTEM, blaSHV, blaTEM, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-24a     | blaCTXM, blaTEM, blaSHV | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-30a     | blaTEM, tet(A), blaSHV | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-32a     | blaTEM, blaSHV, qnrS, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-32b     | blaTEM, blaSHV, qnrS, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-35a     | blaTEM, aac(6’)-ib-cr, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-46a     | blaTEM, qnrS | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-52a     | blaTEM, tet(A), tet(B), qnrS, blaSHV, blaTEM, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-54b     | blaTEM, qnrS, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |
| Rh-56a     | blaTEM, blaTEM, tet(A), qnrS, blaSHV, ISEcp1 | Resistant: DO, COT, C, IM, FO, GEN | A           |

Isolates with multidrug-resistant are in bold and underlined in the table. COT: Cotrimoxazole; IPM: Imipenem; FO: Fosfomycin; NX: Norfloxacin; TE: Tetracyclin; GEN: Gentamicin; AK: Amikacin; DO: Doxycyclin; C: Chloramphenicol; PIT: Piperacillin + Tazobactam, and NIL: No antibiotics was intermediate sensitive.

Fig. 5: A 2% (w/v) agarose gel of blaCTXM gene fragments amplified from E. coli isolated from dogs. Lanes 1-6: blaCTXM gene specific PCR amplicons, Lane 7: 100 bp DNA ladder, and Lane 8: No DNA template control.

producing E. coli isolates were analyzed by PCR for the presence of different resistance genes (Table 2). The major beta-lactamase genes were amplified using PCR methods for the detection of blaCTXM, blaTEM, and blaSHV genes. The most predominant resistance gene was blaCTXM which was detected in all the isolates (100%) (Fig. 5), followed by the blaTEM gene in 6 isolates (28.57%) whereas the blaSHV gene was detected in two E. coli isolates (9.50%). In our study, we observed that distinct beta-lactamase genes coexisted within the same isolates. The blaCTXM with blaTEM was the most common combination with or without blaSHV in this study (Table 3).

A separate multiplex PCR was implemented for the detection of minor beta-lactamase genes blaGES, blaPER, and blaVEB type of ESBL resistance gene in E. coli isolates. Seven isolates (33.33%) indicated PCR amplification for the blaVEB type of ESBL gene although none of the isolates was found to be positive for blaGES or blaPER type of ESBL gene. Tetracycline resistance tet(A) and tet(B) gene was detected in 11 (52.38%) and 6 (28.57%) E. coli isolates, respectively whereas other tetracycline...
resistance genes for instance tet(C), tet(D), and tet(E) were not detected in our study. Two separate multiplex PCR assays were applied for the identification of fluoroquinolone-resistant genes. Out of total 21 isolates, 14 (66.67%) isolates showed resistant to qnr(S) gene while 5 (23.8%) and 3 (14.29%) isolates were resistance to qnr(A) and qnr(B) gene, respectively. Other two fluoroquinolone-resistant genes such as qnr(C) and qnr(D) were not recognized in our samples. Another plasmid-mediated resistant gene of fluoroquinolones namely aac(6')-ib-cr was detected in 6 isolates (28.57%) by PCR-based detection. The presence of integrin 2 was reported in one isolate (5.8%); however, integrin 1 was not noticed in the current study. The mobile genetic element, ISEcp1 was detected in 17 (80.95%) E. coli isolates.

| Resistance gene (cassette) | Number of isolates | Percentage |
|----------------------------|--------------------|------------|
| blaCTX-M                   | 21                 | 100.00     |
| blaTEM                     | 6                  | 28.57      |
| blaSIV                     | 2                  | 9.52       |
| blaVEB                     | 7                  | 33.33      |
| tet(A)                     | 11                 | 52.38      |
| tet(B)                     | 6                  | 28.57      |
| qnrS                       | 14                 | 66.67      |
| qnrA                       | 5                  | 23.81      |
| qnrB                       | 3                  | 14.29      |
| aac(6')-ib-cr              | 6                  | 28.57      |
| Integron 2                 | 1                  | 4.76       |
| ISEcp1                     | 17                 | 80.95      |
| blaAmpC                    | 18                 | 85.71      |
| blaCIT                     | 1                  | 4.76       |

Table 2: Antibiotic resistance gene profiling of ESBL/AmpC-producing E. coli isolates from dogs in Kolkata, India

PCR-based identification assay was implemented for genotypic characterization of AmpC producing E. coli. blaAmpC gene was detected in 18 (85.71%) (Fig. 6) isolates; although 19 (90.48%) isolates were phenotypically confirmed as AmpC producers. The AmpC gene, blaCIT was detected in 1 isolate (4.75%) but other AmpC genes like FOX, MOX, DHAM, MIR, and ACC were not detected in this study.

**Phylogrouping of isolates**

Previously, E. coli strains were divided into four main phylogenetic groups (A, B1, B2, and D) by multilocus enzyme electrophoresis or ribotyping (Abram et al., 2021). Later PCR-based phylogenetic grouping was implemented according to the Clermont method (2000) to assign phylogroups of the E. coli isolates. In the case of positive amplification triplex PCR will generate 279 bp, 211 bp, and 152 bp PCR fragments for chuA, yjaA, and TspE4C2 regions, respectively (Clermont et al., 2000) (Fig. 7). Among 21 E. coli isolates, chuA specific PCR amplificon was detected in 5 isolates. However, none of these isolates were positive for yjaA specific PCR amplificon. Only one isolate (Rh-24a) amplified two DNA fragments in PCR assay, 279 bp fragment of chuA and 152 bp fragment of TspE4C2. Among all E. coli isolates phylogenetic group A was the predominant phylogenetic group (16 isolates, 76%) whereas no isolate was identified for both phylogroup B1 and B2. Rest 5 isolates (24%) were categorized in phylogenetic group D (Fig. 8). The detailed antibiogram, resistance genes profiling, and phylogenetic grouping of E. coli isolates were summarized and presented in Table 3.

Fig. 6: A 2% (w/v) agarose gel of blaAmpC gene fragments amplified from E. coli isolated from dogs. Lanes 3-8: blaAmpC gene-specific PCR amplicons, Lane 2: 100 bp DNA ladder, and Lane 1: No DNA template control

Fig. 7: Multiplex PCR profiles showing Clermont phylo-typing method of E. coli isolated from dogs. Lanes 1-5: chuA and TspE4C2 gene-specific PCR amplicons, Lane 7: No DNA template control, and Lane 6: 100 bp DNA ladder

Fig. 8: Frequency of the different phylogroup of ESBL-producing E. coli isolated from dogs suffering from diarrhea

| Patterns of ESBL genotype | No. of isolates | Percentage |
|---------------------------|-----------------|------------|
| blaCTX-M + blaTEM + blaSIV| 1               | 4.76       |
| blaCTX-M + blaTEM          | 5               | 23.81      |
| blaCTX-M + blaSIV          | 1               | 4.76       |
| blaCTX-M only              | 14              | 66.67      |

Table 3: Distribution of different patterns of ESBL genotypes among ESBL-producing E. coli isolates from dogs in Kolkata, India
Discussion

Monitoring antimicrobial resistance trends among antimicrobial resistance (AMR) bacteria isolated from dogs need to be conducted for guiding antimicrobial usage in canine practice. Despite the possible threat to human and animal health, research outcomes on antimicrobial-resistant bacteria in canine over and above companion animals are very limited. Numbers of studies have been already documented on the prevalence of ESBL-producing *E. coli* in humans (Day et al., 2019). However, reports on the prevalence of ESBL-producing *E. coli* in dogs are inadequate (Deepthi et al., 2020; Salgado-Caxito et al., 2021). The resistance profile of multi-drug resistance bacteria and the potential risk of resistance transmission is important for assessing the risk of transmission from dogs to humans. The present study was designed to investigate the prevalence of ESBL/AmpC-producing *E. coli*, antibiotics resistance patterns in different isolates, the identification of resistance genes, and the phylogenetic grouping of *E. coli* isolate recovered from dogs suffering from diarrhea in Kolkata, India.

In present experiment, we observed a higher proportion (30%) of *E. coli*-positive for ESBL than the 22% reported at a veterinary teaching hospital in South Africa (Qekwana et al., 2018) or the 27% reported in the USA (Stiffler et al., 2006). Although the prevalence rate was not as much as 56% reported at veterinary teaching hospital, USA (Seguin et al., 2003). Differences in results could be attributed to differences in study designs or sample collection methods. In the Netherlands, an earlier study reported a high level of ESBL-producing *E. coli* in both healthy dogs (45%) and diarrheic dogs (55%) (Hordijk et al., 2013). The increasing rate of prevalence of ESBL-producing *E. coli* is creating an alarming situation that can impact animal morbidity as well as mortality. A previous study in human patients witnessed a very low proportion (68, 2.46%) of ESBL-producing *E. coli* recovered from 2,755 *E. coli* cultures from vaginal or newborn samples (Birgy et al., 2013). The prevalence of ESBL-producing *E. coli* was 62% among the patient and *bla*<sub>CTX-M</sub> (63.1%) was the highest prevalence ESBL gene detected by PCR (Mohmid et al., 2013). This detection of ESBL-producing isolates in dogs in our study could represent a public health concern if transmitted to humans.

Antibiogram study shows 85.7% isolates were resistance to norfloxacin, followed by 61.90% to tetracycline, 57.14% to doxycycline, 52.38% to piperacillin/tazobactam, 47.62% to cotrimoxazole, 42.62% to gentamicin, 23.81% to amikacin, and 19.05% to chloramphenicol. Similar results were reported for tetracycline, gentamycin, and chloramphenicol resistance in cats and dogs in Switzerland (Zogg et al., 2018). Human and companion animals are usually treated therapeutically for a particular time for bacterial infections, hence reflecting high resistance. The degree of resistance reported in each paper corresponds to the frequency and magnitude of their use in different countries. The major risks that are associated with MDR (multi-drug-resistant) *E. coli* in dogs include underlying disease conditions and high usage antimicrobial agents.

The present study revealed a high prevalence of ESBL- and AmpC-producing *E. coli* in dogs. Among the 21 ESBL-producing *E. coli* isolates, 19 isolates were positive for AmpC production which ascertained co-production of ESBL and AmpC in *E. coli*. Co-production of ESBL and AmpC is significant as it can inactivate the potentiated cephalosporins prescribed for ESBL infections.

Our study revealed that the commonest ESBLs coding gene was *bla*<sub>CTX-M</sub> (100%), followed by *bla*<sub>TEM</sub> (33.33%) and *bla*<sub>PER</sub> (28.57%). Low levels of *bla*<sub>SHV</sub> (9.52%) and no *bla*<sub>BES</sub> and *bla*<sub>BER</sub> were reported in our study. The present study exhibits a high rate of occurrence of CTX-M type ESBLs among all ESBL-positive isolates which is the following previous reports (Canton and Coque, 2006; Rossolini et al., 2008). Antibiotic abuse and overuse may play a role in the selection and spread of ESBL-positive *E. coli* (Zeynudin et al., 2018). A study conducted in the German community reported that the number of *E. coli* strains expressing ESBL was extremely low (6.3%), and majority (95.2%) of isolates harbored the CTX-M type gene as the most common type ESBL gene (Valenza et al., 2014). Another study also detected *bla*<sub>CTX-M</sub> genes as major ESBL genes where 84.3% of isolates were positive for a particular CTX-M type gene (Birgy et al., 2013). In Sweden, a study of human households carried a similar strain of ESBL producing *E. coli* to the isolates found in household dogs, confirming transfer between humans and dogs (Ljungquist et al., 2016). Zogg et al. (2018) reported that the proportion of ESBL-producers was much greater in dog isolates than in cat isolates, and major genotypes were *bla*<sub>CTX-M</sub>-1, *bla*<sub>CTX-M</sub>-14, *bla*<sub>CTX-M</sub>-27, *bla*<sub>CTX-M</sub>-55, and *bla*<sub>SHV</sub>-12 genotypes in those ESBL-*E. coli* isolates. The β-lactamase CTX-M-15 represents the most commonly reported ESBL type in canine and feline *E. coli* isolates (O’Keefe et al., 2010; Shaheen et al., 2011; Huber et al., 2013).

Phylogroup A was the most abundant phylogenetic group among all ESBL/AmpC-producing *E. coli* isolates in our survey (16 isolates, 76%), followed by phylogroup D (5 isolates, 24%), however, phylogroup B1 and B2 were not reported in our study. A previous study reported that phylogroups B2 and D of *E. coli* strains carried more virulence factors than the phylogroups A and B1 (Johnson et al., 2001). According to a previous study, resistant animal isolates exhibited phylogenetic shifts toward group A and away from groups B1 and B2, but not toward virulence-associated group D. However in human isolates, trends toward non-B2 phylogenetic groups (particularly groups A and D) were detected (Johnson et al., 2003). Previously, the ESBL-producing *E. coli* isolates (129) from humans were classified as phylogenetic group B2 (36.4%), phylogenetic group D (25.5%), and phylogenetic groups A and B1 (27.9% and 10%, respectively) where phylogenetic distribution shifts toward non-B2 phylogenetic groupings, particularly
groups D and A, were related to ESBL production in *E. coli* (Branger et al., 2005). Phylogenetic shifts toward group A and group D were seen in this investigation, which could be linked to the production of ESBL in *E. coli* isolates of canines.

Several previous studies have documented that extraintestinal pathogenic strains usually followed phylogroup B2 and D (Picard et al., 1999; Johnson and Stell, 2000), the commensal strains to groups A and B1 (Nowrouzian et al., 2019), whilst the intestinal pathogenic strains fit into groups A, B1, and D (Pupo et al., 1997). In our research, we found similar results. Five isolates with phylogroup D, which are considered pathogenic *E. coli* strains, maintain the overall virulence level and are the primary threat to both pet owners and the environment (Walk et al., 2007). Antibiotic resistance of phylogroups A may even provide a substantial benefit to the pathogen in immuno-compromised hosts.

In the present study, only dogs suffering from canine diarrhea were examined, screening of healthy controls was not performed. Diarrhea appears to have an important factor for more shedding of ExPEC and thus can contribute to the environmental dissemination of resistant flora and resistant genes. The high rates of ESBL/AmpC-producing *E. coli* in dogs argue for considerable ESBL carrier rates among pet dogs as well as companion animals. Higher antimicrobial usage, particularly the use of higher generation antibiotics may be accountable for such a higher resistance rate.

Our results reveal a high prevalence of ESBL/AmpC-producing *E. coli* isolates from dogs which are frightening for the animal as well as human health. The co-existence of the three primary ESBL genotypes, *bla* _CTX-M, bla* _TEM, and _bla* _SHV in *E. coli* isolates, as well as co-resistance to a different group of antibiotics, is concerning. In addition, to demonstrate the rapid emergence and dissemination of multi-resistant ESBL/AmpC-producing *E. coli* in dogs and other companion animals, a surveillance research with a large sample size is required. Furthermore, our findings highlight the importance of diagnostic antimicrobial susceptibility testing for effective antimicrobial therapy and to prevent the emergence of antimicrobial resistance in Kolkata and the surrounding area.

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**Conflict of interest**

The authors declare no competing interests.

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