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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 (*bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$) 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%), gentamicin (42.62%), amikacin (23.81%), and chloramphenicol (19.05%). Major resistance genes included *bla*$_{CTX-M}$ (100%), *bla*$_{TEM}$ (28.57%), and *bla*$_{SHV}$ (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 β-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 (Babic *et al*., 2006).

AmpC is mainly found in Gram-negative bacilli which are resistant to broad-spectrum antimicrobials that include penicillin, cephalosporin, monobactams, and cephamycins. AmpC is easily impacted by cloxacillin, oxacillin, and aztreonam, but other enzyme inhibitors, such as clavulanic acid, salbactum, and tazobactam have a limited effect on them (Jacoby, 2009). Organisms over-expressing AmpC are unaffected by beta-lactam drugs except for cefepime, cefpirome, and carbapenem (Perez-Perez and Hanson, 2002). AmpC-producing *Entero-bacteriaceae* 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 (Ljungquist *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 β-lactamases followed by TEM and SHV β-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; Ljungquist *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 among 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 a 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 $MgCl_2$, 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 *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ (Bhattacharjee *et al*., 2007), and bla$_{AmpC}$ (Shahid *et al*., 2012), minor ESBLs genes *bla*$_{VEB}$, *bla*$_{GES}$, and *bla*$_{PER}$ (Dallenne *et al*., 2010), plasmid-mediated fluoroquinolone resistance genes *aac(6')-Ib-cr*, *qnr*A, *qnr*B, and *qnr*S (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, ISEcp1 (Saladin *et al*., 2002), integron1, and integrin2 (Machado *et al*., 2005), and pAmpC gene groups, *bla*$_{ACC}$, *bla*$_{FOX}$, *bla*$_{MOX}$, *bla*$_{DHAM}$, *bla*$_{CIT}$, and *bla*$_{MIR}$ (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.

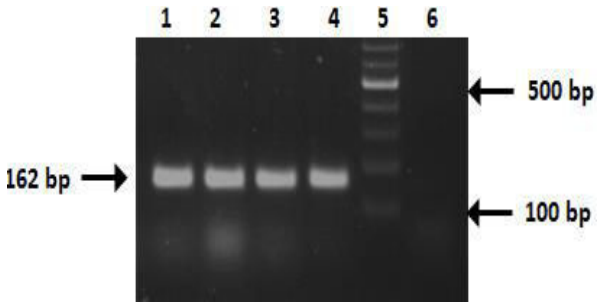


Fig. 1: A 2% (w/v) agarose gel of *UidA* gene fragments amplified from *E. coli* isolated from dogs. Lanes 1-4: *UidA* gene specific PCR amplicons, Lane 5: 100 bp DNA ladder, and Lane 6: No DNA template control

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 $MgCl_2$, 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*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 *uid*A gene for molecular documentation of *E. coli*. u*idA* gene encodes β-glucoronidase enzyme which is considered a signpost for the *E. coli* isolates. 162 bp PCR amplicons of *uid*A 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).

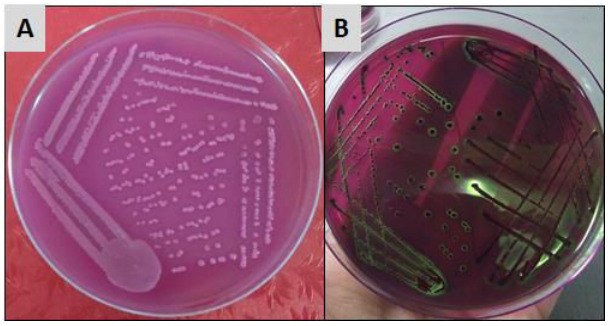


Fig. 2: Colonial morphology of *E. coli* isolated from dogs. (**A**) *E. coli* isolates displayed rose pink dotted colonies in MacConkey agar, and (**B**) *E. coli* isolates demonstrated metallic green colonies in EMB agar

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.

Molecular characterization of antimicrobial resistance genes

Among the 21 phenotypically confirmed ESBL-

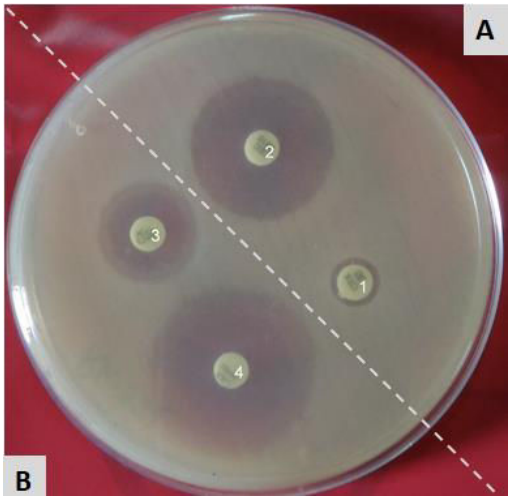
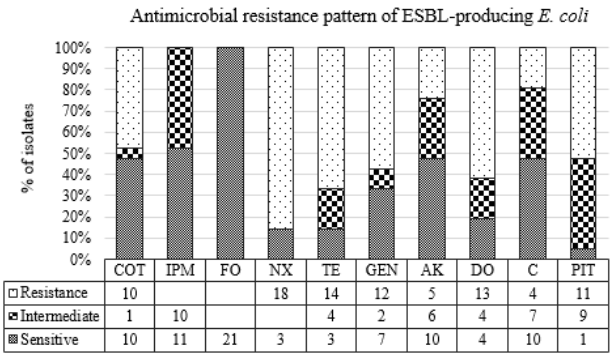


Fig. 3: Phenotypic characterization of *E. coli* isolated from dogs. (**A**) Detection of ESBL production based on zone of inhibition in Double Disc Diffusion test, and (**B**) Detection of AmpC production based on zone of inhibition in cefoxitin-cloxacillin Double Disc Synergy test (CC-DDST).



	COT	IPM	FO	NX	TE	GEN	AK	DO	C	PIT
Resistance	10			18	14	12	5	13	4	11
Intermediate	1	10			4	2	6	4	7	9
Sensitive	10	11	21	3	3	7	10	4	10	1

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: Doxycyclin, 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
		Resistant	Sensitive	Intermediate	
Sec-5	*bla*$_{CTX-M}$, *bla*$_{TEM}$, *tet(A)*, *aac(6')-ib-cr*, *bla*$_{AmpC}$, ISEcp1	DO, COT, C, AK, PIT, NX, TE	IPM, FO, GEN	NIL	A
Rh-2g	*bla*$_{CTX-M}$, *tet(A)*, *qnrS*, *bla*$_{AmpC}$, ISEcp1	DO, GEN, PIT, TE, NX	C, FO, COT	IPM, AK	A
Rh-2h	*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{VEB}$, *tet(A)*, *tet(B)*, *qnrS*, *qnrA*, *aac(6')-ib-cr*, *bla*$_{AmpC}$, integrin 2, ISEcp1	DO, C, PIT, TE, NX	FO, COT, AK	IPM, GEN	A
Rh-3a	*bla*$_{CTX-M}$, ISEcp1	PIT, TE, NX	FO, GEN, AK, IPM	DO, COT, C	A
Rh-5a	*bla*$_{CTX-M}$	DO, AK, PIT, NX	FO, COT, C, GEN, IPM	TE	A
Rh-15b	*bla*$_{CTX-M}$, *tet(B)*, *bla*$_{AmpC}$, ISEcp1	PIT, NX	FO, COT, GEN, IPM, C	DO, AK, TE	A
Rh-17a	*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{VEB}$, *tet(A)*, *qnrS*, *bla*$_{AmpC}$, ISEcp1	DO, GEN, AK, PIT, TE, NX	FO, COT, C, IPM	NIL	D
Rh-17b	*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{VEB}$, *tet(A)*, *qnrS*, *qnrA*, *bla*$_{AmpC}$, ISEcp1	DO, COT, GEN, PIT, NX, TE	FO, C	IPM, AK	D
Rh-20a	*bla*$_{CTX-M}$, *bla*$_{VEB}$, *tet(A)*, *tet(B)*, *qnrS*, *qnrA*, *qnrB*, *aac(6')-ib-cr*, *bla*$_{AmpC}$, ISEcp1	DO, COT, GEN, NX, TE	FO, AK, C	IPM, PIT	A
Rh-20b	*bla*$_{CTX-M}$, *bla*$_{VEB}$, *tet(A)*, *tet(B)*, *qnrS*, *qnrA*, *qnrB*, *aac(6')-ib-cr*, *bla*$_{AmpC}$, ISEcp1	DO, COT, PIT, NX, TE	FO, C, GEN, AK	IPM	A
Rh-22a	*bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{VEB}$, *tet(A)*, *tet(B)*, *qnrS*, *qnrA*, *qnrB*, *aac*(6')-*ib-cr*, *bla*$_{AmpC}$, ISEcp1	GEN	DO, COT, C, FO, IPM, NX, AK	PIT, TE	A
Rh-23a	*bla*$_{CTX-M}$, *bla*$_{SHV}$, *qnrS*, *bla*$_{AmpC}$, ISEcp1	COT, C, GEN, AK	FO, IPM, PIT, NX, TE	DO	A
Rh-24a	*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{AmpC}$	GEN, NX	FO, AK, COT, DO	C, IPM, PIT, TE	D
Rh-30a	*bla*$_{CTX-M}$, *tet*(A), *bla*$_{AmpC}$	DO, COT, GEN, AK, PIT, TE, NX	FO	C, IPM	A
Rh-32a	*bla*$_{CTX-M}$, *bla*$_{AmpC}$, *qnrS*, ISEcp1	DO, COT, TE, NX, C	FO, GEN, IPM	AK, PIT	D
Rh-32b	*bla*$_{CTX-M}$, *bla*$_{AmpC}$, *qnrS*, ISEcp1	NX, TE	DO, FO, COT, GEN, IPM	PIT, AK, C	D
Rh-35a	*bla*$_{CTX-M}$, *aac*(6')-*ib*-cr, *bla*$_{AmpC}$, ISEcp1	COT, GEN, NX	DO, FO, TE	C, IPM, PIT, AK	A
Rh-46a	*bla*$_{CTX-M}$, *qnrS*	GEN	FO, COT, C, IPM, AK, TE, NX	DO, PIT	A
Rh-52a	*bla*$_{CTX-M}$, *tet*(A), *tet*(B), *qnrS*, *bla*$_{AmpC}$, *bla*$_{CIT}$, ISEcp1	DO, COT, GEN, PIT, TE, NX	FO, AK	C, IPM	A
Rh-54b	*bla*$_{CTX-M}$, *qnrS*, *bla*$_{AmpC}$, ISEcp1	DO, NX, TE	FO, COT, AK	C, GEN, IPM, PIT	A
Rh-56a	*bla*$_{CTX-M}$, *bla*$_{VEB}$, *tet(A)*, *qnrS*, *bla*$_{AmpC}$, ISEcp1	DO, COT, GEN, NX, TE	FO, C, IPM, AK	PIT	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: Piperacilin + Tazobactam, and NIL: No antibiotics was intermediate sensitive

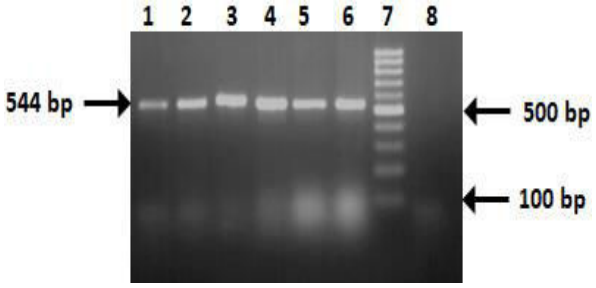


Fig. 5: A 2% (w/v) agarose gel of *bla*$_{CTX-M}$ gene fragments amplified from *E. coli* isolated from dogs. Lanes 1-6: *bla*$_{CTX-M}$ gene specific PCR amplicons, Lane 7: 100 bp DNA ladder, and Lane 8: No DNA template control

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 *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ genes. The most predominant resistance gene was *bla*$_{CTX-M}$ which was detected in all the isolates (100%) (Fig. 5), followed by the *bla*$_{TEM}$ gene in 6 isolates (28.57%) whereas the *bla*$_{SHV}$ gene was detected in two *E. coli* isolates (9.50%). In our study, we observed that distinct β-lactamase genes coexisted within the same isolates. The *bla*$_{CTX-M}$ with *bla*$_{TEM}$ was the most common combination with or without *bla*$_{SHV}$ in this study (Table 3).

A separate multiplex PCR was implemented for the detection of minor β-lactamase genes *bla*$_{GES}$, *bla*$_{PER}$, and *bla*$_{VEB}$ type of ESBL resistance gene in *E. coli* isolates. Seven isolates (33.33%) indicated PCR amplification for the *bla*$_{VEB}$ type of ESBL gene although none of the isolates was found to be positive for *bla*$_{GES}$ or *bla*$_{PER}$ 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.

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

Resistance gene (cassette)	Number of isolates	Percentage
$bla_{\text{CTX-M}}$	21	100.00
bla_{TEM}	6	28.57
bla_{SHV}	2	9.52
bla_{VEB}	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
bla_{AmpC}	18	85.71
bla_{CIT}	1	4.76

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

Patterns of ESBL genotype	No. of isolates	Percentage
$bla_{\text{CTX-M}}$ + bla_{TEM} + bla_{SHV}	1	4.76
$bla_{\text{CTX-M}}$ + bla_{TEM}	5	23.81
$bla_{\text{CTX-M}}$ + bla_{SHV}	1	4.76
$bla_{\text{CTX-M}}$ only	14	66.67

PCR-based identification assay was implemented for genotypic characterization of AmpC producing *E. coli*, bla_{AmpC} gene was detected in 18 (85.71%) (Fig. 6) isolates; although 19 (90.48%) isolates were phenotypically confirmed as AmpC producers. The AmpC gene, bla_{CIT} 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 amplicon was detected in 5 isolates. However, none of these isolates were positive for *yjaA* specific PCR amplicon. 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.

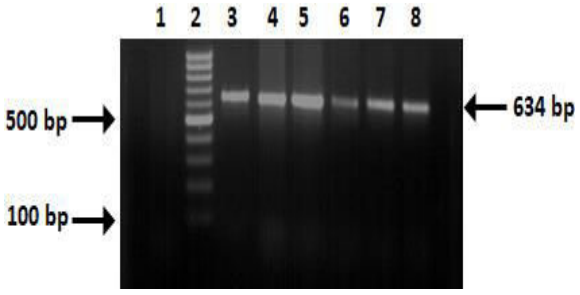


Fig. 6: A 2% (w/v) agarose gel of bla_{AmpC} gene fragments amplified from *E. coli* isolated from dogs. Lanes 3-8: bla_{AmpC} gene-specific PCR amplicons, Lane 2: 100 bp DNA ladder, and Lane 1: No DNA template control

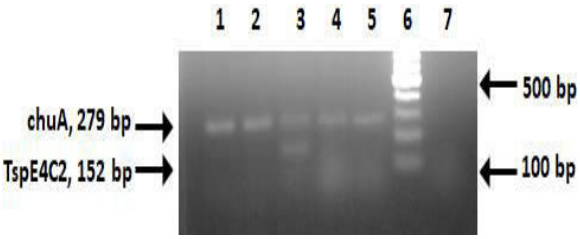


Fig. 7: Multiplex PCR profiles showing Clermont phylo-typing method of *E. coli* isolated from dogs. Lanes 1-5: *chuA* and *TspE4C2* gene-specific PCR amplicons, Lane 7: No DNA template control, and Lane 6: 100 bp DNA ladder

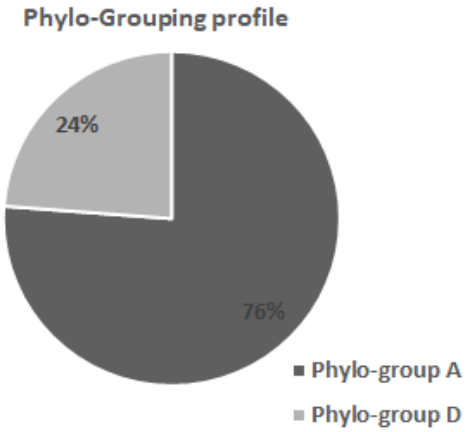


Fig. 8: Frequency of the different phylogroup of ESBL-producing *E. coli* isolated from dogs suffering from diarrhea

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 $bla_{\text{CTX-M}}$ (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 $bla_{\text{CTX-M}}$ (100%), followed by bla_{VEB} (33.33%) and bla_{TEM} (28.57%). Low levels of bla_{SHV} (9.52%) and no bla_{GES} and bla_{PER} 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 $bla_{\text{CTX-M}}$ 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 $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-27}}$, $bla_{\text{CTX-M-55}}$, and $bla_{SHV\text{-}12}$ 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, $bla_{\text{CTX-M}}$, 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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