

High rates of CTX-M group-I extended-spectrum β -lactamases producing *Escherichia coli* from pets and their owners in Faisalabad, Pakistan

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Purpose: Pet animals have been considered a potential carrier of clinically important multidrug-resistant *Escherichia coli*. However, little is known about the role of pets as reservoirs of extended-spectrum β -lactamase (ESBL) producing *E. coli* in Pakistan. This study was designed to determine the prevalence and genetic relatedness of ESBL-producing multidrug-resistant *E. coli* in pets, their owners, and veterinary professionals.

Methods: A total of 105 fecal samples were collected from dogs, cats, their owners, and veterinary professionals from veterinary clinics. Isolates of ESBL-producing *E. coli* were subjected to antimicrobial susceptibility testing. The presence of *bla*_{CTX-M} genes and CTX-M groups I and II in multidrug-resistant *E. coli* was detected using PCR. Clonal diversity was checked using BOX-PCR.

Results: Of the 105 fecal samples screened, 73 (69.5%) were found to contain ESBL-producing *E. coli*. The percentage of ESBL-producing *E. coli* isolates in dogs and dog owners was found to be 81.8% (18/22) and 59% (13/22), respectively. In cats, this percentage was 73.9% (17/23) and in cat owners, 56.5% (13/23). Furthermore, 80% (12/15) of *E. coli* isolates in veterinary professionals were ESBL producers. Of these 73 ESBL-producing *E. coli* isolates, 23 isolates exhibited a multidrug-resistant phenotype. The most prevalent multidrug-resistant pattern (17.4%) identified was resistant to ampicillin, cefotaxime, ciprofloxacin, and nitrofurantoin. In the multidrug-resistant *E. coli*, *bla*_{CTX-M} was identified as the most common ESBL-producing genotype (19/23), with *bla*_{CTX-M-1} dominating in all 19 isolates. Furthermore, BOX-PCR analysis exhibited genetically diverse clonal groups among isolates of the CTX-M-1 group.

Conclusion: Our results provide important baseline information on the potential burden of multidrug-resistant *E. coli* among companion animals in Pakistan. Further studies are needed to understand the drivers of antimicrobial resistance at human–animal–environmental intersections.

Keywords: antimicrobial resistance, ESBL-producing *E. coli*, pets, zoonosis, multidrug resistance, one-health

Introduction

Antimicrobial resistance has become a global challenge for public health. The extended-spectrum cephalosporins are considered critically important antibiotics in both human and veterinary medicine. However, resistance to extended-spectrum cephalosporins mediated by extended-spectrum β -lactamase (ESBL) enzymes has become a well-recognized problem among Gram-negative bacteria. The main ESBL enzymes include CTX-M, TEM, and SHV classes.¹ However, the dominance of CTX-M type ESBLs in human hospitals and community settings, and its occurrence in different

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animal hosts, suggests a one-health approach to determine the genetic relationship between *Escherichia coli* isolates from different hosts.²

Household pets could be potential causes of the spread of antimicrobial resistance, owing to the extensive use of antimicrobial agents in these animals and their close contact with human beings. The relationship between pet animals and human beings has drastically changed, with dogs in increasingly close contact with their owners. Nevertheless, the use of antimicrobial agents in companion animals implies selection as well as the potential to harbor and spread antimicrobial drug resistance, which constitutes a potential risk to public health.³ *E. coli* is a Gram-negative bacterium commonly present in the gastrointestinal tracts of human beings and animal species. The emergence of multidrug resistance in *E. coli* isolates of human and animal origin has raised serious concerns over the increasing risk of therapeutic failure.^{4,5} ESBL-producing *E. coli* has been considered an indicator for the transmission of antimicrobial resistance at the human–animal interface.⁶ Previous reports have suggested pets as reservoirs of clinically relevant ESBL-producing *E. coli*.⁷

High rates of ESBL-producing human clinical *E. coli* isolates have been reported in numerous studies from Pakistan in the last decade,^{8,9} but there is a scarcity of data on antimicrobial resistance among pet animals and their putative zoonotic connections. The objective of this study was to determine the occurrence and genetic relationship between ESBL-producing *E. coli* from pets, their owners, and veterinary professionals.

Materials and methods

Sample collection

A total of 105 fecal sample pairs from pets including dogs and cats (n=23 and n=22, respectively) and their owners (n=45) and veterinary professionals (n=15) were collected from July to December 2016 from the Veterinary Teaching Hospital, University of Agriculture, Faisalabad, and two private veterinary clinics in the Faisalabad district of Pakistan. These samples were transferred to the Zoonotic and Food Pathogen Research Laboratory, Institute of Microbiology, University of Agriculture, Faisalabad, under refrigerated conditions for further processing.

Isolation of ESBL-producing *E. coli*

All 105 rectal swabs were streaked directly onto Chrom agar ESBL (CHROMagar, Paris, France) for presumptive detection of ESBL-producing *E. coli*. Biochemical identification of *E. coli* was achieved using the RAPID kit identification

system (Remel, Kent, UK) according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of presumptive ESBL-producing *E. coli* isolates was determined on Muller Hinton agar (Oxoid, Basingstoke, UK) by the disk diffusion method, following the Clinical and Laboratory Standards Institute criteria with 30 µg each of ceftazidime, cefotaxime (CTX), and ceftriaxone. Phenotypic detection of ESBL production was achieved using the double-disk synergy test according to Clinical and Laboratory Standards Institute guidelines.¹⁰ Additionally, antimicrobial susceptibility to the following antimicrobials was tested: ampicillin (10 µg), CTX (30 µg), ertapenem (10 µg), ciprofloxacin (5 µg), tobramycin (30 µg), tigecycline (15 µg), nitrofurantoin (100 µg), and chloramphenicol (30 µg). The reference strain *E. coli* ATCC 25922 was used for quality control in antimicrobial susceptibility testing. The results were compared with Clinical and Laboratory Standards Institute standards; this strain was found to be susceptible to the given classes of antibiotics. Multidrug resistance was defined as phenotypic resistance to at least three antimicrobial drugs belonging to three different classes.¹¹

Detection of *bla*_{CTX-M} gene and *bla*_{CTX-M} groups using PCR

DNA extraction was done manually by phenol-chloroform method.¹² ESBL-producing *E. coli* isolates with multidrug-resistant phenotypes were investigated for the *bla*_{CTX-M} gene with the primers and thermal cycler conditions reported previously.¹³ *bla*_{CTX-M}-positive *E. coli* were further tested for the detection of major *bla*_{CTX-M} groups, ie, CTX-M-1, CTX-M-2, and CTX-M-9, using a multiplex PCR approach.¹⁴ The primer sequence, gene target, amplified product size, and annealing conditions are listed in Table S1.

Plasmid extraction and replicon typing

Plasmid was extracted using Plasmid DNA kit (GeneJET plasmid miniprep kit; Fermentas, Glen Burnie, MD, USA), and PCR-based replicon typing was performed to determine plasmid incompatibility (Inc) groups.¹⁵ In addition, we further detected ESBL *bla*_{CTX-M} gene on the extracted plasmids.

Genetic relationship between ESBL-producing *E. coli* isolates

To determine the clonal relationship between ESBL-producing *E. coli* with multidrug resistance, BOX-PCR was performed, as described earlier.¹⁶ The BOX-AIR primer

5'-CTA CGG CAA GGC GAC GCT GAC G-3' was used with amplification conditions as follows: 1 cycle of 95°C for 4 minutes, 30 cycles of 94°C for 30 seconds, 92°C for 30 seconds, 50°C for 1 minute, 65°C for 3 minutes, and 1 cycle of 65°C for 8 minutes. The PCR products were run on 1% agarose gel at 90 V for ~1.5 hours. Gels were made and run in premade 1X TBE buffer. After gel electrophoresis, amplicons were visualized under an ultraviolet transilluminator (Dolphin-Doc, Wealtec, NV, USA). The BOX-PCR fingerprints were analyzed using Bionumerics software (Applied Maths, Kortrijk, Belgium). A dendrogram was constructed according to the unweighted pair group method, based on Dice coefficients. Isolates with a Dice similarity index $\geq 80\%$ were considered the same clone.

Results

Prevalence of ESBL-producing *E. coli*

A total of 73/105 (69.5%) ESBL-producing *E. coli* isolates were detected in this study. The maximum percentage of ESBL-producing *E. coli* isolates was observed in dogs (18/22, 81.8%), followed by cats (17/23, 73.9%), whereas

the detection rate in dog and cat owners was 13/22 (59.1%) and 13/23 (56.5%), respectively. Furthermore, 12/15 (80%) veterinary professionals carried ESBL *E. coli*.

Multidrug resistance pattern

The multidrug resistance phenotype, defined as resistance to three or more antimicrobial classes, was detected in 23/73 (31.5%) isolates. The most common multidrug-resistant phenotype was resistance to ampicillin, CTX, ciprofloxacin, and nitrofurantoin (Figure 1).

Frequency of *bla*_{CTX-M} gene and CTX-M group

The overall prevalence of the *bla*_{CTX-M} gene in ESBL-producing multidrug-resistant *E. coli* was 19/23 (82.6%). Of these, results showed that seven out of eight isolates of dog origin and three out of four isolates from dog owners carried the *bla*_{CTX-M} gene. In cats 4/5 of isolates and in cat owners 3/4 of isolates harbored the *bla*_{CTX-M} gene. All of the isolates (2/2) from veterinary professionals were found to carry the *bla*_{CTX-M} gene. All CTX-M-positive multidrug-resistant

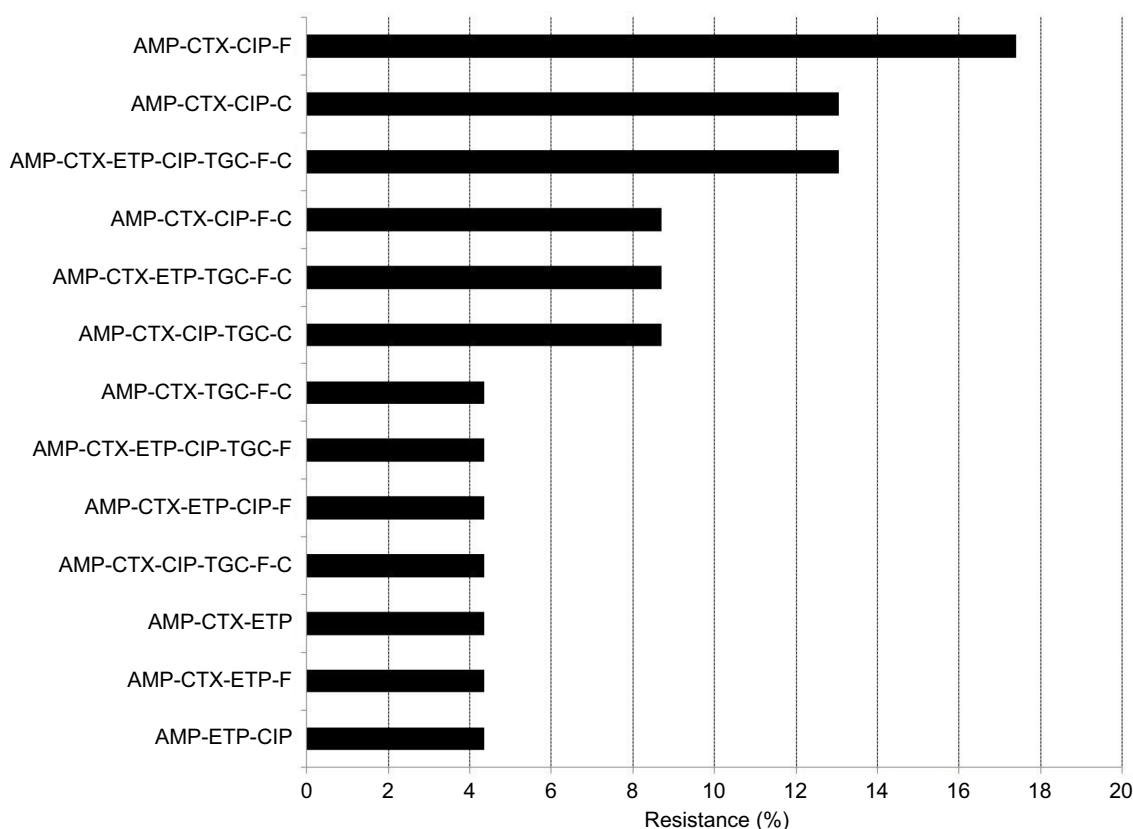


Figure 1 Multidrug resistance phenotypes.

Abbreviations: AMP, ampicillin; C, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; ETP, ertapenem; F, nitrofurantoin; TGC, tigecycline.

ESBL-producing *E. coli* belonged to the CTX-M-1 group; both CTX-M-2 and CTX-M-9 groups were absent (Table 1).

Plasmid replicon types

Overall, replicon typing of the 23 ESBL-producing *E. coli* plasmids showed the predominant presence of IncFII (n=14) and IncFIA (n=8). One isolate carried IncB/O (Table 1). The ESBL encoding gene *bla*_{CTX-M} was detectable from all the extracted plasmids.

Genetic diversity

A large genetic diversity was found among ESBL-producing *E. coli* isolates in this study. Of 23 multidrug-resistant ESBL-producing *E. coli* isolates, 21 different clonal groups were identified using BOX-PCR analysis. The Dice's coefficient among isolates varied from 0.3 (high genomic dissimilarity) to 0.8 (full genomic similarity), demonstrating the high polymorphism of the analyzed genotypes. Two clonal groups were found to have high genetic relatedness. The first clonal group consisted of two isolates of dog origin and the second comprised one isolate from a cat and one from a dog (Figure 2).

Discussion

Antimicrobial resistance is an ongoing public health problem of global dimensions. In this study, we detected

multidrug-resistant ESBL-producing *E. coli* from pets, their owners, and veterinary professionals, and determined their genetic relationships for the first time, to our knowledge, in Pakistan.

Our results demonstrated high rates of CTX-M-producing *E. coli* with multidrug resistance phenotypes in pets and their owners in Pakistan. Previously, several studies have reported the presence of ESBL-producing *Enterobacteriaceae* in clinical samples of human origin in Pakistan. One of these studies, probing ESBL-producing isolates among tertiary care hospital laboratories in Pakistan, detected 41% of ESBL-producing *E. coli* among 2,840 samples.¹⁷ Another study conducted on urinary tract pathogens showed high rates of ESBL-producing *E. coli* (53.4%). Most of these isolates were of multidrug-resistant pathogens.¹⁸ However, there is a lack of data on ESBL-producing *E. coli* among companion animals in Pakistan.

Globally, various studies have been conducted on the zoonotic impact of multidrug-resistant *E. coli* in pets and owners and their associated environments. We found 23/73 (31.5%) isolates of multidrug-resistant ESBL-producing *E. coli* from different sources, with the highest prevalence in dogs (8/18, 44.4%), followed by dog owners (4/13, 30.77%), cat owners 4/13 (30.8%), cats (5/17, 29.4%), and veterinary professionals (2/12, 16.7%). In a previous study conducted on dogs and

Table 1 Distribution of CTX-M gene and groups and plasmid replicon types

Sample ID	Origin	CTX-M	CTX-M-1	CTX-M-2	CTX-M-9	Replicon type
D115	Dog	+	+	-	-	IncFIA
D302	Dog	+	+	-	-	IncFII
D306	Dog	-	-	-	-	IncFII
D108	Dog	+	+	-	-	IncFII
D103	Dog	+	+	-	-	IncFIA
D104	Dog	+	+	-	-	IncFIA
D105	Dog	+	+	-	-	IncFII
D101	Dog	+	+	-	-	IncFIA
O105	Dog owner	+	+	-	-	IncFIA
O109	Dog owner	+	+	-	-	IncFIA
O114	Dog owner	+	+	-	-	IncFIA
O301	Dog owner	-	-	-	-	IncFII
C203	Cat	+	+	-	-	IncFII
C207	Cat	+	+	-	-	IncFII
C211	Cat	-	-	-	-	IncFII
C312	Cat	+	+	-	-	IncFII
C317	Cat	+	+	-	-	IncFII
O202	Cat owner	+	+	-	-	IncFII
O204	Cat owner	+	+	-	-	IncFIA
O315	Cat owner	+	+	-	-	IncB/O
O320	Cat owner	-	-	-	-	IncFIA
Vp106	Veterinary professional	+	+	-	-	IncFII
Vp108	Veterinary professional	+	+	-	-	IncFIA

Notes: In the first column, the letter represents the sources of the isolate and the numbers represent the isolate numbers.

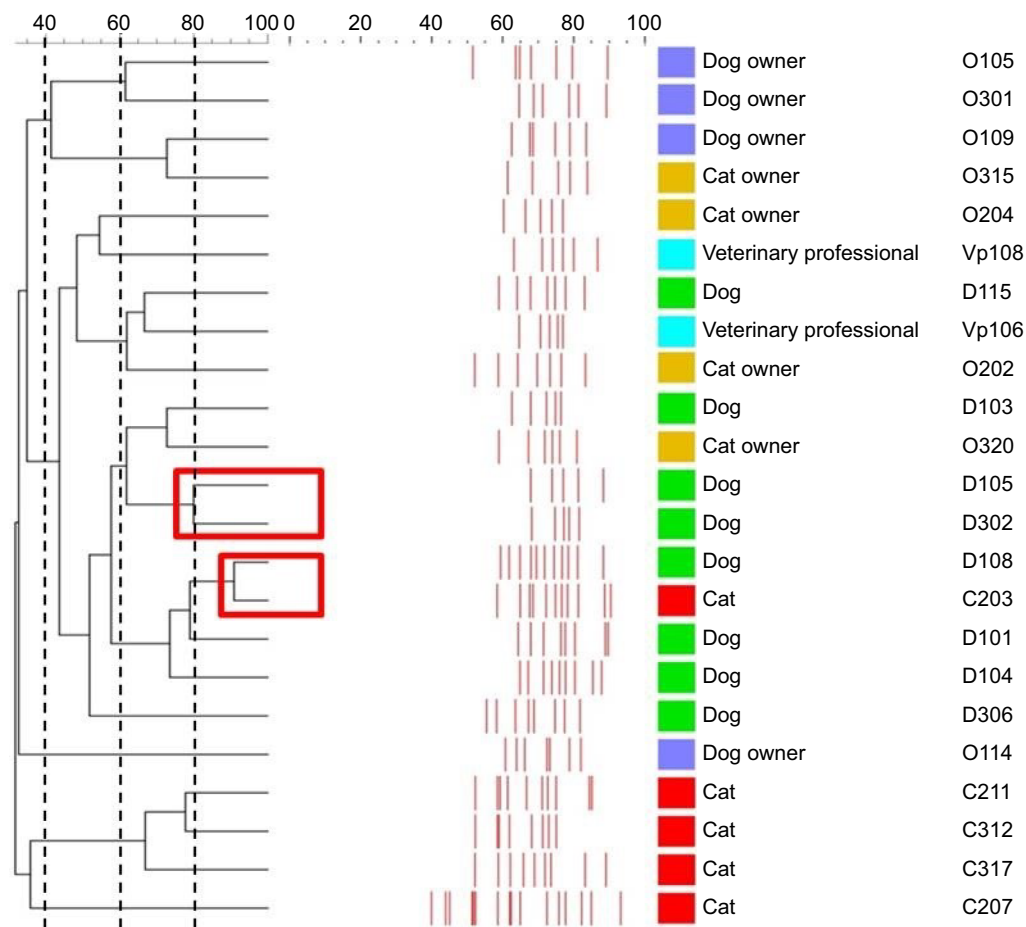


Figure 2 Genetic relationship between multidrug-resistant *Escherichia coli* isolates.

Notes: Dendrogram constructed using Bionumerics v 6.0 (Applied Maths) using Dice similarity coefficient. Isolates with a Dice similarity index $\geq 80\%$ were considered to belong to the same clone.

dog owners, 31.3% of the total number of isolates collected contained multidrug-resistant *E. coli*.¹⁹ In another study, the prevalence of multidrug-resistant *E. coli* was reported as 8% in dogs and cats, which is quite low compared with our findings.²⁰ This low occurrence of multidrug-resistant *E. coli* can be attributed to the antibiotic treatment of animals prior to the study.²¹ The persistence of multidrug resistance in dogs or dog owners has been variably reported in different studies. A study concluded that dogs and their owners harbor less percentage of multidrug-resistant isolates than the control groups.²² The current situation of resistance to antibiotics has reached a serious point in urinary tract infections. Presently, multidrug-resistant bacteria, including ESBL-producing bacteria, can be readily encountered in clinics,²¹ and animals presented for medical attention in hospitals are at greater risk of acquiring the antimicrobial-resistant bacteria.²³

In our study, the highest frequency of resistance was found against ampicillin (80.8%) followed by CTX (39.7%)

and nitrofurantoin (34.2%), while the least resistance was observed against ertapenem (12.3%) and tigecycline (19.1%) among *E. coli* isolates, while none of the isolates was resistant to tobramycin. Many studies reported resistance phenotype patterns similar to our results and described ampicillin and CTX as the most resistant phenotypes detected in pets.^{4,17} Almost similar but more drastic patterns of resistance have been reported in the clinical isolates of humans and animals by Tuerena et al.²⁴ The use of antimicrobials in companion animals not only implies selection but also the potential to carry and spread antimicrobial drug resistance, which is a probable risk to public health.

We detected the globally prevalent type of multidrug-resistant gene, *bla*_{CTX-M}, in our study. We found 19/23 (82.6%) of the multidrug-resistant isolates carrying the *bla*_{CTX-M} gene in dogs, cats, pet owners, and veterinary professionals. Cluster analysis of the isolates revealed that all the CTX-M-producing isolates had the CTX-M-1 genotype. Previous reports also suggest that

CTX-M-1 is the most prevalent genotype to be isolated from multidrug-resistant and ESBL-producing isolates.²⁵ It has been reported that CTX-M was the most common (73.5%) genotype to be isolated from dogs and cats in the USA,²⁶ which was comparable to our study. Another study illustrated the presence of CTX-M-1 in pets,²⁷ although the prevalence was quite low when compared with our findings; similarly, veterinary professionals also carried the CTX-M-1 genotype. The results of our study suggest and favor the idea that CTX-M-1 is not only limited to human beings or animals in Pakistan, but is more prevalent than any other type of multidrug-resistant genotype in human beings and animals globally. In this study, plasmid replicon typing showed two common plasmids, FII and FIB. Similarly, in other Asian countries, IncFII plasmids associated with CTX-M genotypes have been reported from pets.^{28,29}

Clonal diversity of the multidrug-resistant isolates was checked by BOX-PCR to determine any link between pets and pet owner's isolates. Repetitive element sequence-based PCR fingerprinting (eg, BOX-PCR) has been shown to be a valuable tool for classifying and typing a variety of Gram-negative and several Gram-positive genera.³⁰ The results of that study suggest that multidrug-resistant isolates of pets were genetically more similar to each other than those of pet owners or veterinary professionals. Interestingly, most of the multidrug-resistant isolates showed almost similar phenotypic profiles and resistant genotypes, while they were different genetically. This type of pattern has already been observed in studies of pets and pet owners in Brazil;¹⁹ it was elucidated that only the isolates from pets were genetically similar, rather than owners, but another study reported the genetical similarity of pets' and pet owners' isolates, based on pulsed-field gel electrophoresis.²² This inconsistency in genetic linkage between isolates from pets and pet owners may be because different *E. coli* strains acquire the antibiotic-resistant phenotypes and genotypes from the same source or because the same strain carrying multidrug-resistant phenotypes and genotypes was transmitted to both pets and pet owners.³¹ Transmission dynamics of antimicrobial resistance is complex and need more in-depth studies including multilocus sequence typing or whole genome sequencing to establish epidemiological evidence of transmission of ESBL-producing *E. coli* between human and animals.

Conclusion

Although the sample size of this study was limited, our results provide important baseline information on the potential burden of multidrug-resistant *E. coli* among the companion

animals in Pakistan. Further studies are needed to understand the epidemiological and molecular characteristics of CTX-M type ESBL-producing *E. coli* in the companion animals for infection control and prevention in veterinary as well as in human medicine.

Ethics approval

The Institutional Bioethics and Biosafety Committee of the University of Agriculture, Faisalabad, has approved this study (D. No. 43/ORIC). This study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all human participants.

Acknowledgments

We thank all pet owners and veterinary professionals involved in this study for their cooperation and help in sampling. This work was supported by the Federal German Foreign Office (grant reference number AA-OR 12–370.43), BIOS FLI PAK, and the Higher Education Commission, Pakistan (NRPU Grant number 4681).

Author contributions

Dr Mashkoor Mohsin is responsible for the experimental plan and is the corresponding author. All authors contributed to data analysis, preparing, and drafting the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Primers used in study with target gene and amplicon size

Target gene	Primer name	Sequence	Product size	Reference
<i>bla</i> _{CTX-M}	CTX-M-F CTX-M-R	5'SCS ATG TGC AGY ACC AGT3' 5'ACC AGA AYV AGC GGB GC3'	585 bp	13, 14
<i>bla</i> _{CTX-M-1}	CTX-M-1-F CTX-M-1-R	5'GCG TGA TAC CAC TTC ACC TC3' 5'TGA AGT AAG TGA CCA GAA TC3'	260 bp	13, 14
<i>bla</i> _{CTX-M-2}	CTX-M-2-F CTX-M-2-R	5'TGA TAC CAC CAC GCC GCT C3' 5'TAT TGC ATC AGA AAC CGT GGG3'	341 bp	13, 14
<i>bla</i> _{CTX-M-9}	CTX-M-9-F CTX-M-9-R	5'ATC AAG CCT GCC GAT CTG GTT A3' 5'GTA AGC TGA CGC AAC GTC TGC3'	293 bp	13, 14

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