



Detection and Plasmid Profiling of Extended Spectrum Beta Lactamase Producing *Escherichia coli* from Diarrheic Calves

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10.18805/IJAR.B-5584

ABSTRACT

Background: Cephalosporins are major antimicrobials used to treat serious infections. However, their effectiveness is being compromised by the emergence of Extended Spectrum Beta Lactamases (ESBL). The study was aimed to perform plasmid profiling of ESBL producing *Escherichia coli* from diarrheic calves.

Methods: A total of 104 rectal swabs were collected from diarrheic calves (Less than 1 year old) from dairy farms (buffalo calves) and gaushalas (cattle) in and around Jabalpur (M.P.). Total of 81 *E. coli* isolates were isolated. The ESBL screening being conducted by using cefpodoxime, ceftazidime, cefepime and aztreonam antibiotics for initial screening. The phenotypic confirmation of the ESBL producing strains was performed through ESBL phenotypic identification kit and Double Disc Diffusion Test (DDDT). While, for genotypic detection PCR was performed for *bla* TEM, *bla* CTX-M and *bla* AmpC. Plasmid profiling of resistant isolates was performed.

Result: Out of the 81 *E. coli* isolated, 07 (8.64%) were phenotypically confirmed as ESBL strains. PCR amplification revealed 03 (60%) isolates positive for *bla* TEM (867bp) and one (20%) isolate as *bla* CTX-M (540bp) genes, respectively. *bla* AmpC gene was not detected from these isolates. Co expression of *bla* TEM and *bla* CTX-M was recorded in only one diarrheic buffalo calf. Plasmid profiling of the isolates showed that all the isolates were harboring plasmids of varying size and number. The plasmid number among the isolates ranged from 3-4 plasmids per isolate and plasmid size varied from 900bp to >10kb.

Key words: Calves, Diarrheic, ESBL, *Escherichia coli*, Plasmid.

INTRODUCTION

In 2015, World Health Organization (WHO) highlighted antimicrobial resistance (AMR) as a significant threat to food security, development and global health. The rise of antimicrobial resistance in bacteria, particularly in *Escherichia coli*, presents a major challenge to both veterinary and human medicine. The increasing prevalence of *E. coli* strains resistant to important antimicrobials, including first-line drugs such as cephalosporins and fluoroquinolones, complicates the treatment of infections. The WHO's designation of ESBL-producing Enterobacteriales as a critical priority on the global priority list of antibiotic-resistant bacteria highlights the urgency of addressing this issue (Tacconelli *et al.*, 2017). This situation leads to greater morbidity, mortality, higher treatment costs and production losses in livestock, making it a significant public health and economic issue (de Been *et al.*, 2014). Beta-lactam antimicrobial drugs, such as cephalosporins, penicillins and monobactams, are commonly used to treat bacterial infections. However, the emergence of extended-spectrum beta-lactamases (ESBLs), which confer resistance to third- and fourth-generation cephalosporins, has become a significant concern (Pitout *et al.*, 2005 and Himani *et al.*, 2025). ESBLs are divided into several groups, with the TEM, SHV and CTXM types being the most prominent (Singh *et al.*, 2017). ESBL-producing bacteria, especially members of the Enterobacteriaceae family, have been identified as major concern in the spread of resistance. These ESBLs are

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How to cite this article: Himani, K.M., Nayak, A., Jogi, J., Rai, A., Gupta, V., Shakya, P., Shukla, S. and Garg, A. (2025). Detection and Plasmid Profiling of Extended Spectrum Beta Lactamase Producing *Escherichia coli* from Diarrheic Calves. *Indian Journal of Animal Research*. 1-6. doi: 10.18805/IJAR.B-5584.

Submitted: 19-03-2025 **Accepted:** 30-08-2025 **Online:** 13-10-2025

often plasmid-associated, which means that the resistance genes can be easily transferred between bacterial species, further exacerbating the problem. Detection of ESBL-producing *E. coli* in food-producing animals and animal products is particularly concerning for consumers, as these strains may enter the food supply, contributing to the spread of antimicrobial resistance (Kar *et al.*, 2015). The increase in the emergence and spread of antimicrobial resistance, especially among Enterobacteriaceae like *E. coli*, represents one of the most significant health challenges worldwide. This growing antibiotic resistance is largely driven by the widespread prevalence of ESBL-producing strains (Lade *et al.*, 2025).

MATERIALS AND METHODS

The present study was carried out in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Jabalpur (M.P.).

Collection of samples

Total 104 rectal swabs from diarrheic calves consisting of 51 buffalo calves from Instructional Livestock Farm Complex, Adhartal, Private dairy farms and 51 cattle calves from Gaushalas, Jabalpur less than one year of age were collected between month of January to April. Samples collected in transport media and incubated at 37°C for 18 hrs. All samples were processed for isolation of *E. coli*.

Bacterial isolation

Bacterial isolation was carried out as per Markey *et al.* (2013). The inoculum was inoculated in to selective MacConkey agar and incubated at 37°C for 18 hrs. All the isolates were lactose fermenters as indicated by small bright pink colonies on MacConkey agar medium and the pink colonies were further inoculated on Eosin Methylene Blue agar and incubated at 37°C for 18 hrs. which showed colonies with metallic sheen. Morphologically, all isolates in Gram's staining revealed pink colored Gram negative rods and were confirmed by IMViC pattern as *E. coli*. All the isolates were sent to National Salmonella and Escherichia Serotyping Centre, Central Research Institute, Kasauli for serotyping.

Phenotypic confirmation of ESBL

All the isolates were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method against commonly used antibiotics as per the recommendation of CLSI (2013). The antibiotics used for the study were cefpodoxime, ceftazidime, cefepime and aztreonam (30 mcg) for initial screening. The isolates exhibiting resistance to the extended spectrum cephalosporin and monobactam group of antibiotics were selected for confirmation of ESBLs production by ESBL identification kit and double disc diffusion test (DDDT). The Himedia HX096 hexa disc containing Cefpodoxime 10 mcg and Cefpodoxime/Clavulanic acid 10/5 mcg, Ceftazidime 30 mcg and Ceftazidime/Clavulanic acid 30/10 mcg, Cefotaxime 30 mcg and Cefotaxime/Clavulanic acid 30/10 mcg disc place on the inoculated Mueller Hinton agar plate. It was incubated

overnight and a difference of ≥ 5 mm in the inhibition zone between cephalosporin and its combination with clavulanic acid indicates production of ESBL. ESBL production was analyzed by the DDDT. The central disc was Amoxicillin/Clavulanic acid 20/10 mcg. Four other discs were placed within a 20 mm radius of the first one: Ceftazidime 30 mcg, Ceftriaxone 30 mcg, Cefepime 30 mcg and Aztreonam 30 mcg. Samples were considered positive for ESBL when the inhibition zone around any cephalosporin increased toward the central disc with AMX/AC and when the inhibition zone around at least one of the cephalosporins were smaller than 19 mm.

Extraction of genomic DNA and plasmid DNA

The chromosomal DNA was extracted as per the method of Wilson (1987) with a slight modification. The extracted DNA samples were stored at -20°C for further molecular analyses. Plasmid from each tested ESBL producing isolates were extracted as per the method described by Sambrook and Russel, (2001).

Molecular identification of *E. coli*

All the 81 isolates of *E. coli* were genotypically characterized for 16S rRNA by PCR assay using specific primer as mentioned in Table 1. PCR was carried out in a thermal cycler and the cycling condition for 16S rRNA gene was: initial denaturation at 95°C for 5 min followed by 30 cycles of amplification with denaturation at 94°C for 30 s, annealing at 69°C for 30 s and extension at 72°C for 2 min, ending with a final extension at 72°C for 10 min. (Shrivastava, 2016).

PCR detection of ESBL gene

All the phenotypic isolates of *E. coli* along with positive for ESBLs production were tested for the presence of *bla* CTX-M, *bla* TEM and *bla* AmpC genes by PCR assay using specific primers (Table 1). Total DNA (3 µl) was used in a 25 µl reaction mixture that contained 12.5 µl of Dream Taq Green PCR master mix (2X) (Thermo Fisher Scientific, UK) (containing Dream Taq TM DNA polymerase, optimized Dream Taq Green buffer, 0.4mM of each of the dNTPs, 4 mM MgCl₂), 7.5 µl of nuclease free water and 1 µl of each primer. PCR was carried out in a thermal cycler and the cycling condition for *bla* CTX-M was: initial denaturation at 94°C for 1 min followed by 35 cycles of amplification with

Table 1: Details of the primers used for the detection of *bla* CTX-M and *bla* TEM genes.

Gene	Primer	Product size	Reference
<i>E. coli</i> 16S rRNA	F- TGGGAACGGCGAGTCGGAATAC R- GGGCGCAGGGGATGAACTCAAC	1476 bp	(Shrivastav, 2016)
<i>bla</i> CTXM	F- CAATGTGCAGCACCAAGTAA R- CGCGATATCGTTGGTGGTC	540 bp	(Datta <i>et al.</i> , 2013)
<i>bla</i> TEM	F- ATGAGTATTCAACATTTCCG R- CTGACAGTTACCAATGCTTA	867 bp	(Bhattacharjee <i>et al.</i> , 2007)
<i>bla</i> AmpC	F- CCCCCTTATAGAGCAACAA R- TCAATGGTCGACTTCACACC	631 bp	(Shahid <i>et al.</i> , 2012)

denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, ending with a final extension at 72°C for 10 min. For *bla* TEM gene, initial denaturation at 95°C for 5 min followed by 35 cycles of amplification with denaturation at 95°C for 30 s, annealing at 45°C for 1 min and extension at 72°C for 1 min, ending with a final extension at 72°C for 10 min. For *bla* AmpC gene, initial denaturation at 94°C for 3 min followed by 25 cycles of amplification with denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, ending with a final extension at 72°C for 7 min.

RESULTS AND DISCUSSION

Today, livestock production has become a significant source of antimicrobial resistance. In the past few decades, ESBL-carrying pathogens have increased significantly in both humans and animals (Mills *et al.*, 2020). ESBL are rapidly spreading worldwide and are frequently isolated from animals and humans Enterobacteriaceae isolates. In the present study 104 rectal swabs were collected from diarrheic calves in and around Jabalpur for characterization of ESBL producing *Escherichia coli*. A total 81 (77.88%) samples yielded *E. coli* which include 51 (98.07%) from organized buffalo farm diarrheic calf and 30 (57.69 %) from gaushalas diarrheic cattle calves (Table 2). In this study, simplex PCR was performed for targeting the highly conserved gene 16S rRNA for confirmation and rapid diagnosis of *E. coli*. All the 81 (100 %) isolates produced 1476 bp of amplicon and were confirmed as *E. coli*.



Fig 1: Initial screening of *Escherichia coli* isolates for ESBL.

The present study shows much higher isolation rate than Masud *et al.* (2012) (30.71%) and Gebregiorgis and Tessema (2016) (36.8 %). The reason why the result of the current study varies from the other reports might be due to variations in farm management conditions, the season in which sample were collected. The present study findings corroborate with the result of Pereira *et al.* (2014); Awosile *et al.* (2018) and Putra *et al.* (2020), they isolated and identified *E. coli* from calf and confirmed *E. coli* in 96%, 88.1% and 100%, respectively. *E. coli* Prevalence of *E. coli* was lower in diarrheic calves (77.88%) compared to other studies. The differences in the prevalence rates of *E. coli* among diarrheic calves may be attributed to the geographical locations of the farm, management practice and hygienic measures (El-Seedy *et al.*, 2016).

Of the 81 isolates, 07 (8.64%) *E. coli* isolates (06 from buffalo calves and 01 from cattle calf) showed reduced susceptibility to one or more antimicrobials of initial screening (Fig 1). Total 05 (10.20%) isolates (04 from buffalo calves and 01 from cattle calf) were phenotypically confirmed as ESBL-producers by using ESBL identification kit and double disc diffusion test (Table 3, Fig 2). Out of the 05 phenotypically positive isolates (DDDT) screened for the presence of *bla* genes by PCR, 03 (60%) isolate were found to be positive for *bla* TEM gene (02 from buffalo calves and 01 from cattle calf) and 01 (20%) isolates for *bla* CTX-M gene (buffalo calves). None were positive for *bla* AmpC gene. Coexpression of *bla* TEM and *bla* CTXM was recorded in 01 (20%) isolate (Table 4) from diarrheic buffalo calf. Phenotypic methods may miss low-level ESBL producers so all the *E.coli* were tested for ESBL genes. Some ESBL producers may not express sufficient activity to be detected phenotypically. Mutations or co-expression of AmpC may mask the ESBL phenotype.

Findings of present study are supported by observations of Liu *et al.* (2018) from China using double disc diffusion test reported 9.60% *E. coli* isolates from pigs as ESBL producer. In France, Haennia *et al.* (2014) reported prevalence of ESBL *E. coli* to be 29.40% in calves fecal flora. Hiroi *et al.* (2011) screened 16 *E. coli* isolates, out of which two isolates (12.50%) were phenotypically confirmed as ESBL producers. Similar findings of lower prevalence

Table 2: Isolation of *Escherichia coli* from calves.

Particulars	Sample size	Total <i>E. coli</i> isolated	Total 16S rRNA
Diarrheic buffalo calves from organized dairy farms	52	51 (98.07%)	51 (100%)
Diarrheic cattle calves from gaushalas	52	30 (57.69%)	30 (100%)
Total	104	81 (77.88%)	81 (100.%)

Table 3: Phenotypic confirmation of ESBL producing *Escherichia coli* isolates from calves.

Particulars	<i>E. coli</i> Isolates	Initial screening of <i>E. coli</i> isolates	No. of ESBL producing <i>E. coli</i>
Diarrheic buffalo calves from organized dairy farms	51	06 (11.76%)	04 (7.84%)
Diarrheic cattle calves from gaushalas	30	01 (3.33%)	01 (3.33%)
Total	81	07 (8.64%)	05 (6.17%)

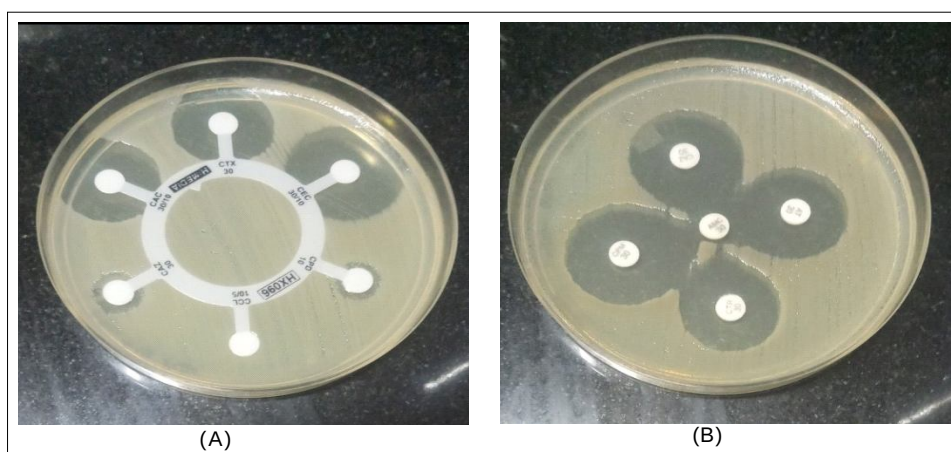


Fig 2: Phenotypic identification of ESBL producing (A) ESBL identification kit (B) Double disc diffusion test.

Table 4: Genotypic characterization of ESBL producing *Escherichia coli*.

Particulars	No. of ESBL producing <i>E. coli</i> isolates	No. of isolates showing amplified product of <i>bla</i> TEM gene	No. of isolates showing amplified product of <i>bla</i> CTX-M gene
Diarrheic buffalo calves from organized dairy farms	04	02	01
Diarrheic cattle calves from gaushalas	01	01	00
Total	05	03	01

of ESBL producer *E. coli* were observed in the present study it might be due to differences in the detection methods. As documented in the Schmid *et al.* (2013) using enrichment and selective media (MacConkey agar containing cefotaxime) for isolation of ESBL producing *E. coli*. Olowe *et al.* (2015) performed PCR in *E. coli* isolates obtained from animal fecal samples in Nigeria and detected *bla* TEM and *bla* CTX gene in 48 (42.10%) and 51 (44.70%) isolates, respectively. Liu *et al.* (2018) from China reported 9.60% *E. coli* isolates from pigs as ESBL producer harbored at least one type of beta lactamase, with *bla* CTX-M, *bla* TEM, being detected in 90.90% and 68.18 %, respectively. The present study revealed *bla* TEM shows higher prevalence which is similar to the finding of Montso *et al.* (2019) from South Africa screened 53.1% *E. coli* isolates as ESBL producers. The *bla* TEM and *bla* CTX-M genes were detected in 85.5% and 58.00%, respectively. Tekiner and Ozpinar (2016) from Turkey detected *bla* TEM and *bla* CTX-M 96.40% and 53.70%, respectively in foods of animal origin. In present study occurs of ESBL resistance was higher in dairy buffalo calves in comparison to gaushala cattle calves. In dairy farms for treatment and prophylactic purpose antibiotic are use, while in gaushalas abandoned cattle from rural area are kept hence less chance of resistance.

The plasmid profile of 05, ESBL producing *E. coli* isolates from diarrheic calves. Plasmid profiling of ESBL producing *E. coli* isolates from diarrheic calves were observed by agarose gel electrophoresis which showed plasmid bands in different combinations. The average

plasmid number among the isolates was 3.4 and ranging from 3-4 plasmids per isolate. Two isolates (40%) were harboring 03 plasmids whereas three isolates with 04 plasmids which were highest number of plasmid. The plasmid size was found in different combinations which ranged from 900bp to >10 kbp in all isolates. pQE-30 Xa vector was used as positive control in our study.

In *E. coli*, the antimicrobial resistance genes reside in plasmids which are responsible for resistance to numerous antimicrobial agents. We recorded smaller plasmids of <20 kb in size. Existence of common plasmid among the isolates implies the spread of resistant plasmid in the community. Gupta *et al.* (2014) reported smaller size plasmid ranging from 3 kbp to 8 kbp and their number also varied from one to four. Whereas Gohar *et al.* (2015) reported plasmid size from 100 bp to 12 kbp. Furthermore, the similarity in plasmids among different isolates suggested plasmid movement between bacteria.

CONCLUSION

In the present study detection of antibiotic resistance genes showed the occurrence of ESBL producing *E. coli* higher in buffalo calves in comparison to cattle calves. Existence of common plasmids among isolates implies the spread of resistant plasmid in the community.

ACKNOWLEDGEMENT

The present study was supported by College of Veterinary Science and A.H., Jabalpur, Madhya Pradesh.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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