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Characterization of Extended Spectrum Beta Lactamase Producing *Escherichia coli* from Calves

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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 present study was aimed to characterize ESBL producing *Escherichia coli* from healthy and diarrheic cattle calves.

Methods: A total of 52 rectal swabs were collected from healthy and diarrheic cattle calves between August 2019 to March 2020. Phenotypically *E. coli* were characterized based on morphology, microscopy and biochemical tests. Extended Spectrum Beta Lactamases (ESBL) *E. coli* initial screening was performed by using cefpodoxime, ceftazidime, cefepime and aztreonam antibiotics. The confirmation of the ESBL producing strains was conducted through ESBL phenotypic identification kit and double disc diffusion test (DDDT). Molecular characterization of ESBL *E. coli* was conducted by PCR amplification of blaCTX-M, *bla* TEM and *bla* AmpC genes. Plasmid profiling of the ESBL *E. coli* isolates was carried out.

Result: Phenotypically, out of the 51 *E. coli* isolated, 05 (9.8%) *E. coli* isolates were confirmed as ESBL strains. PCR amplification revealed 01 (20%), 04 (80%) and 03 (60%) isolates positive for *bla*CTX-M, *bla* TEM and *bla* AmpC genes, respectively. Plasmid profiling of the isolates showed that 60% isolates were harboring plasmids of varying size and number. The plasmid number among the isolates ranged from 0-4 plasmids per isolate and plasmid size varied from 900 bp to >10 kb. The relatively high rates of ESBL producers in diarrheic calves were found than healthy calves. The study highlights the existence of ESBL producing *E. coli* isolates with certain strains carrying mobile genetic elements in livestock and probable disseminators of resistance, thus imposing public health threat.

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

INTRODUCTION

Neonatal calf diarrhea is multifactorial symptom which despite of continuous research is major cause of poor growth in young calves. Gram negative bacteria are a major therapeutic challenge in both livestock and human beings. The rapid global rise of *Escherichia coli* infections that are resistant to therapeutically important antimicrobials, including first line drugs such as cephalosporins and fluoroquinolones, is of serious concern, as it hampers treatment of infections leading to significant morbidity, mortality, treatment costs as well as production losses in livestock (de Been *et al*., 2014).

Beta lactam antimicrobial drugs exhibit the most common treatment for bacterial infections. Extended spectrum beta lactamases (ESBL) are classified into several groups, the prominent among them are TEM, SHV and CTXM types (Singh *et al*., 2017). Members of the family Enterobacteriaceae commonly produce ESBLs that confer resistance to the 3rd and 4th generation of cephalosporins, penicillins and monobactams. ESBLs are of great concern because they are plasmid associated. ESBL genes are situated on plasmids that can be easily transferred between and within bacterial species. Some ESBL genes are mutant derivatives of established plasmid mediated beta lactamases (*e.g*. bla TEM/SHV) and others are mobilized from environmental bacteria (*e.g*. *bla* CTXM). *E. coli* has a very wide natural distribution and a propensity of plasmid carriage (Sherley *et al.,* 2003). Detection of ESBL producing *E. coli* in food producing animals and edible animal products has become a serious cause of concern for the consumers (Kar *et al*., 2015). The increase, emergence and spread of antimicrobial resistance among *E. coli* are the most important health problems worldwide. This increased antibiotic resistance is mainly due to increased prevalence of ESBL producing Enterobacteriaceae (Canton *et al*., 2008).

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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.). A total 52 rectal swabs were collected from Instructional Livestock Farm Complex, Adhartal, Private dairy farms and Gaushala of Jabalpur district betweenthe month of October 2019 to February 2020, in transport media and incubated at 37°C for 18 hrs. All samples were processed for isolation of *E. coli*. 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.The pink colonies were further inoculated on Eosin Methylene Blue agar and incubated at 37°C for 18 hrs resulted in metallic green sheen colonies. Identification of *E. coli* was done by Gram's staining, catalase, oxidase, indole, methyl red, Voges Proskauer and citrate utilization tests.

Extended spectrum beta lactamase production was studied as per the recommendations 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). HX096 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 was placed on the inoculated Mueller Hinton agar plate. It was incubated overnight and difference in zone of inhibition of ≥5 mm of cephalosporin and their cephalosporin plus clavulanic acid containing disc indicate 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.

All the *E. coli* isolates obtained from different samples were investigated for presence of ESBL genes namely *bla* CTX-M, *bla* TEM and *bla* AmpC. 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).

All the 51 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 16 SrRNA 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. All the isolates found tobe positive for ESBLs production phenotypically 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 DreamTaqTM DNA polymerase, optimized DreamTaq Green buffer, 0.4 mM of each of the dNTPs, 4 mM $MgCl_2$), 7.5 µl of nuclease free water and 1 µl of each primer. PCR was carriedout 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 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

Isolation and Identification of *E. coli*

The emergence of multidrug resistant bacteria is a global health concern. ESBL are rapidly spreading worldwide and

Table 1: Details of the primers used for the detection of ESBL genes.

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

are frequently isolated from animals and humans Enterobacteriaceae isolates. In the present study 52 rectal swabs of healthy as well as diarrheic cattle calves in and around Jabalpur were collected for characterization of ESBL producing *Escherichia coli*. A total 51 (98.07%) samples yielded *E. coli* which include 27(100%) from non diarrheic cattle and 24 (96%) from diarrheic cattle calves (Table 2). In this study, singlex PCR was performed for targeting the highly conserved gene 16S rRNA for confirmation and rapid diagnosis of *E. coli*. Total 39 (76.47%) isolates produced 1476 bp of amplicon and were confirmed as *E. coli* (Fig 3a).

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 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* are the normal commensal bacteria of intestine so they were present in all the non diarrheic calves as stated by Saidani *et al*. (2017). Prevalence of *E. coli* was lower in diarrheic calves (88.46%) 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).

ESBL production

Out of 51 *E. coli* isolates, 07 (13.72%) *E. coli* isolates showed reduced susceptibility to one or more antimicrobials of initial screening (Fig 1). Total 05 (9.8%) isolates were phenotypically confirmed as ESBL producing *E. coli* by using ESBL identification kit and double disc diffusion test (Table 3, Fig 2). Out of the 05 phenotypically positive isolates screened for the presence of *bla* genes by PCR, 01 (20%) isolate was found to be positive for *bla* CTX-M gene (Fig 3b), 04 (80%) isolate was found to be positive for *bla*TEM gene (Fig 3c) and 03 (60%) isolates were positive for *bla* AmpC gene (Fig 3d). Coexpression of *bla* TEM and *bla* AmpC was recorded in 03 (60%) isolates (Table 4).

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 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. Dewangan *et al*. (2017) from Chhattisgarh detected 39.4% isolates were found to harbour

Table 2: Isolation of *Escherichia coli.*

Particulars	Sample size	Total *E. coli* isolated	Total 16S rRNA
Non diarrheic cattle calves	27	27 (100%)	24 (88.88%)
Diarrheic cattle calves	25	24 (88.46%)	15 (62.50%)
Total	52	51 (94.23%)	39 (76.47%)

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

Particulars	*E. coli* Isolates	Initial screening of *E. coli* isolates	No. of ESBL producing *E. coli*
Non diarrheic cattle calves	27	01 (3.70%)	01 (3.70%)
Diarrheic cattle calves	24	06 (25%)	04 (16.66%)
Total	51	07 (13.72%)	05 (9.80%)

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

Particulars	No. of ESBL producing *E. coli* isolates	No. of isolates showing amplified product of *bla* AmpC gene	No. of isolates showing amplified product of *bla* TEM gene	No. of isolates showing amplified product of *bla* CTX-M gene
Non diarrheic cattle calves	01	01	01	00
Diarrheic cattle calves	04	02	03	01
Total	05	03	04	01

the *bla*TEM gene on their plasmid DNA indicating the presence of multidrug resistant ESBL producing *E. coli* in foods of animal origin and human samples.Tekiner and Ozpınar (2016) from Turkey detected *bla*TEM and *bla*CTX-M 96.40% and 53.70%, respectively in foods of animal origin.

Plasmid profiling

The plasmid profile of 05, ESBL producing *E. coli* isolates from healthy and diarrheic cattle calves are presented in Table 5. Plasmid profiling of ESBL producing *E. coli* isolates from healthy and diarrheic cattle calves were observed by agarose gel electrophoresis which showed plasmid bands in different combinations (Fig 4). The average plasmid number among the isolates was 2.4 and ranging from 0-4 plasmids per isolate. Three isolates (60%) were harboring 04 plasmids which were highest number of plasmid and no plasmid was observed in two isolates (40%). The plasmid size was observed in different combinations which ranged from 900 bp 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. From ESBL resistant isolates, 40% of the isolates we couldn't recover plasmid although the concentration of plasmid was visible in spectrophotometer but on electrophoresis, we were not able to amplifying it. It may be due to low copy number or loss of plasmid during curing in laboratory procedure.

Table 5: Plasmid profiles of ESBL producing *Escherichia coli* isolates.

Sample	Number of plasmid	Mol. Wt. of plasmid (bp)
NDC23	-	-
DC6	04	1300, 2100, 8000, > 10000
DC8	04	1300, 2100, 8000, > 10000
DC9	-	-
DC10	04	900, 1200, 8000, >10000



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

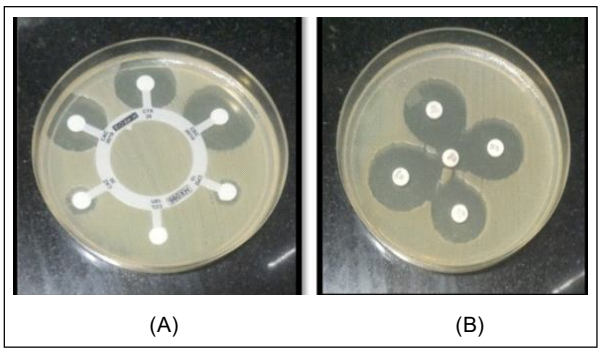


Fig 2: Phenotypic identification of ESBL producing (A) ESBL identification Kit (B) Double Disc Diffusion Test.

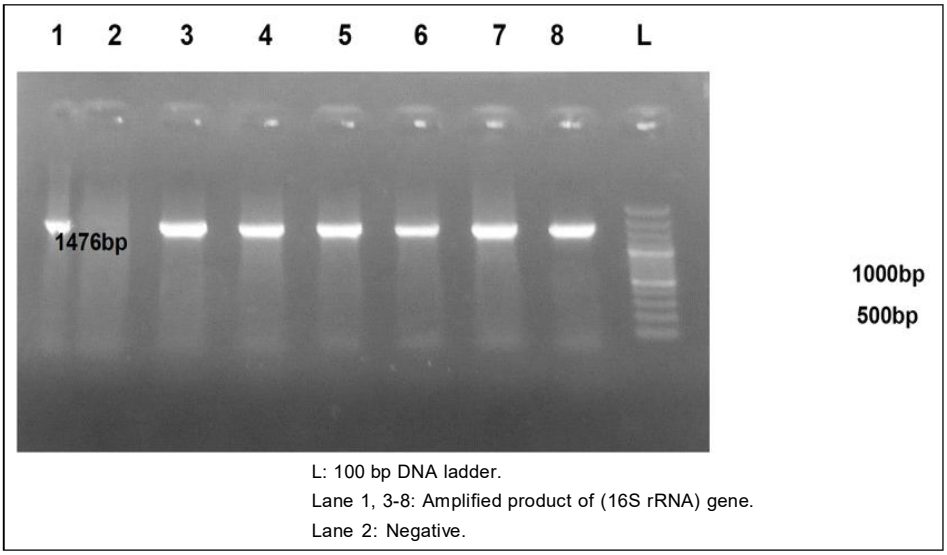
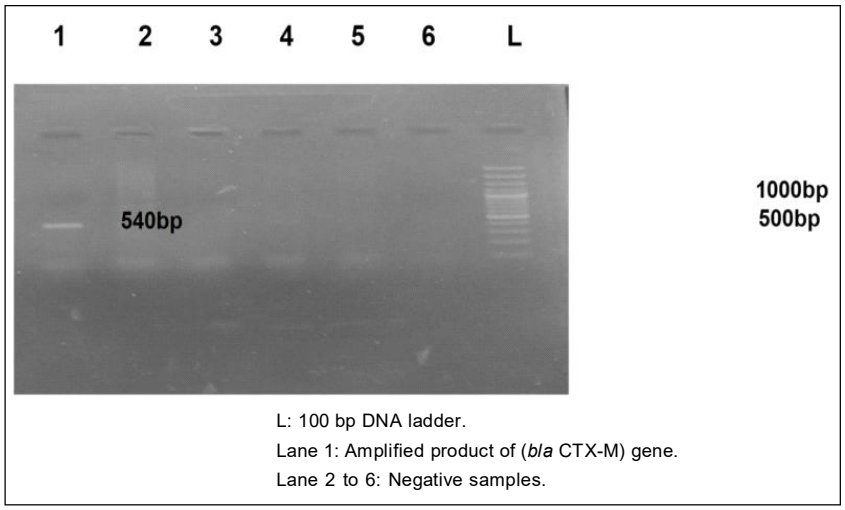
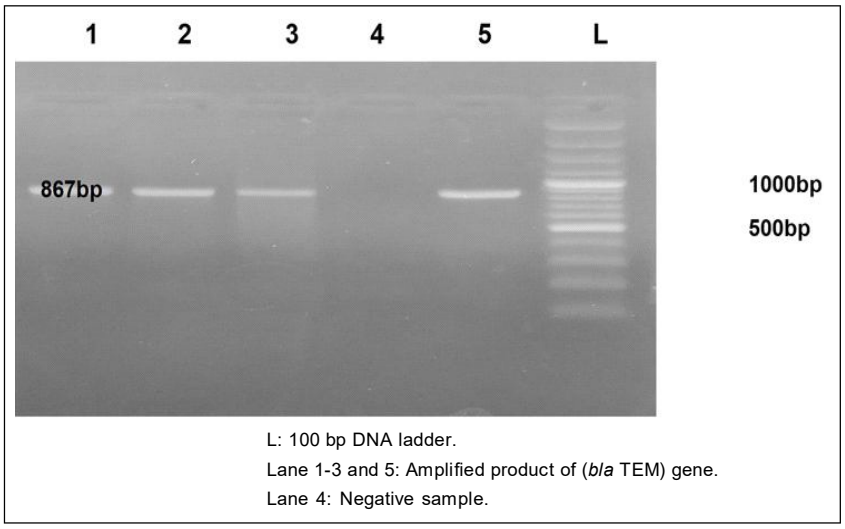


Fig 3a: Agarose gel electrophoresis showing amplified product (1476 bp) of 16S rRNAgene of *Escherichia coli* isolates.



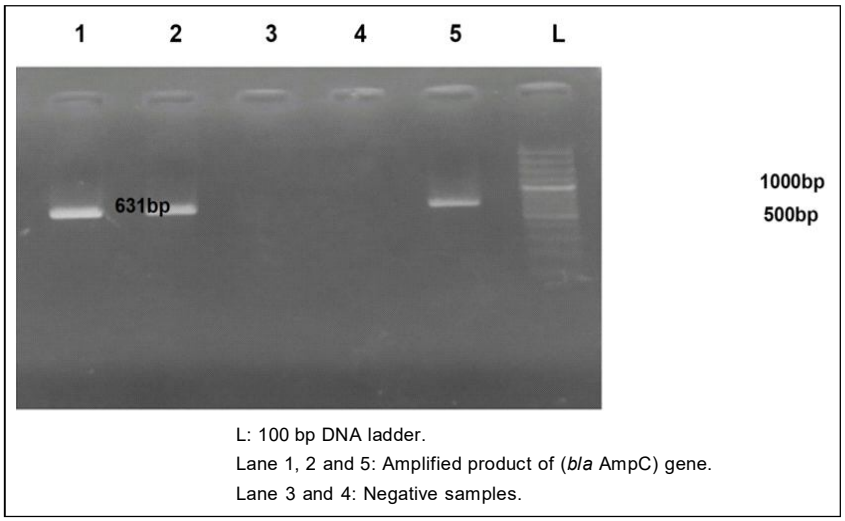
L: 100 bp DNA ladder.
Lane 1: Amplified product of (*bla* CTX-M) gene.
Lane 2 to 6: Negative samples.

Fig 3b: Agarose gel electrophoresis showing amplified product (540 bp) of *bla* CTX-M gene of *Escherichia coli* isolates.



L: 100 bp DNA ladder.
Lane 1-3 and 5: Amplified product of (*bla* TEM) gene.
Lane 4: Negative sample.

Fig 3c: Agarose gel electrophoresis showing amplified product (867 bp) of *bla* TEM gene of *Escherichia coli* isolates.



L: 100 bp DNA ladder.
Lane 1, 2 and 5: Amplified product of (*bla* AmpC) gene.
Lane 3 and 4: Negative samples.

Fig 3d: Agarose gel electrophoresis showing amplified product (631 bp) of *bla* AmpC gene of *Escherichia coli* isolates.

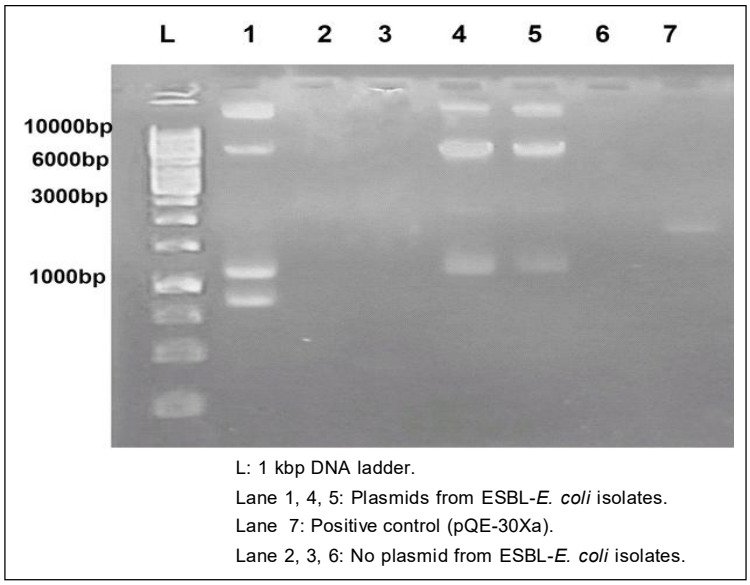


Fig 4: Agarose gel electrophoresis (0.8%) showing ESBL producing *Escherichia coli* plasmids.

CONCLUSION

In the present study detection of antibiotic resistance genes showed prevalence of ESBL producing *E. coli* in cattle calves in and around Jabalpur. Existence of common plasmids among isolates implies the spread of resistant plasmid in the community.

Conflict of interest

All authors declare that they have no conflict of interest.

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