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Detection and Molecular Characterization of Extended-spectrum β-lactamase Producing *E. coli* and *Klebsiella* spp. Isolates of Cattle Origin in Eastern Plain Zone of Uttar Pradesh

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ABSTRACT

Background: Antibiotics are widely used in animals and human for treatment without referring an antibiogram that resulted into the proliferation of ESBL producing strains. Nowadays resistance to β-lactam groups of antibiotic is expanding rapidly worldwide and threatening the public healthcare due to limited treatment options. Therefore this study aimed to detect ESBL resistance genes in *E. coli* and *Klebsiella* spp. isolated from various sources of cattle in this area of study.

Methods: Total 240 samples were collected during August, 2019 to June, 2020, from two districts of Eastern plain zone of Uttar Pradesh. *E. coli* and *Klebsiella* spp. isolates were confirmed using *uidA* and 16S *rRNA* gene respectively. *In vitro* antibiotic sensitivity test was performed using disc diffusion method. ESBL producing isolates was confirmed by DDST, ESBL E-strip and PCR analysis by targeting (*bla*-$_{CTX-M-1}$, *bla*-$_{CTX-M-9}$, *bla*-$_{TEM}$ and *bla*-$_{SHV}$) genes.

Result: PCR analysis of these isolates confirmed 135(56.25%) as *E. coli* and 16(6.67%) as *Klebsiella* spp. Antibiotics found to be most resistant were ampicillin (89.40%) followed by cefotaxime (72.18%), cefpodoxime (71.52%), ceftriaxone (66.9%), aztreonam (61.0%) and ceftazidime (54.30%). Total 101(66.88%) isolates were confirmed as ESBL producers using DDST and 92(60.92%) by ESBL E-strip test. ESBL genes were detected in 87(57.61%) isolates by PCR analysis and among them, *bla*-$_{CTX-M-1}$ was found most dominant gene.

Key words: Antibiogram, Cattle, *E. coli*, ESBL, *Klebsiella*.

INTRODUCTION

The emergence of antimicrobial resistance (AMR), especially among *Enterobacteriaceae* has been increasingly problematic and posses serious threat for both human and animal health (WHO, 2013). It is directly related with the use of these drugs in animal husbandry at insufficient doses and unprofessional choice which limit the treatment options. Resistant bacteria include both pathogenic and commensal organisms, with the later serving as a potential reservoir for mobile resistance elements (Khachatryan *et al*., 2004). Resistance among ESBL organism is mediated by enzyme Extended-spectrum β-lactamases (ESBLs) that hydrolyses most of the β-lactam antibiotics and mediates resistance against penicillins, 3rd and 4th generation cephalosporins (Saravanan *et al*., 2018). Among *Enterobacteriaceae, E. coli* and *Klebsiella* spp. are major ESBL producers and have been identified as global threat due to their increasing prevalence in livestock in last few years (Reuland *et al*., 2013). ESBL has been also reported in various niches as commensal in human, animals and environment. Such ecological niches may serve as reservoir and vehicle for transmission and dissemination in production animals due to their direct contact with food chain (Madec *et al*., 2017). Since no in depth study has been done on distribution of ESBL among cattle in this area of study and also their possible role in development of resistance to other species or pathogens. The present study will provide database to

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help the field veterinarians, pharmaceuticals and policy makers in proper selection of antibiotics for treatment.

MATERIALS AND METHODS

Study area

This study was carried out in the Department of Veterinary Microbiology, C.V. Sc. and A.H. Kumarganj, Ayodhya. The samples were collected from Ayodhya and Sultanpur district of Eastern Plain Zone of Uttar Pradesh, India. The study was conducted between August 2019 and December 2020.

Sample collection

Total 240 samples (120 milk samples and 120 faecal samples) were collected from 5 tehsils of Ayodhya and 3 tehsils of Sultanpur district. Samples were collected randomly and sampling consisted of 10 normal and 5 mastitic milk samples from each of the tehsil. Likewise, 10 normal and 5 diarrhoeic faecal samples from above mentioned regions. California Mastitis Test was used for screening of mastitic milk samples. Approximately 5 ml of milk was collected into sterilized test tubes and faecal samples were collected by swab technique. All collected samples were immediately transported to bacteriology laboratory in an icebox.

Isolation and Identification

All samples were enriched with 2 ml nutrient broth and incubated for 24 hrs at 37°C. A loopful of inoculum was directly streaked on MacConkey agar plates added with 2mg/L cefotaxime and incubated at 37°C for 24 hr. Plates showing pink colonies were picked up and streaked on Eosine Methylene Blue agar plates. Colonies showing greenish metallic sheen were tentatively considered as *E. coli* while dark centred light purple colonies with mucoid appearance were suspected as *Klebsiella* spp. A single pure colony was picked up and transferred to nutrient agar slant. Further identification of the isolates was done by various biochemical tests *viz.* IMViC pattern, catalase test, nitrate reduction, urease test, triple sugar iron agar and sugar fermentation reaction as per the method of Edward and Ewing (1972) and PCR analysis.

Extraction of genomic DNA

The DNA templates were prepared by using snap-chill method as described by Franco *et al.* (2008).

Molecular identification of *E. coli* and *Klebsiella* spp.

All presumptively positive *E. coli* isolates were confirmed by PCR amplification using species specific *uidA* and *Klebsiella* spp. by bacteria specific 16S rRNA gene as per method described by Anbazhagan *et al.* (2010) and Andersson *et al.* (2008) respectively (Table 1). PCR reaction was carried out in total 25 µl volume constituted 12.5 µl of 2X EmeraldAmp GT Master Mix, 8.5 µl nuclease free water, 1 µl mixture of the forward and reverse primers (0.5 µl each primer, conc. 0.5 µM each primer) and 3.0 µl of template DNA. Amplification was performed using thermal cycler (Bio-Rad, USA). The cycling conditions of PCR are mentioned in Table 1.

Antibiotic sensitivity testing

All the confirmed isolates (151) were subjected to in vitroantibiotic sensitivity testing against 13 antibiotics of HiMedia mentioned in Table 4. It was performed by disc diffusion method (Bauer *et al.*, 1966) on Muller Hinton agar (MHA) (HiMedia) plates inoculated with$1.5x10^8$ organism/ml and incubated at 37°C for 24 hrs and isolates were classified as susceptible and resistant based on interpretation criteria of Clinical Standard Laboratory Institute (2019). The isolates showing reduced susceptibility towards cefotaxime, cefpodoxime, ceftazidime, ceftriaxone and aztreonam were screened as ESBL producers.

Confirmation of ESBL producing *E. coli* and *Klebsiella* spp. by phenotypic methods

Double disc synergy test (DDST)

The screened isolates were further confirmed by DDST using ESBL kit 1 and Kit 3 (Hi-media) (Fig 3). The commercially available discs were placed at 25 mm apart on MHA plates inoculated with$1.5x10^8$ organism/ml and incubated at 37°C for 24 hrs. The results were interpreted as per CLSI guidelines (2019).

Minimum inhibitory concentration (MIC) ESBL E-test

This test was done by placing E-strip on MHA plates inoculated with$1.5x10^8$ organism/ml and incubated at 37°C

Table 1: Oligonucleotide primer sequences used for amplification of *uidA* and 16S *rRNA* genes and PCR cycling conditions used.

Targeted gene	Primer sequence (5′-3′)	Amplicon size (bp)	PCR conditions and cycles	References
uidA	F-5′CTGGTATCAGCGCGAAGTCT3′ R-5′AGCGGGTAGATATCACACTC3′	556	1 cycle of 5 minutes at 95°C, 35 cycles of 45 seconds at 95°C, 55 seconds at 56°C, 1 minutes at 72°C, 1 cycle of 7 minutes at 72°C	Anbazhagan *et al.*, 2010
784F 1061R	F-5′AGGATTAGATACCCTGGTA3′ R-5′CRRCACGAGCTGACGAC3′	265	1 cycle of 5 minutes at 95°C, 35 cycles of 50 seconds at 95°C, 45 seconds at 54°C, 1 minutes at 72°C, 1 cycle of 7 minutes at 72°C	Andersson *et al.*, 2008

Table 2: Detail of primers and PCR conditions used detection of ESBLs genes in isolates of *E. coli* and *Klebsiella* spp.

Targeted genes	Primer sequence (5'-3')	Amplicon size (bp)	PCR conditions and cycles	References
bla-$_{CTX-M-1}$gp	F-5'TTAGGAARTGTGCCGCTGYA3' R- 5'C GATATCGTTGGTGGTRCCAT3'	688	1 cycle of 10 minutes at 94°C, 30 cycles of 40 sec. at 94°C, 40 sec. at 60°C, 1 miutes at 72°C, 1 cycle of 7 minutes at 72°C	Dallenne *et al.*, 2010
bla-$_{CTX-M-9}$gp	F-5' TCAAGCCTGCCGATCTGGT3' R-5' TGATTCTCGCCGCTGAAG3'	561	1 cycle of 10 minutes at 94°C, 30 cycles of 40 sec. at 94°C, 40 sec. at 60°C, 1miutes at 72°C, 1 cycle of 7 minutes at 72°C	Dallenne *et al.*, 2010
bla-$_{TEM}$	F-5'CATTTCCGTGTCGCCCTTATTC3' R-5'CGTTCATCCATAGTTGCCTGAC3'	800	1 cycle of 10 minutes at 94°C, 30 cycles of 40 sec. at 94°C, 40 sec. at 61°C, 1 miutes at 72°C, 1 cycle of 7 minutes at 72°C	Dallenne *et al.*, 2010
bla-$_{SHV}$	F-5'AGGATTGACTGCCTTTTTG3' R-5' ATTTGCTGATTTCGCTCG3'	393	1 cycle of 10 minutes at 94°C, 40 cycles of 40 sec. at 94°C, 40 sec. at 60°C, 1miutes at 72°C, 1 cycle of 7minutes at 72°C	Bhattacharjee *et al.,* 2007

Table 3: Isolation rate of *E. coli* and *Klebsiella* spp. from apparently healthy and clinical samples of cattle.

Samples (Source /Origin)		Presumptive positive isolates (Biochemical tests)			Confirmed positive isolates (PCR analysis)		
		E. coli	*Klebsiella* spp.	Total	*E. coli*	*Klebsiella* spp.	Total
	Normal milk (n=80)	16 (20.0%)	8 (10.0%)	24 (30.0%)	12 (15.0%)	5 (6.25%)	17 (21.25%)
	Mastitic milk (n=40)	24 (60.0%)	6 (15.0%)	30 (75.0%)	18 (45.0%)	4 (10.0%)	22 (55.0%)
Cattle	Normal faecal (n=80)	77 (96.25%)	7 (8.75%)	84 (105.0%)	72 (90.0%)	5 (6.25%)	77 (96.25%)
	Diarrhoeic faecal (n=40)	35 (87.5%)	3 (7.5%)	38 (95.0%)	33 (82.5%)	2 (5.0%)	35 (87.5%)
Total	240	152 (63.33%)	24 (10.0%)	176 (73.33%)	135 (56.25%)	16 (6.67%)	151 (62.91%)

Table 4: *In vitro* antimicrobial drug resistance pattern of *E. coli* and *Klebsiella* spp. Isolates.

Antimicrobial agents	*E. coli* (n=135)	*Klebsiella* spp. (n=16)	Total (151)
Conc. (µg/disc)	Resistance	Resistance	Resistance
Imepenem (10)	12 (8.89%)	3 (18.75%)	9.9%
Meropenem (10)	5(3.7%)	2(12.50%)	4.6%
Cefotaxime (10)	95(70.37%)	14 (87.5%)	72.18%
Cefpodoxime (10)	97 (71.85%)	11(68.75%)	71.52%
Ceftazidime (30)	73 (54.07%)	9 (56.25%)	54.30%
Ceftriazone (30)	90 (66.7%)	11(68.75%)	66.9%
Aztreonam (30)	54 (40.0%)	7 (43.75%)	61.0%
Cefoxitin (30)	14 (10.37%)	5(31.25%)	12.58%
Ampicillin (25)	120(88.88%)	15 (93.75%)	89.40%
Gentamicin (10)	0(0.0%)	0(0.0%)	0.0%
Amikacine (30)	0 (0.0%)	0 (0.0%)	0.0%
Enrofloxacin (10)	17 (12.59%)	1 (12.5%)	11.92%
Chloramphenicol (30)	0 (0.0%)	1(16.66%)	0.7%

for 24 hrs. The result was interpreted as per CLSI guidelines (2019) (Fig 4).

Detection of ESBL genes by polymerase chain reaction

Extraction of plasmid DNA

Single pure colony of ESBL positive isolates were inoculated into 10 ml of Luria-Bertani (LB) broth medium (HiMedia, India) and incubated at 37°C for 18 hrs in shaking incubator. After that plasmid DNA was isolated using GeneJet plasmid Miniprep kit (Thermo Scientific) as per the instruction of the manufacturers.

Detection of CTX-M genes (*bla*-$_{CTX-M-1}$, *bla*-$_{CTX-M-9}$), *bla*-$_{TEM}$ and *bla*-$_{SHV}$ genes

Genotypic confirmation of ESBL genes was done in a total reaction volume of 25 µl for CTX-M and *bla*-$_{TEM}$ as per method described by Dallenne *et al.* (2010) and *bla*-$_{SHV}$ genes by Bhattacharjee *et al.* (2007). Amplicon size, primer sequence

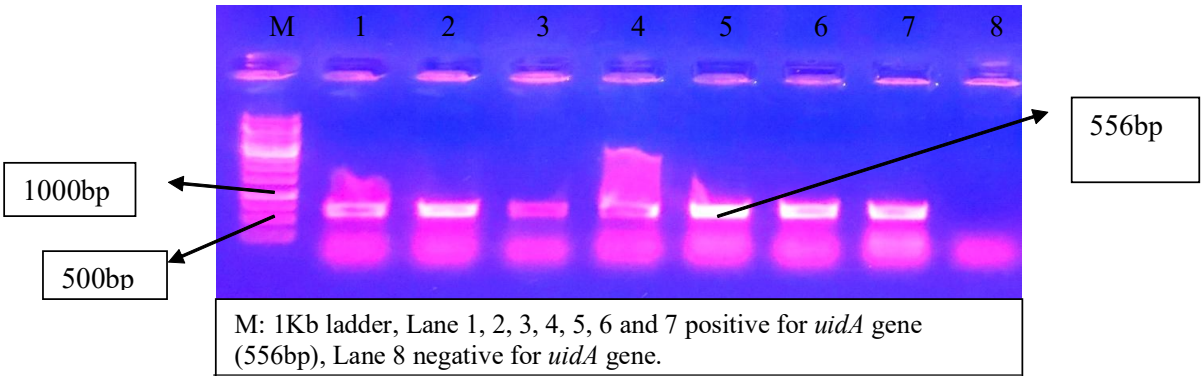

Fig 1: PCR amplification of *uidA* gene (556bp).

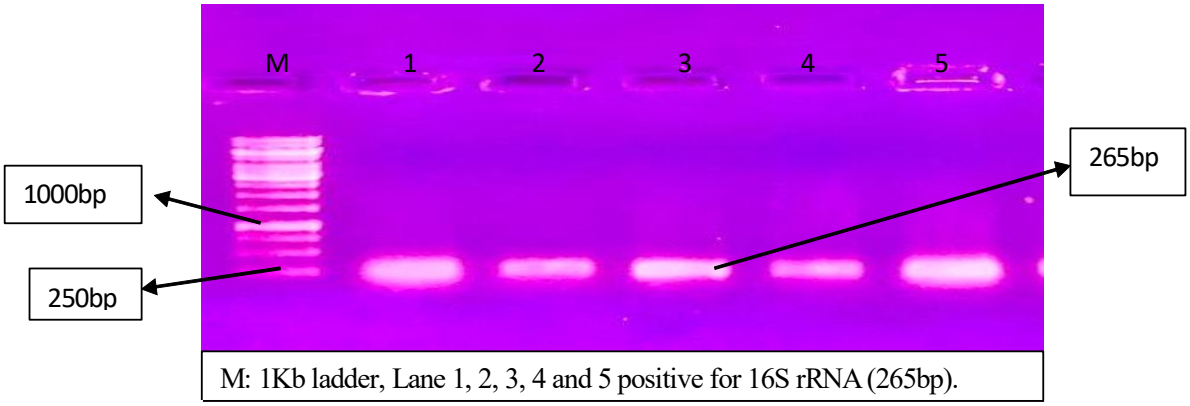

Fig 2: PCR amplification of 16S rRNA gene (265bp).

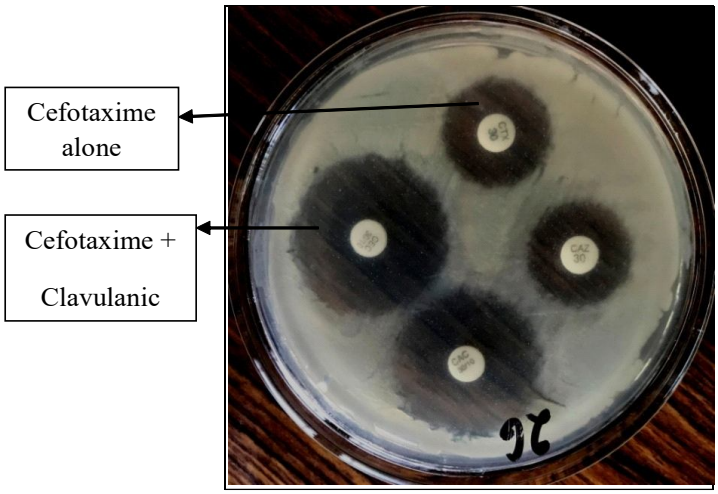

Fig 3: DDST for confirmation of ESBL producing *E. coli* and *Klebsiella* spp.

of targeted genes and cyclic conditions of PCR are mentioned in Table 2. Multiplex PCR was performed for CTX-M genes and simplex PCR was performed for *bla*-$_{TEM}$ and *bla*-$_{SHV}$ genes. Amplified products (5 µl) were mixed with 3µl of bromophenol blue dye (6X) and electrophoresis was done in 2% agarose gel for CTX-M genes using 50bp ladder and in 0.8% gel for *bla*-$_{TEM}$ and *bla*-$_{SHV}$ genes using 1 kb ladder at 60-70 mA for 40 min and gel was visualized using the UV illuminator (GeNei Bangalore, India).

RESULTS AND DISCUSSION

A total 240 samples of milk and faeces were collected, out of which 152 (63.33%) isolates were presumptively identified as *E. coli* and 24 (10.0%) as *Klebsiella* spp. on the basis of morphological and biochemical characteristics. Further PCR analysis of these isolates confirmed 135 (56.25%) as *E. coli* and 16 (6.67%) as *Klebsiella* spp. (Table 3, Fig 1 and 2). In the present study, *E. coli* was predominant among isolates

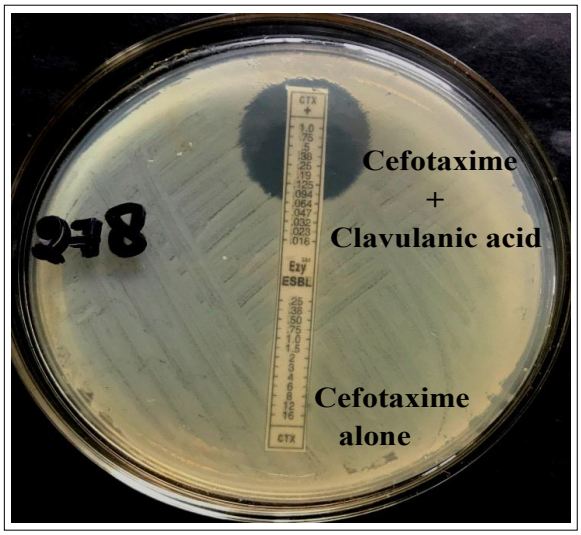

Fig 4: ESBL E-strip test for confirmation of ESBL producing *E. coli.* and *Klebsiella* spp.

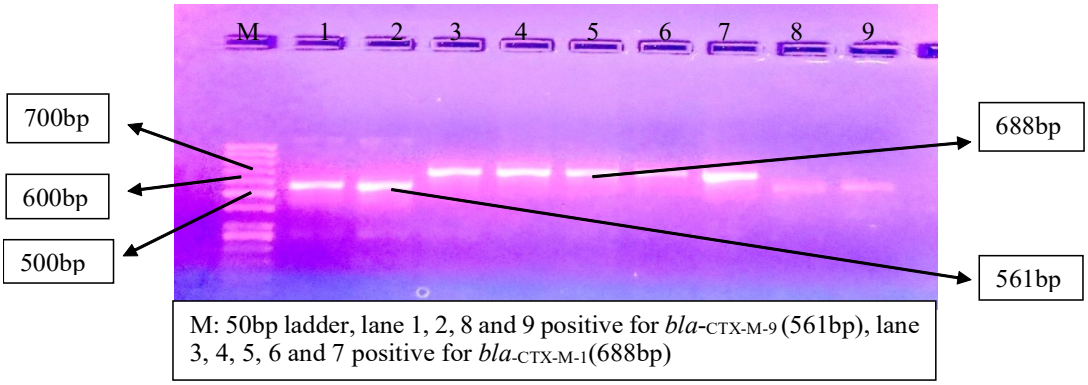

Fig 5: Multiplex PCR amplification of *bla*$_{-CTX-M-1}$(688bp), and *bla*-$_{CTX-M-9}$(561bp).

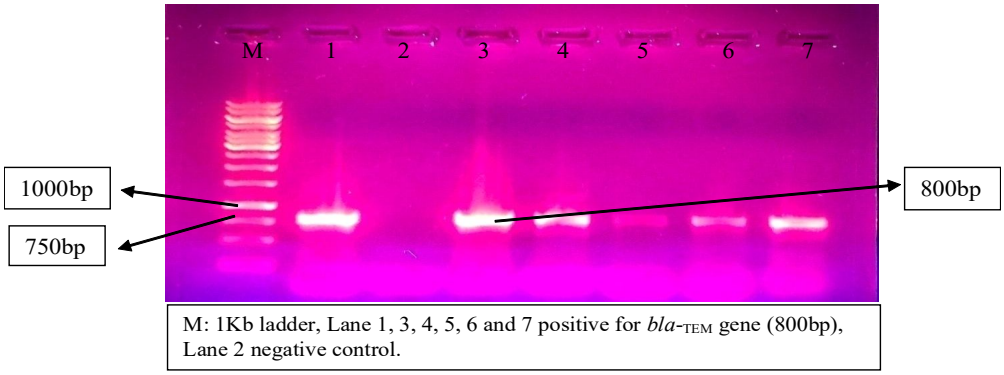

Fig 6: PCR amplification of *bla*-$_{TEM}$ gene (800bp).

Table 5: Distribution of ESBL positive *E. coli* and *Klebsiella* spp. isolates according to screening, phenotypic and genotypic confirmation tests.

Tests	Positive ESBL		
	E. coli (135)	*Klebsiella spp.*(16)	Total (151)
Screening test (AST)	115/135=85.18%	14/16=87.5%	129/151=85.43%
Double disc synergy test (DDST)	93/135=68.88%	08/16=50.0%	101/151=66.88%
ESBL-E test	86/135=63.70	06/16=37.5%	92/151=60.92%
PCR	82/135=60.74%	05/16=31.25%	87/151=57.61%

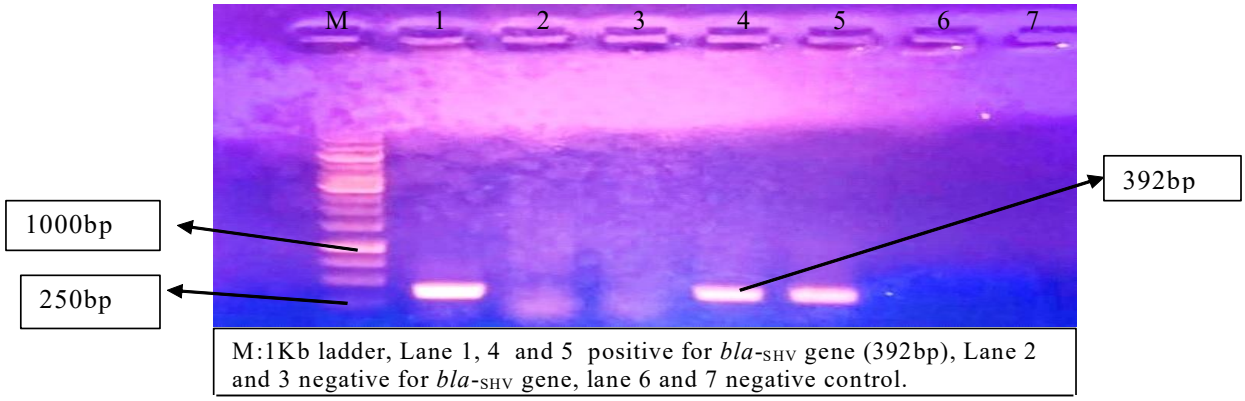

Fig 7: PCR amplification of *bla*-$_{SHV}$ gene (392bp).

Table 6: Distribution of ESBL genes among ESBL positive isolates.

No. of genes	Bla-genes	Positive isolates (n=92)	
		Number	Per cent
Single gene	*bla*-$_{CTX-M-1}$	60	65.21%
	bla-$_{CTX-M-9}$	38	41.30%
	bla-$_{TEM}$	29	31.52%
	bla-$_{SHV}$	25	27.17%
Multiple genes	*bla*-$_{CTX-M-1,}$ *bla*-$_{CTX-M-9}$	7	7.60%
	bla-$_{CTX-M-9,}$ *bla*$_{-SHV}$	05	5.43%
	bla-$_{CTX-M-1,}$ *bla*-$_{TEM}$	18	19.57%
	bla-$_{CTX-M-1,}$ *bla*$_{-SHV}$	08	8.7%
	bla-$_{CTX-M-9,}$ *bla*-$_{TEM}$	03	3.26%
	bla-$_{CTX-M-1,}$ *bla*-$_{CTX-M-9,}$ *bla*-$_{SHV}$	06	6.52%
	bla-$_{CTX-M-1,}$ *bla*-$_{CTX-M-9,}$ *bla*-$_{TEM}$	08	8.7%

recovered from both milk and faecal samples. These finding were in concordance with the findings of Ibrahim *et al.* (2018) and Kotsoana *et al.* (2019). Higher isolation rate of *E. coli* in this study might be attributed to high prevalence of *E. coli* in the gut flora of ruminants.

The results of in vitro antimicrobial susceptibility test (AST) are mentioned in Table 4. The AST revealed that most resistant antibiotics were ampicillin (89.40%) followed by cefotaxime (72.18%), cefpodoxime (71.52%), ceftriaxone (66.9%), aztreonam (61.0%) and ceftazidime (54.30%) but drugs such as gentamicin, amikacin and chloramphenicol were found to be 100% sensitive. There are several reports that corroborate with the finding of this study (Ramasamy *et al.*, 2021; Batabyal *et al.*, 2018; Badri *et al.* 2017). In this study, carbapenem antibiotics like imipenem and meropenem also showed resistance against these isolates, 18.75% to 12.50% for *Klebsiella* spp. and 8.89 to 3.7% for *E. coli* respectively (Table 4). Although these antibiotics are not allowed to use in animal husbandry practices anywhere in the country, even then resistance in animal isolates may emerged and propagated as a result of clinical use in human medicine and transfer of these resistant genes to zoonotic pathogens (Bhardwaj, 2015). In this study 81.52% (75/92) isolates were found to be multidrug resistant (MDR), which highlighted a potential threat to human health and thereby limiting the therapeutic options.

The present study also aimed to determine the proportion of ESBL phenotypes among clinical and apparently healthy samples. Total 151 isolates (135 *E. coli* and 16 *Klebsiella* spp.) were subjected to screening and confirmatory phenotypic tests. Out of 151 isolates, 129 (85.43%) were presumed as ESBL producer by AST. In phenotypic confirmatory testing, 101 (66.88%) isolates were confirmed as ESBL positive by DDST and 92(60.92% by ESBL-E strip test. Final confirmation was done by PCR analysis which revealed 87 (57.61%) ESBL positive isolates (Table 5). There was little difference in the sensitivity of both phenotypic confirmatory tests and PCR analysis and this observation corroborated with the findings of Badri *et al.* (2017) and Olowe *et al.* (2015).

Gene distribution study of ESBL positive isolates was carried out using PCR by targeting CTX-M (*bla*-$_{CTX-M-1}$, *bla*-$_{CTX-M-9)}$, *bla*-$_{TEM}$ and *bla*-$_{SHV}$ genes (Fig 5, 6, 7). Over all prevalence of ESBL gene was found to be 65.21% for *bla*-$_{CTX-M-1}$ followed by *bla*-$_{CTX-M-9}$, *bla*-$_{TEM}$ and *bla*-$_{SHV}$ with 41.30%, 31.52% and 27.17%, respectively. The present finding revealed predominance of *bla*-$_{CTX-M-1}$ gene in this area. Similarly various co-workers across the world have also

reported high frequency of *bla*-$_{CTX-M}$ gene in different sample sources (Badri *et al.*, 2017; Ibrahim *et al.*, 2018; Paghdar *et al.*, 2020; Olowe *et al.*, 2015; Yadav *et al.*, 2019; Schmid *et al.*, 2013). Multiple co-existences of *bla* genes were also observed which has been mentioned in Table 6. Similar to this finding, co-existence of *bla* genes was also reported by various workers (Yadav *et al.*, 2019; Tekinar and Ozpinar 2016).

CONCLUSION

This study highlighted, higher occurrence of ESBL producing *E. coli* than *Klebsiella* spp. and most of the isolates showed resistance to ampicillin, 3rd and 4th generation cephalosporins, which is an alarming situation for this area. Despite this, some isolates of *E. coli* and *Klebsiella* spp. also exhibited resistance against carbapenems, even without its use in animal husbandry practices, which are not a good sign from public health point of view. Therefore, a specific study on rational use of antibiotics and continuous monitoring for resistance genes against these antibiotics in livestock is warranted.

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