RESEARCH ARTICLE

Isolation, Identification, Molecular Characterization and Antimicrobial Resistance Profiling of ESBL Positive *E. coli* Isolates of Cattle in Eastern Zone of Uttar Pradesh

Vibha Yadav[1*], Rajesh K. Joshi[2], Namita Joshi[3], Amit Kumar[4], Mrinal Srivastva[5], Satyavrat Singh[6], Pankaj K. Chaudhary[7]

Abstract

In this present study total of 240 milk and fecal samples were collected from two districts of the plain Eastern zone of Uttar Pradesh (India). Among 240 samples, a total of 135 (56.25%) isolates were confirmed as *E. coli* using a species-specific uidA gene oligonucleotide primer. Eighty-six (63.70%) ESBL producing *E. coli* isolates were recovered out of 135 isolates using confirmatory phenotypic methods. The antimicrobial resistance pattern revealed that all isolates showed (60-100%) resistance against cefotaxime, cefpodoxime, ceftriaxone, ampicillin, ceftazidime, aztreonam, etc. and 100% sensitivity against gentamicin, amikacin, chloramphenicol, and polymyxin-B. The multi-drug resistance (MDR) pattern of ESBL organisms was assessed by using the multiple antibiotic resistance (MAR) index. Out of 86 ESBL positive isolates, 70 (81.39%) *E. coli* was showed multi-drug resistance in the study. MAR index of these isolates was in the range from 0.25 to 0.75. The most frequently observed MAR index was 0.30 against 28 isolates. Antibiotic resistance patterns against non-β-lactam antibiotics were also observed, and 51 isolates showed resistance against both β-lactam and non-β-lactam antibiotics, while 19 isolates showed resistance against only β-lactam antibiotics of different groups.

Keywords: Antibiogram, Cattle, *E. coli*, ESBLs, MAR index, uidA gene.

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Introduction

Antimicrobial resistance (AMR) has been a global problem contributing to rise in mortality and cost of treatment. Antibiotics are widely used in animals and humans to fight bacterial infections. However, their indiscriminate use leads to the emergence of multidrug-resistant bacteria, limiting the treatment option (Saidani *et al.,* 2017). *E. coli* is the average inhabitant of the intestinal microbiota of animals and mammals and plays an essential role in host metabolism, immunology, and nutrition.

Production of Extended-spectrum β-lactamases (ESBLs) is the most common resistance mechanism to third-generation cephalosporins among Enterobacteriaceae, including E. coli *Klebsiella* species. β-lactams are the most commonly prescribed antibiotics against bacterial infections. The emergence of ESBL producing strains has limited the use of broad-spectrum cephalosporins in the management of serious infections caused by these pathogens, which results in therapeutic failures and a high mortality rate (Roy *et al*., 2013).

Currently, there is a paucity of information on ESBL producing *E. coli* from animals in this region and their possible contributions to the development of resistance to other species or pathogens. Hence, surveillance-based study, antimicrobial resistance profiling, searching for an alternative antibiotic is the need of the hour to address the global issue of antimicrobial resistance. Hence, in this study, isolation, biochemical analysis, molecular characterization by uidA gene, detection of ESBL positive isolates by phenotypic methods, and antibiotic resistance profiling of ESBL positive

[1-2]Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj-224229, Ayodhya, UP, India.

[3]Department of Veterinary Public Health & Epidemiology, College of Veterinary Science & Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj-224229, Ayodhya, UP, India.

[4-5]Department of Immunology & Defense mechanism, College of Biotechnology, SVP University of Agriculture & Technology, Meerut-250110 (UP), India.

[6]Department of Veterinary Medicine, College of Veterinary Science & Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj-224229, Ayodhya, UP, India

[7]Department of Veterinary Physiology & Biochemistry, College of Veterinary Science & Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj-224229, Ayodhya, UP, India.

Corresponding Author: Vibha Yadav, Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj-224229, Ayodhya, UP, India, e-mail: vibhavet2005@gmail.com

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isolates from milk and fecal samples of healthy and diseased cattle was done.

Materials and Methods

Sample Collection

A total of 240 samples were collected from 5 tehsils of Ayodhya and 3 tehsils of Sultanpur district of plain Eastern zone of Uttar Pradesh (India). The samples were collected randomly from both clinical and non-clinical cases, and it consisted of 10 regular and 5 mastitic milk samples from each of the tehsils. Likewise, 10 normal and 5 diarrhoeic fecal samples were collected from the regions mentioned above. All samples collected aseptically were transported to the Veterinary Microbiology Laboratory under a cold chain for further processing.

Isolation and Identification

Samples were enriched with 2mL nutrient broth and incubated for 24hrs at 37°C. A loopful of inoculums was taken and directly streaked on MacConkey agar (MLA) plates, added with 2 mg/L cefotaxime, and incubated at 37^{O}C for 24hrs. Colonies showing lactose fermenting characteristics (rose pink) (Fig. 1) were picked up and streaked on selective media Eosin Methylene Blue (EMB) (Fig. 2) agar plates and primarily identified by the method of Cruickshank *et al*. (1975). The pure colonies were subjected to Gram's staining and biochemical characterization by using a KBM001 HiMotility^{TM} Biochemical kit for *E. coli* (Hi-media, Fig. 3) combination of 12 tests. The standard test procedure was followed as mentioned in the kit. Results were interpreted as per the standards given in the interpretation chart provided by the manufactures of the kit.

Extraction of Genomic DNA

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

Molecular Identification of *E. coli* by PCR Analysis

All identified *E. coli* by conventional methods were subjected to species-specific PCR identification by amplifying the uidA housekeeping genes specific to *E. coli* species (Table 1).

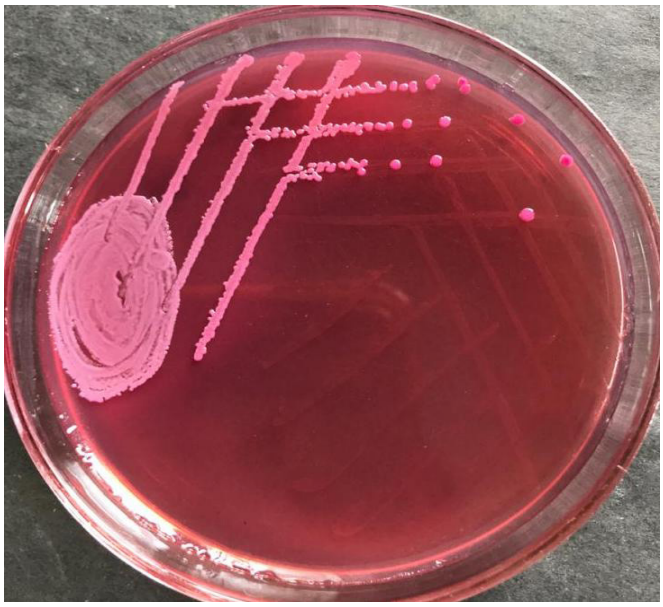

Fig 1: Pink colour colonies of *E. coli* on MLA plate

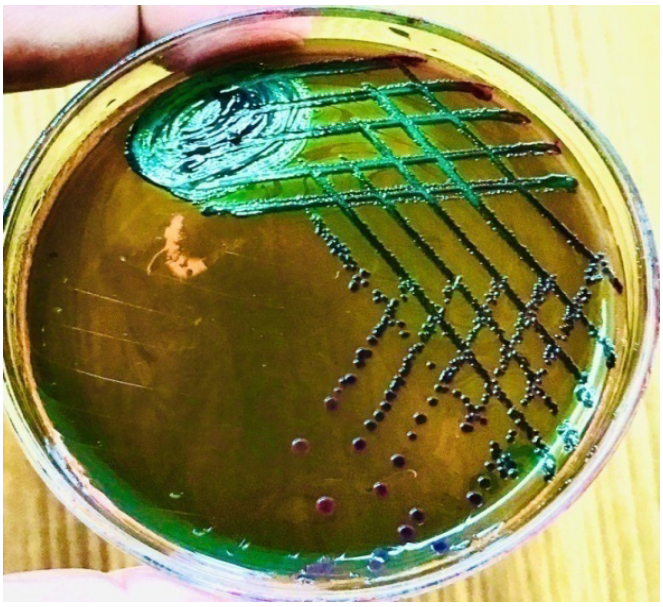

Fig 2: Green metallic sheen of *E. coli* on EMB Agar

Fig 3: Biochemical Test of *E. coli.* isolates using KBM001 HiMotility^{TM} Biochemical kit

Table 1: Detail of primer used for molecular characterization of *E. coli*

Group	*Gene*	*Primer pair*	*Product size (Amplicon)*	*Reference*
E. coli	uidA	F 5'CTG GTA TCA GCG CGA AGT CT-3' R 5'AGC GGG TAG ATA TCA CAC TC-3'	556	Anbazhagan *et al.,* 2010

Sequencing of PCR Amplicons

Two *E. coli* specific uidA gene PCR products were submitted for Sanger sequencing to Biokart India Pvt Ltd, Bengaluru, India, and sequences were subjected to Blast Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the identities of the isolates.

Screening of ESBL Producing Isolates

All the confirmed *E. coli* isolates were subjected to ESBL screening using 3rd, 4th generation cephalosporins and monobactam as per Kirby-Bauer's disc diffusion method (Fig. 5). The results were interpreted as per CLSI (2019) guidelines.

Confirmation of ESBL Producing *E. coli* by Phenotypic Methods

(a) Double disc synergy test (DDST) was performed for confirmation of ESBL production by resistant bacteria using ESBL kit 1 and kit 3 (Hi-media) (Fig. 6). The results were interpreted as per CLSI (2019) guidelines. The test organism was considered ESBL positive if a ≥ 5mm increase in zone diameter was observed for two or more antimicrobial agents tested combined with clavulanic acid versus its zone when tested alone (Fig 6). (b) The minimum inhibitory concentration (MIC) was determined using ESBL E-test strips (Fig. 8) as described by Patwardhan *et al*. (2013). The positive isolates were interpreted as ESBL positive by an eightfold decrease in MIC ratio in the presence of β-lactamase inhibitors (Fig 8).

Study of Multi-Drug Resistance (MDR) Pattern of ESBL Producing *E. coli* Isolates

All the phenotypically confirmed as ESBL resistant were subjected for their resistance or susceptibility, using 20 antibiotics belonging to 12 different groups (CLSI, 2016) (Fig. 7). Isolates showing resistance to at least one antibiotic in three or more classes of the drug were considered as MDR (CLSI, 2016).

Multiple Antibiotic Resistance (MAR) Index

MAR index is the number of antibiotics to which test isolate displayed resistance divided by the total number of antibiotics to which the test organism has been evaluated for sensitivity. MAR index of each isolate was calculated as per the method of Krumpermann (1983).

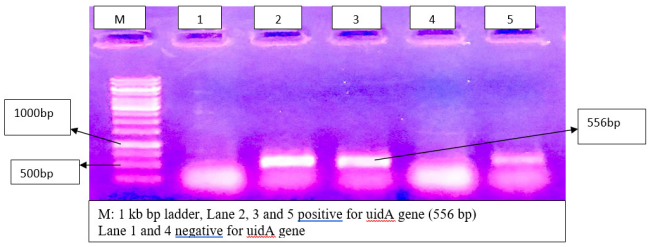

Fig 4: PCR confirmation of uidA gene

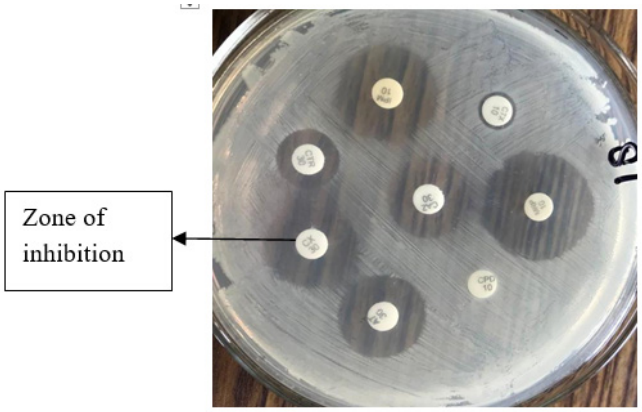

Fig. 5: Kirby Bauer's disc diffusion

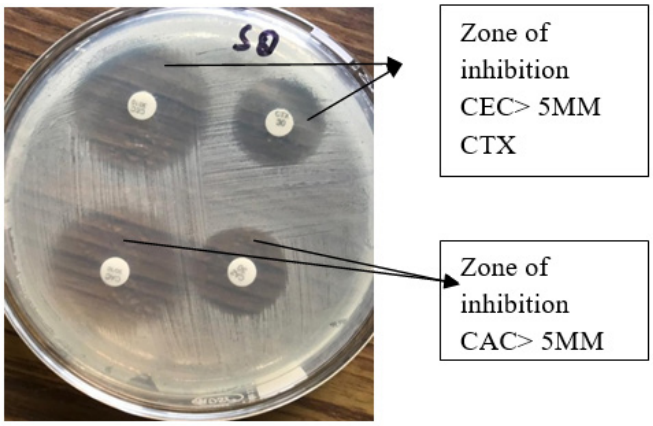

Fig. 6: Double disc synergy test for confirmation of ESBL producing *E. coli*.

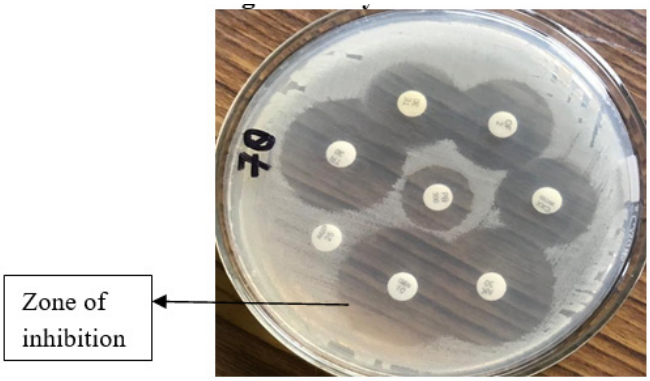

Fig 7: Kirby Bauer's disc diffusion method for ABST of ESBLs.

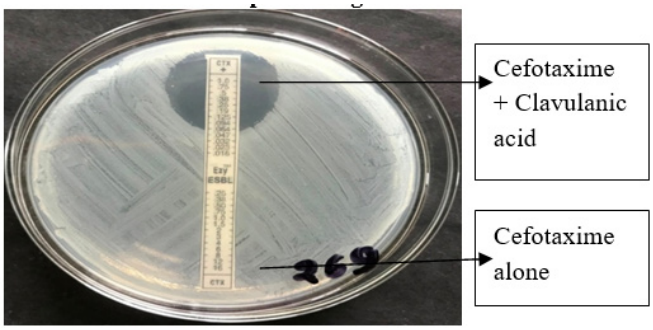

Fig 8: MIC ESBL E- strip test for confirmation of ESBL producing *E. coli*. spp.

Table 2: Isolation rate of *E. coli* in clinical and non-clinical milk and fecal samples of cattle origin

Samples (Source/Origin)	*No of samples*	*Presumptive positive E. coli isolates by conventional method*	*Confirmed E. coli isolates using species specific uidA primer*	*ESBL positive isolates confirmed by Phenotypic methods*
Normal milk	80	18(22.5%)	12(15.0%)	7(8.75%)
Mastitic milk	40	25(62.5)	18(45.0%)	5(12.5%)
Normal faeces	80	79(98.5%)	72(90%)	48(60.0%)
Diarrhoeic faeces	40	37(92.5%)	33(82.5%)	26(65.0%)
Total	240	159(66.25%)	135(56.25%)	86(35.83%)

Table 3: Distribution of ESBL strains, according to screening and phenotypic confirmation test

Source	*Confirmed positive isolates*	*Screened by disc diffusion method*	*Confirmed by phenotypic methods*	
			DDST	*E-strip ESBL test*
Normal milk (n=80)	12/80(15.0%)	11/12(91.7%)	9/12(75.0%)	7/12(58.33%)
Mastitic milk (n=40)	18/40(45.0%)	12/18(66.7%)	7/18(38.9%)	5/18(27.8%)
Normal faeces (n=80)	72/80(90%)	61/72(84.72%)	52/72(72.22%)	48/72(66.7%)
Diarrhoeic faeces (n=40)	33/40(82.5%)	31/33(93.93)	27/33(81.81%)	26/33(78.8%)
Total (240)	135/240(56.25%)	115/135(85.18%)	95/135(70.37%)	86/135(63.70%)

Table 4: Antimicrobial drug resistance profiling in ESBL positive isolates of cattle origin

Antibiotics (Hi-Media)	*Conc. (µg/disc)*	*ESBL isolates of milk*	*ESBL isolates of fecal sample*
		Resistant (%)	*Resistant (%)*
Imepenem (IMP)	10	25.00	12.16
Meropenem (MRP)	10	0.00	6.75
Cefotaxime (CTX)	10	100.00	100.00
Cefpodoxime (CPD)	10	100.00	100.00
Ceftazidime (CAZ)	30	58.33	89.18
Ceftriazone (CTR)	30	100.00	100.00
Aztreonam (AT)	30	41.70	63.51
Cefoxitin (CX)	30	41.70	8.10
Ampicillin (AMP)	30	100.00	98.64
Polymixin B (PB)	300 unit	0.0.00	2.70
Gentamicin (GEN)	10	0.00	0.00
Amikacin (AK)	30	0.00	0.00
Ofloxacin (OF)	2	41.70	20.27
Enrofloxacin (EX)	10	25.00	16.21
Nalidixic acid (NA)	30	25.00	25.67
Tetracycline (TE)	30	16.70	18.91
Trimethoprim (TR)	30	25.00	25.67
Co-trimoxazole (COT)	25 (23.75/1.25)	25.00	24.32
Amoxyclav (AMC)	20/10	0.00	23.00
Chloramphenicol (C)	30	0.00	0.00

Results and Discussion

A total of 240 samples comprising 120 milk samples (80 normal and 40 mastitic milk) and 120 fecal samples (80 normal and 40 diarrhoeic fecal) were processed for isolation and identification. Based on morphological and biochemical characteristics (Fig. 1, Fig. 2), 159 (66.25%) isolates were preliminarily identified as *E. coli.* These isolates were further processed using species-specific uidA gene oligonucleotide primer (Table 1, Fig. 4), and a total of 135 (56.25%) isolates were confirmed as *E. coli*. In this study, the isolation rate observed was 90.0%, 82.5%, 45.0%, and 15.0% in normal fecal, diarrhoeic fecal, mastitic, and normal milk samples, respectively (Table 2). These results were similar to Batabyal *et al*. (2018) and Badri *et al*. (2017), who recorded 12.1% and 38.0% isolation rates of *E. coli* from normal raw milk samples, respectively. Ibrahim *et al*. (2018) also found a 35.0% isolation rate of *E. coli* from mastitic cow milk samples. The isolation rate of *E. coli* from fecal samples was in concordance with the results observed by Gupta *et al*. (2019) and Malik *et al.* 2012). The difference in the isolation rate of *E. coli* from fecal samples of bovine origin may be due to different geographical locations, management, and hygienic practices. In this study, the isolation rate of *E. coli* was 90.0% in normal fecal samples and 82.5% in diarrhoeic fecal samples. The isolation rate of *E. coli* from both normal and diarrhoeic was almost similar and very high compared to milk samples because *E. coli* is a commensal microorganism of the gut flora of cattle.

The species-specific uidA gene sequence data indicated that *E. coli* isolates (Accession No: MW353604) possessed 99.82% similarities to *E. coli* strain K-12 (Accession No: LR881938.1), *E. coli* ST18 strain (Accession No: CP060709.1), *E. coli* O68:H12 strain (Accession No: CP061758.1), *E. coli* strain EC93 (Accession No: CP061329.1), 99.20% with *E. coli* O157:H16 strain (Accession No: CP007592.1) and 99.01% with *E. coli* O145:H28 (Accession No: AP019703.1) beta-D-glucuronidase gene, partial sequence.

Total 135 confirmed isolates were further processed for screening and confirmatory tests, 85.18% were ESBL positive using disc diffusion method (Fig. 5), 70.37% by confirmatory DDST (Fig. 6), and 63.70% by E-strip ESBL test (Fig. 8). The

Table 5: Multiple drug resistance patterns of ESBL positive isolates

No. of isolates	*No. of antibiotics*	*Antibiotic resistance pattern*		
		β-lactam antibiotics	*Non β-lactam antibiotics*	*MAR index (20)*
2	15	IMP,MRP,CPD,CTX,CTR,CAZ,AT,CX,AMP	EX, OF,TE,TR, COT, NA	0.75
1	15	IMP,MRP,CPD,CTX,CTR,CAZ,AT,CX,AMP	EX, OF, TR, NA, PB	0.75
1	14	IMP,MRP,CPD,CTX,CTR,CAZ,AT,AMP	EX, OF, TE, TR, COT, NA	0.70
1	13	IMP,MRPCPD,CTX,CTR,CAZ,AMP	EX, OF, TE, TR, COT, NA	0.65
2	13	IMP,CPD,CTX,CTR,CAZ,AT,CX,AMP	EX, OF, TR, COT, NA	0.65
1	12	MRP,CPD,CTX,CTR,CAZ,AMP	EX, OF, TE, TR, COT, NA	0.60
2	11	CPD,CTX,CTR,CAZ,AT,AMP	EX, OF, TR, COT, NA	0.55
2	10	CPD,CTX,CTR,CAZ,AT,AMC,AMP	OF, TE, TR	0.50
1	10	CPD,CTX,CTR,CAZ,AT,CX,AMP	TE, TR, COT	0.50
3	9	CPD,CTX,CTR,CAZ,AT,CX,AMP	OF, TR	0.45
2	9	CPD,CTX,CTR,CAZ,AT,AMP	OF, TR, COT	0.45
2	9	IMP,CPD,CTX,CTR,CAZ,AT	EX, COT, NA	0.45
1	9	IMP,CPD,CTX,CTR,CAZ,AT,AMP	EX,TE	0.45
1	9	CPD,CTX,CTR,CAZ,AT,AMP	OF,TE,TR	0.45
1	9	CPD,CTX,CTR,CAZ,AT,AMC,AMP	TE,TR	0.45
2	8	CPD,CTX,CTR,CAZ,AT,CX,AMP	TE	0.40
2	8	CPD,CTX,CTR,AT,AMP	TE, TR, COT	0.40
2	8	CPD,CTX,CTR,CAZ,AT,AMC,AMP	EX	0.40
1	8	CPD,CTX,CTR,CAZ,AT,AMC,AMP	NA	0.40
1	8	IMP,CPD,CTX,CTR,CAZ,AT,AMP	OF	0.40
3	7	CPD,CTX,CTR,CAZ,AT,AMC,AMP	-	0.35
2	7	CPD,CTX,CTR,CAZ,AMC,AMP	NA	0.35
2	7	CPD,CTX,CTR,CAZ,AMC,AMP	COT	0.35
1	7	CPD,CTX,CTR,CAZ,AT,AMC,AMP	-	0.35
1	7	CPD,CTX,CTR,CAZ,AT,AMP	NA	0.35
1	7	CPD,CTX,CTR,AT,AMP	PB,NA	0.35
13	6	CPD,CTX,CTR,CAZ,AT,AMP	-	0.30
3	6	CPD,CTX,CTR,AMP	COT, NA	0.30
2	6	CPD,CTX,CTR,CAZ,AMP	TR	0.30
2	6	CPD,CTX,CTR,CAZ,AMP	OF	0.30
2	6	CPD,CTX,CTR,CAZ,AT	NA	0.30
1	6	CPD,CTX,CTR,CAZ,AMP	TE	0.30
1	6	CPD,CTX,CTR,CAZ,AMC,AMP	-	0.30
1	6	CPD,CTX,CTR,AT,AMP	COT	0.30
1	6	IMP,CPD,CTX,CTR,CAZ	NA	0.30
1	6	CPD,CTX,CTR,CAZ,AMP	NA	0.30
1	6	CPD,CTX,CTR,CAZ,CX,AMP	-	0.30
1	5	CPD,CTX,CTR,AMP	PB	0.25

distribution of ESBL positive isolates in normal milk samples was 91.7%, 75.0%, and 58.3%, and in mastitic milk, 66.7%, 38.9%, and 27.8% by disc diffusion, DDST, and E-strip test, respectively (Table 3). Badri *et al*. (2017) also reported similar results with 89%, 74.0%, and 68.0% positive ESBLs in raw milk by screening, DDST, and PCR analysis, respectively. In our

study, ESBL positive isolates observed were 84.72%, 72.22%, and 66.7% from normal fecal samples, while 93.93%, 81.81%, and 78.8% from diarrhoeic fecal samples using disc diffusion, DDST, and E-strip test, respectively (Table 3). Total 86(35.83%) isolates were ESBL positive, comprising 7 (8.75%), 5 (12.5%), 48 (60.0%), and 26 (65.0%) from normal milk, mastitic milk, normal fecal and diarrhoeic fecal samples, respectively (Table 2).

Antibiotic resistance, currently a severe problem, is an increasing global threat to human and animal health. In the present study, the antibiogram pattern of all ESBL positive isolates was recorded against 20 antibiotics. The most frequently observed resistance phenotypes in all isolates of milk samples was 100% against 4 antibiotics, *viz*., cefotaxime, cefpodoxime, ceftriaxone, and ampicillin, while 100% sensitive against meropenem, polymixin-B, gentamicin, amikacin, amoxiclav, and chloramphenicol (Table 4, Fig. 7). Similar findings were also reported by Badri *et al*. (2017). Batabyal *et al*. (2018) found 60-100% resistance against cefotaxime, ceftazidime, and 100% sensitivity against colistin and imipenem in normal milk samples of bovine origin. Ibrahim et al. (2018) reported 100% sensitivity against imipenem and meropenem in mastitic milk samples. Geser *et al*. (2012) reported 100% resistance against ampicillin and cefpodoxime in mastitic milk samples.

All isolates from fecal samples showed 60-100% resistance against cefotaxime, cefpodoxime, ceftriaxone, ampicillin, ceftazidime, and aztreonam while 100% sensitive against gentamicin, amikacin, chloramphenicol followed by 92.0% to 97.3% against polymixin-B, meropenem, and cefoxitin (Table 4). Gupta *et al*. (2019) and Geser *et al*. (2012) reported more or less similar results for isolates of fecal samples of cattle. Mahato *et al*. (2019) reported 100% resistance against ampicillin and cefpodoxime, followed by 85.7% against ceftazidime, cefotaxime, and ceftriaxone. In this study, resistance against imipenem was also found to be 25.0% in milk isolates and 12.16% in fecal isolates; however, these antibiotics are not used to treat animals, which may be attributed to horizontal transfer of these genes from human to animals. Plaucible factors for a high degree of resistance against 3rd and 4th generation cephalosporins in this study may be due to inappropriate and continuous use of these antibiotics without referring to an antibiogram or acquired from farmworkers, environmental sources, and other livestock, or due to production of ESBLs.

Multi-drug resistant (MDR) bacteria are of serious concern, limiting the treatment options and may pose severe health complications to humans. Out of 86 ESBL positive isolates, 70(81.39%) of *E. coli* had shown multi-drug resistance in our study (Table 5). A similar finding (85.7%) was observed by Mahato *et al*. (2019**).** MAR index 0.75, 0.65 and 0.5 was observed for 3 isolates each, 0.70, 0.60 and 0.25 for one isolate each, 0.55 for 2 isolates, 0.45 and 0.35 for 10 isolates each, 0.40 for 8 isolates, 0.30 for maximum 28 isolates **(**Table 5**).** Out of 70 ESBL positive MDR 19 (27.14%) isolates showed resistance against only β-lactam antibiotics, while 51(72.85%) isolates showed resistance against both β-lactam and non-β-lactam antibiotics of different groups. The genes responsible for ESBL positive isolates are linked to other resistant genes to non-β-lactam antibiotics.

Conclusions

The present study focussed on the occurrence of ESBL producing *E. coli* in milk and fecal samples of cattle. The overall prevalence of ESBL producing *E. coli* was 86 (35.86%), with 31(38.75%) in diseased cattle and 55 (34.38%) in apparently healthy cattle. Hundred percent resistance was observed against cefotaxime, cefpodoxime, ceftriaxone, and ampicillin since these antimicrobial agents are extensively used as the preferred drug in the treatment of various infections in livestock of this area. Findings of the study related to the sensitivity of the isolates suggest that aminoglycosides, polypeptides, chloramphenicol are the most influential group of antibiotics. More than 81.39% ESBL positive isolates showed multiple drug resistance, suggesting a very close link in antibiotic exposure histories. This study showed high resistance of *E. coli* to antibiotics, particularly third-generation cephalosporins, for which regular monitoring, appropriate and regulated use of antibiotics after referring antibiogram should be encouraged.

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