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Prevalence of Extended-spectrum β-lactamase Producing *E. coli* and *Klebsiella* spp. Isolated from Buffaloes in Eastern Plain Zone of Uttar Pradesh

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ABSTRACT

Background: Antimicrobial resistance (AMR) due to emergence and spread of Extended-spectrum β-lactamase (ESBL) producing bacteria are point of discussion in both human as well as in animals across the world as it is one of the latest challenges faced by scientific community. The present study highlighted the prevalence of ESBL producing *E. coli* and *Klebsiella* spp. isolated from buffaloes in Uttar Pradesh.

Methods: Total 240 samples were collected from two district of eastern plain zone of Uttar Pradesh during January 2020 to March 2021. *E. coli* and *Klebsiella* spp. isolates were confirmed by cultural characteristics in selective media and biochemical tests. ESBL producing isolates were confirmed by DDST, ESBL E-strip and PCR analysis using specific primer for (*bla*-$_{CTX-M-1}$, *bla*-$_{CTX-M-2}$, *bla*-$_{CTX-M-9}$, *bla*-$_{TEM}$ and *bla*-$_{SHV}$). Antibiogram of all confirmed ESBL producing isolates was performed against 20 antimicrobials under 12 different classes.

Result: In the present study, a total of 147 (61.25%) *E. coli* and 19 (7.91%) *Klebsiella* spp. were isolated and identified based on cultural and biochemical characteristics. A total of 104 (62.65%) and 99 (59.63%) isolates were confirmed as ESBL producers using DDST and ESBL E-strip tests respectively. PCR analysis, revealed the presence of selected ESBL genes in 92 (55.42%) isolates, among them *bla*-$_{CTX-M-1}$ was found most dominant gene (69.41%) in *E. coli* and (71.42%) in *Klebsiella* spp. All the ESBL positive *E. coli* and *Klebsiella* spp. isolates were found to be 99-100% and 86-100% sensitive to chloramphenicol, polypeptides and aminoglycosides classes respectively. Both isolates of *E. coli* and *Klebsiella* spp. showed 100% resistance to cefotaxime, cefpodoxime ceftriazone and ampicillin, 76.47% and 71.42% to ceftazidime respectively and 81.52% isolates were found to be MDR.

Key words: Buffaloes, *E. coli*, ESBL, *Klebsiella* spp.

INTRODUCTION

Extended-spectrum β-lactamase (ESBL) producing organisms are expanding rapidly throughout the world and have become a major problem in both human and veterinary medicine. Among *Enterobacteriaceae*, *E. coli* and *Klebsiella* spp. are major ESBL producers and have been identified as an emerging global threat due to their increasing prevalence in livestock during last few years (Reuland *et al*., 2013) and are main environmental pathogens, associated with various illnesses. ESBL are enzymes that hydrolyse most of β-lactam antibiotics and mediate resistance against penicillins, 3rd and 4th generation cephalosporins (Saravanan *et al*., 2018). The genes encoding for these enzymes are commonly located on mobile genetic elements, among the species belonging to family *Enterobacteriaceae,* which are horizontally transferred between a close families of bacteria and also help to spread the AMR gene in the environment (Ansari *et al*., 2018). As a result, ESBLs have emerged as a cause of resistance in *Enteribacteriaceae*, particularly *E. coli* and *Klebsiella* spp. Extensive and indiscriminate use of antibiotics leads to emergence of resistance against a variety of antibiotics called MDR organisms, which poses significant challenges for both scientists and clinicians (Kappeli *et al*., 2019). MDRs in *Enterobacteriaceae* is increasing day by day, leads to limited antimicrobial

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therapeutic options and major cause of morbidity and mortality worldwide (WHO, 2016).

Since no in depth study has been done on prevalence and distribution of ESBL genes (*bla*-$_{CTX-M}$, *bla*-$_{TEM}$ and *bla*-$_{SHV}$) among *E. coli* and *Klebsiella* spp isolated from buffaloes in this area of study and their importance in development of resistance to other species or pathogens. Molecular characteristics and AMR pattern, will help the researchers, field veterinarians and policy makers to developed a guideline to scrutinize the use of antibiotics for therapeutic purpose and also helpful in controlling the spread of these bacteria in community setting.

MATERIALS AND METHODS

Study area

The study was carried out in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya. The samples were collected from Ayodhya and Sultanpur districts of eastern plain zone of Uttar Pradesh, India. The study was conducted between January 2020 and March 2021.

Sample collection

Total 240 samples (120 faecal and 120 milk) were collected from 5 tehsils of Ayodhya district and 3 tehsils of Sultanpur district. Sampling was done randomly and consisting of 10 apparently normal healthy and 5 diarrhoeic animals from each of the tehsil. Likewise, 10 apparently healthy and 5 clinical mastitic milk samples from same regions. California Mastitis Test was used for screening of mastitic milk samples. Faecal samples were collected by swab technique and approximately 5 ml of milk samples were collected in sterilized test tubes. All collected samples were transported immediately to Bacteriology Laboratory under cold chain.

Isolation and identification

All samples were enriched with 2ml nutrient broth and incubated at 37°C for 24 hrs. A loopful inoculum was taken and directly streaked on MacConkey agar plates added with 2 mg/L cefotaxime and incubated at 37°C for 24 hrs. Colonies with rose pink colouration were picked up and streaked on Eosine Methylene Blue agar plates. Colonies showing greenish metallic sheen (Fig 1) were tentatively considered as *E. coli* while light purple colonies with dark centred and mucoid appearance (Fig 2) were suspected as *Klebsiella* spp. After this, pure colonies were taken onto sterilized nutrient agar slant and further identification was done by various biochemical tests *viz.* IMViC pattern, catalase test, (Fig 3) nitrate reduction, urease test, triple sugar iron agar and sugar fermentation reaction as per the method of Edward and Ewing (1972).

Screening of ESBL producing isolates

All confirmed isolates of *E. coli* and *Klebsiella* spp. were subjected to screening of ESBLs, using 3rd, 4th generation cephalosporins, monobactam and carbapenems as per Kirby-Bauer's. The results were interpreted as per CLSI guidelines (2019). Each isolates which showed resistant to one or more of these antibiotics, were screened for ESBL production.

Confirmation of ESBL producing isolates by phenotypic methods

Double disc synergy test (DDST)

All screened isolates were further confirmed by using ESBL kit 1 and kit 3 (Hi-media) (Fig 4). These commercially available discs were placed on MHA plates, infused with 1.5×10^8 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 performed by placing E-strip on MHA plates infused with 1.5×10^8 organism/ml and incubated at 37°C for 24 hrs. The results were interpreted as per CLSI guidelines (2019) (Fig 5).

Molecular characterization of ESBL producing *E. coli* and *Klebsiella spp*.

Extraction of plasmid DNA

Plasmid DNA was extracted from phenotypically confirmed isolates using GeneJet plasmid Miniprep kit (Thermo Scientific) as per the protocol of the manufacturers.

Fig 1: Metallic sheen of *E. coli* on EMB agar.

Fig 2: Purple dark mucoid colony of *Klebsiella* spp. on EMB agar.

Molecular characterization of CTX-M genes (*bla*-$_{CTX-M-1}$, *bla*-$_{CTX-M-2}$, *bla*-$_{CTX-M-2}$), *bla*-$_{TEM}$ and *bla*-$_{SHV}$ gene

ESBL gene detection was carried out 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*-$_{SHV}$ by Bhattacharjee *et al*. (2007). The primer sequence of targeted genes and amplicon size are listed in Table 1. Visualization of PCR product was done by mixing 5 µl of amplified products with 3 µl of bromophenol blue dye (6X) and electrophorased in 0.8% agarose gel in 1X TAE buffer mixed with 1 µl (5µg/ml) ethidium bromide, using 1kb DNA ladder (Thermo Scientific # SM 0311) at 60-70 mA for 40 min and gel was visualized using the UV illuminator (GeNei Bangalore, India).

***In vitro* antibiotic sensitivity test of ESBL producing isolates**

Antibiogram of all phenotypically confirmed *E. coli* and *Klebsiella* spp. was performed using 20 antibiotics of 12 classes (Hi-Media), mentioned in Table 6. It was done by disc diffusion method on Muller Hinton Agar (MHA, Hi-Media) plates inoculated with 1.5×10^8 organism/ml and incubated at 37°C for 24 hrs and isolates were classified as susceptible and resistant as per interpretation criteria of CLSI (2019)

Fig 3: Catalase positive test.

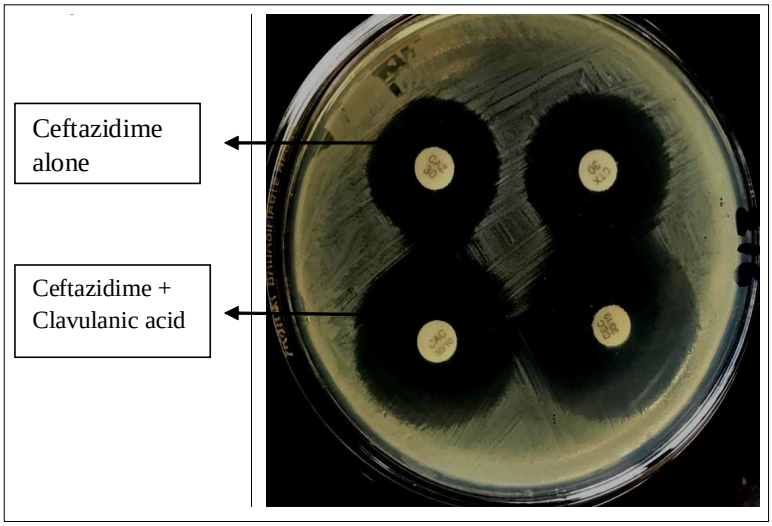

Fig 4: DDST for confirmation of ESBL producing *E. coli* and *Klebsiella* spp.

Table 1: Detail of primers used for molecular characterization of ESBLs genes in isolates of *E. coli* and *Klebsiella* spp.

Genes	Primers pair	Product size (bp)	References
bla-$_{CTX-M-1gp}$	F-5′ TTAGGAARTGTGCCGCTGYA3′	688	Dallenne *et al*., 2010
	R-5′ CGATATCGTTGGTGGTRCCAT3′		
bla-$_{CTX-M-2gp}$	F-5′ CGTTAACGGCACGATGAC3′	404	Dallenne *et al*., 2010
	R-5′ CGATATCGTTGGTGGTRCCAT3′		
bla-$_{CTX-M-9gp}$	F-5′ TCAAGCCTGCCGATCTGGT3′	561	Dallenne *et al*., 2010
	R-5′ TGATTCTCGCCGCTGAAG3′		
bla-$_{TEM}$	F-5′ CATTTCCGTGTCGCCCTTATTC3′	800	Dallenne *et al*., 2010
	R-5′ CGTTCATCCATAGTTGCCTGAC3′		
bla-$_{SHV}$	F-5′ AGGATTGACTGCCTTTTTG3′	393	Bhattacharjee *et al.,* 2007
	R-5′ ATTTGCTGATTTCGCTCG3′		

and those organisms showing resistance to at least one antibiotic of three or more classes, were called Multi-drug resistant (MDR) bacteria.

RESULTS AND DISCUSSION

A total 240 milk and faecal samples were collected from different tehsils of Ayodhya and Sultanpur districts. Out of them 147 (61.25%) isolates were identified as *E. coli* and 19 (7.91%) as *Klebsiella spp*. on the basis of their morphology, cultural characteristics in selective media and biochemical tests (Table 2, Fig 1, Fig 2 and Fig 3). In this study, isolates of *E. coli* was found to be predominate among isolates recovered from both milk and faecal samples. This finding was also supported by findings of various workers (Ibrahim *et al.,* 2018; Kotsoana *et al*., 2019). Higher isolation rate of *E. coli* in faecal samples may be attributed to high prevalence of *E. coli* in GIT flora of ruminants.

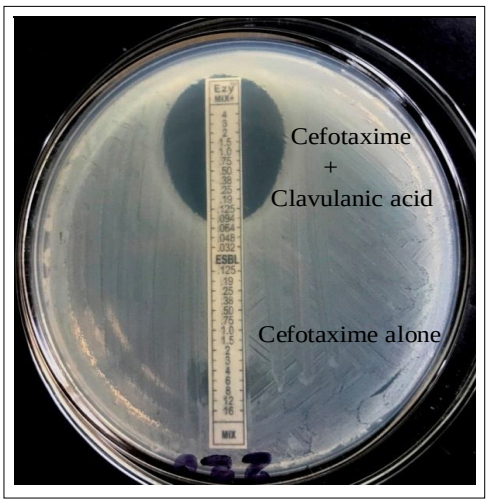

Fig 5: ESBL E-strip test for confirmation of ESBL producing *E. coli* and *Klebsiella* spp.

To study the prevalence of ESBL producing isolates among clinical and apparently healthy isolates, total 166 (147 *E. coli* and 19 *Klebsiella* spp.) were subjected to screening, confirmatory phenotypic tests and PCR analysis. On preliminary screening, 79.5% isolates presumed as ESBL producer. Using phenotypic confirmatory testing, 62.65% isolates were confirmed as ESBL by DDST and 59.63% by ESBL-E strip test and final confirmation was done by PCR analysis, which revealed 55.42% ESBL positive isolates (Table 3). Total 92 (38.33%) were ESBL positive comprising 05 (1.25%), 16 (40.0%), 51 (63.75%) and 20 (50.0%) from normal milk, mastitic milk, normal faecal and diarrhoeic faecal samples, respectively (Table 2). These results were in conformity with findings of various workers (Kotsoana *et al*., 2019; Yadav *et al*., 2019). The prevalence of ESBL producers was found much higher in mastitic milk than normal milk irrespective of pathogen, which may be attributed to indiscriminate and irrational use of antibiotics for treating mastitis in Eastern Plain Zone of Uttar Pradesh.

Genotypic analysis of confirmed ESBL producing isolates (85 *E. coli* and 07 *Klebsiella* spp.) was done by targeting *bla*-$_{CTX-M}$ (*bla*-$_{CTX-M-1}$, *bla*-$_{CTX-M-2}$, *bla*-$_{CTX-M-9}$,) *bla*-$_{TEM}$ and *bla*-$_{SHV}$ genes (Fig 6, 7, 8, 9, 10). The present study showed the overall predominance of *bla*-$_{CTX-M-1}$ (69.56%) followed by *bla*-$_{CTX-M-9}$, *bla*-$_{SHV,}$ *bla*-$_{TEM}$ and *bla*-$_{CTX-M-2}$ with 40.21%, 33.69%, 32.60% and 4.34% respectively (Table 4). This study discloses the predominance of *bla*-$_{CTX-M-1}$ gene in this area. Likewise various co-workers across the world have also reported the high frequency of this gene in different sample sources (Ibrahim *et al*., 2018; Paghdar *et al*., 2020; Yadav *et al*., 2019). Overall distribution of genes according to organisms, *E. coli* revealed highest (69.41%) *bla*-$_{CTX-M-1}$ followed by *bla*-$_{CTX-M-9}$ (42.35%), *bla*-$_{TEM}$ (32.94%) *bla*-$_{SHV}$ (32.94%) and *bla*-$_{CTX-M-2}$ (4.71%) where as *Klebsiella spp*. revealed highest (71.42%) *bla*-$_{CTX-M-1}$ followed by *bla*-$_{SHV}$ (42.85%), *bla*-$_{TEM}$ (28.57%) and *bla*-$_{CTX-M-9}$ (14.28%) (Table 5). It was notable in this study that *bla*-$_{CTX-M-1}$ and *bla*-$_{CTX-M-9}$ was

Table 2: Prevalence of ESBL producing of *E. coli* and *Klebsiella* spp. among various sources.

Samples (Source/Origin)		*E. coli* isolates	ESBL positive *E. coli*	*Klebsiella* spp. isolates	ESBL positive *Klebsiella* spp.	Total ESBL positive isolates
Normal milk	(n=80)	15 (18.75%)	04 (5.0%)	05 (6.25%)	1 (1.25%)	05 (1.25%)
Mastitic milk	(n=40)	21 (52.5%)	12 (30.0%)	08 (20.0%)	4 (15.0%)	16 (40.0%)
Normal faeces	(n=80)	73 (91.25%)	50 (62.5%)	05 (6.25%)	1 (2.50%)	51 (63.75%)
Diarrhoeic faeces	(n=40)	38 (95.00%)	19 (47.5%)	03 (7.50%)	1 (5.0%)	20 (50.0%)
Total N=240		147 (61.25%)	85 (35.41%)	19 (7.91%)	07 (2.91%)	92 (38.33%)

Table 3: Distribution of ESBL strains, according to screening, phenotypic and genotypic confirmatory tests.

Tests	Positive ESBLs		
	E. coli (147)	*Klebsiella spp.*(19)	Total (166)
Screening test	118/147=80.27%	14/19 =73.68%	132 (79.5%)
Double disc synergy test (DDST)	92/147=62.58%	12/19 =63.15%	104(62.65%)
ESBL-E test	88/147=59.86%	11/19 =57.89%	99(59.63%)
PCR	85/147=57.82%	7/19 =36.84%	92(55.42%)

present in both isolates but *bla*-$_{CTX-M-2}$ gene was only present in *E. coli* isolates. Multiple co-existence of *bla* genes were also reported which has been mentioned in Table 4. Similar to this finding, multiple co-existence of *bla* genes were also noticed by various workers (Yadav *et al*., 2019; Tekinar and Ozpinar, 2016).

Antimicrobial resistance is currently a most serious problem that received the attention of larger scientific

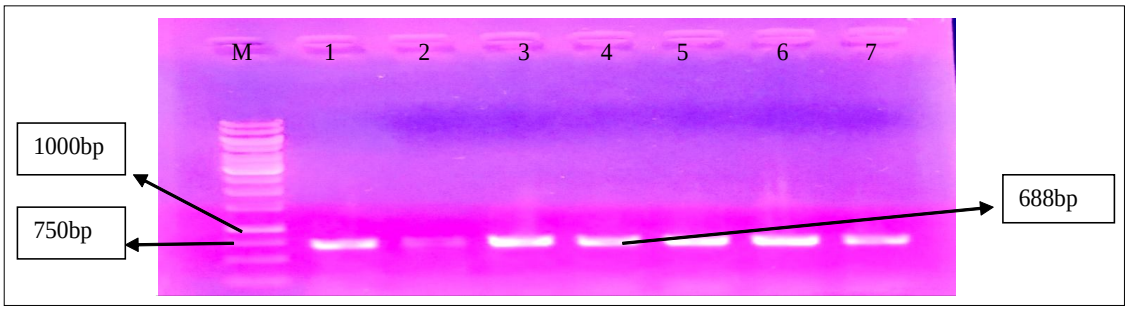

Fig 6: PCR amplification of *bla*-$_{CTX-M-1}$ gene (688bp).
M: 1kb ladder, Lane 1, 3, 4, 5, 6 and 7 positive for *bla*-$_{CTX-M-1}$ (688bp).

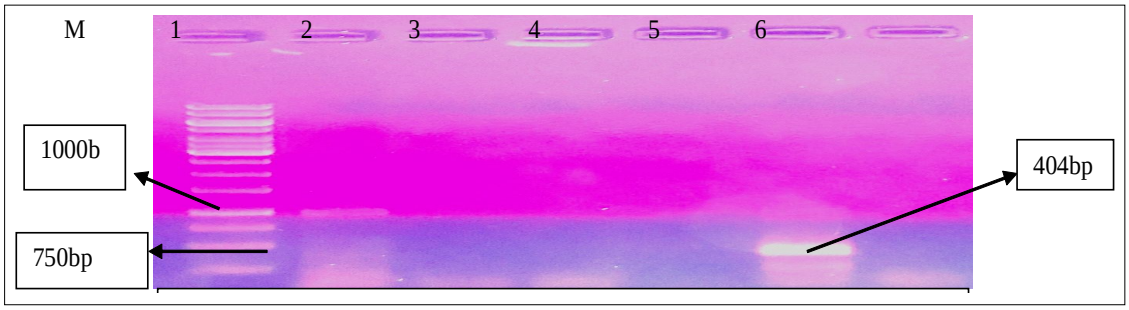

Fig 7: PCR amplification of *bla*-$_{CTX-M-2}$ gene (404bp).
M: 1Kb ladder, Lane 5 positive for *bla*-$_{CTX-M-2}$ gene (404bp), Lane 1, 2, 3 and 4 negative for *bla*-$_{CTX-M-2}$ gene, Lane 6 negative control.

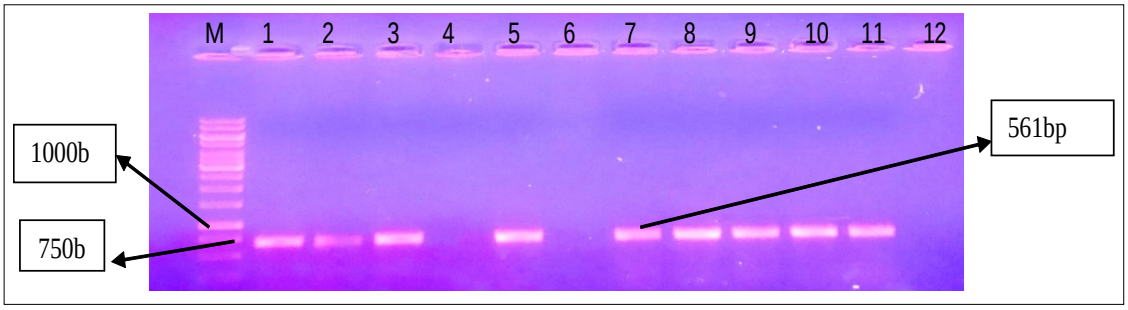

Fig 8: PCR amplification of *bla*-$_{CTX-M-9}$ gene (561bp).
M: 1 Kb ladder, Lane 1, 2, 3, 5, 7, 8, 9, 10 and 11 positive for *bla*-$_{CTX-M-9}$ gene (561bp), Lane 4 and 6 negative, Lane 12 negative control.

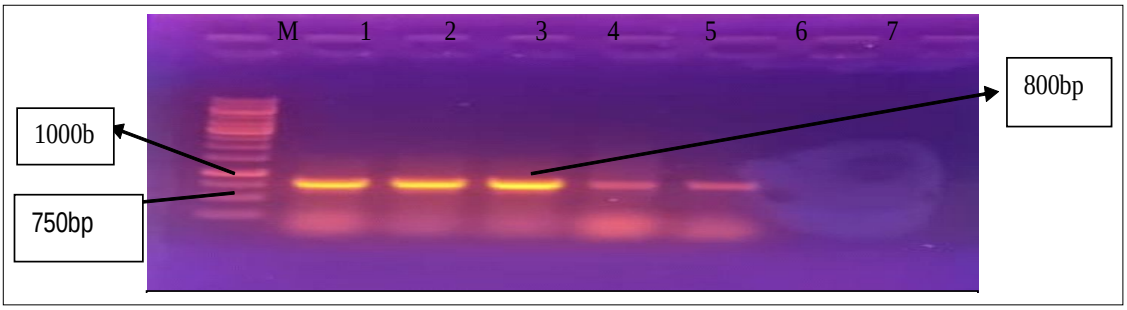

Fig 9: PCR amplification of *bla*-$_{TEM}$ gene (800bp).
M: 1kb ladder, Lane 1, 2, 3, 4, and 5 positive for *bla*-$_{TEM}$ gene, Lane 6 and 7 negative for *bla*-$_{TEM}$ gene.

community across the world. In present study, antimicrobial susceptibility test (AST) of all ESBL positive isolates was performed against 20 antibiotics of 12 different classes. All isolates of *E. coli* and *Klebsiella* spp. were found (70-100%) resistant to 3rd, 4th generation cephalosporins and ampicillin (Table 6). The plausible factors for high degree of resistance against these antibiotics might be due to persistent antibiotic pressure or acquired from horizontal transmission. Susceptibility pattern of these isolates varied with different classes of non-β-lactam antibiotics. *E. coli* isolates were found 98.83% to 100% sensitive against chloramphenicol, polypeptides and aminoglycosides classes respectively while *Klebsiella* spp. was found 85.7% to 100% against chloramphenicol, polypeptides and aminoglycosides (Table 6). There are abundant evidences that corroborate with this finding in India and abroad for both *E. coli* and *Klebsiella* spp. isolated from bovine (Batabyal *et al*.,2018 ; Gupta *et al*., 2019; Ghatak *et al*., 2013; Ibrahim *et al*., 2018). In this study resistance to carbapenem antibiotics also reported, even though these antibiotics are not used in animal husbandry practices across the country, this may be attributed as a result of clinical use in human medicine and transfer of these resistant genes to zoonotic pathogens (Bhardwaj *et al*., 2015).

Presently, Multi-drug resistant (MDR) isolates is a cause of concern, they may possess severe health complications by limiting the treatment options. In this study 81.52% isolates were found to be MDR *i.e.* resistant to at least one antibiotic of three or more classes of antimicrobials, which highlighted the potential threat by limiting the therapeutic options.

Table 4: Prevalence of ESBL genes among ESBL positive isolates.

No of genes	*bla*-genes	Positive isolates (n=92)	
		Number	Per cent
Single genes	*bla*-$_{CTX-M-1}$	64	69.56%
	bla-$_{CTX-M-2}$	04	4.34%
	bla-$_{CTX-M-9}$	37	40.21%
	bla-$_{TEM}$	30	32.60%
	bla-$_{SHV}$	31	33.69%
Multiple genes	*bla*-$_{CTX-M-1}$, *bla*-$_{CTX-M-9}$	05	5.4%
	bla-$_{CTX-M-1}$, *bla*-$_{CTX-M-2}$	01	1.08%
	bla-$_{CTX-M-9}$, *bla*-$_{SHV}$	09	9.78%
	bla-$_{CTX-M-9}$, *bla*-$_{TEM}$	03	3.26%
	bla-$_{CTX-M-1}$, *bla*-$_{CTX-M-9}$, *bla*-$_{SHV}$	07	7.60%
	bla-$_{CTX-M-1}$, *bla*-$_{CTX-M-9}$, *bla*-$_{TEM}$	09	9.78%
	bla-$_{CTX-M-1}$, *bla*-$_{CTX-M-2}$, *bla*-$_{CTX-M-9}$	01	1.08%

Table 5: Distribution of ESBL genes according to organisms.

Organisms	ESBL genes				
	bla-$_{CTX-M}$ group genes			*bla*-$_{TEM}$	*bla*-$_{SHV}$
	bla-$_{CTX-M-1}$	*bla*-$_{CTX-M-2}$	*bla*-$_{CTX-M-9}$		
E. coli (n=85)	59 (69.41%)	04 (4.71%)	36 (42.35%)	28 (32.94%)	28 (32.94%)
Klebsiella spp. (n=07)	05 (71.42%)	Nil	01 (14.28%)	02 (28.57%)	03 (42.85%)

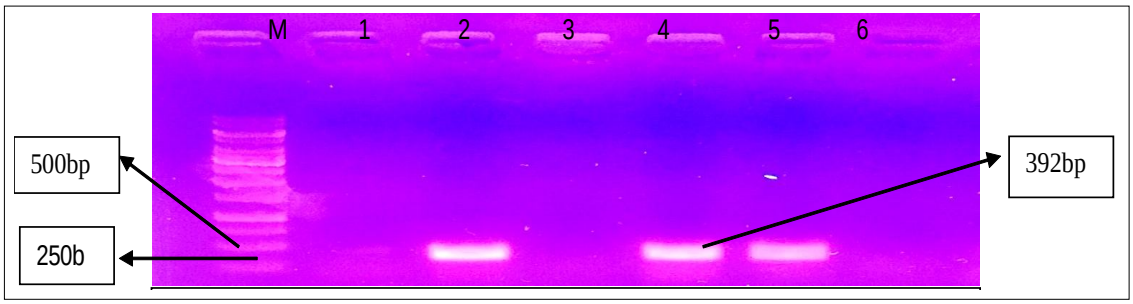

Fig 10: PCR amplification of *bla*-$_{SHV}$ gene (392bp).

M: 1kb ladder, ladder 2, 4 and 5 positive for *bla*-$_{SHV}$ gene (392), 1 and 2 negative for *bla*-$_{SHV}$ gene, Lane 6 negative control.

Table 6: AMR pattern of ESBL positive *E. coli* and *Klebsiella* spp. Isolates.

Group	Antibiotics (Hi-Media)	Conc. (µg/disc)	*E. coli* (n=85)	*Klebsiella* spp. (n=07)
			Resistance	Resistance
Aminogylcosides	Gentamicin (Gen)	10	0.0%	0.0%
	Amikacine (Ak)	30	0.0%	0.0%
Carbapenems	Imepenem (IMP)	10	11.76%	28.57%
	Meropenem (MRP)	10	4.70%	14.28%
3rd and 4th generation cephalosporins	Cefotaxime (CTX)	10	100%	100%
	Cefpodoxime(CPD)	10	100%	100%
	Ceftazidime (CAZ)	30	76.47%	71.42%
	Ceftriazone (CTR)	30	100%	100%
Monobactams	Aztreonam (AT)	30	30.58%	28.57%
2nd generation cephalosporins	Cefoxitin (CX)	30	14.11%	28.57%
Penicillin	Ampicillin (AMP)	25	100%	100%
Polypeptides	Polymyxin-B (PB)	300 unit	0.0%	0.0%
Sulphonamides	Co-trimoxazole (COT)	25	38.82%	71.42%
	Trimethoprim (TR)	30	28.23%	28.57%
Quinolones	Enrofloxacin (EX)	10	23.52%	0.0%
	Ofloxacin (OF)	2	21.17%	14.28%
	Nalidixic acid (NA)	30	44.70%	42.85%
Tetracycline	Tetracycline (TE)	30	22.35%	28.57%
Amoxyclav	Amoxicillin/Clavulanic (AMC)	(20/10)	5.58%	42.85%
Chloramphenicol	Chloramphenicol (C)	30	1.17%	14.28%

CONCLUSION

Present study highlighted the prevalence of ESBL producing bacteria in eastern plain zone of Uttar Pradesh, India. Larger proportion of *E. coli* and *Klebsiella* spp. (81.52%) was found to be MDR. Despite this some isolates of *E. coli* and *Klebsiella* spp. also exhibited resistance against carbapenems, even without its use in animal husbandry practices, which is not a good sign from public health point of view. Taking into consideration, this emerging drug resistance, the practice of routine ESBL testing along with conventional antibiogram would be useful for all cases, which will help in appropriate selection of antibiotic and also prevent further development of AMR. This study also reveals high prevalence of *bla*-$_{CTX-M}$ gene in this area which will help in reliable epidemiological investigation of AMR. These enzymes may be chromosomal or plasmid mediated which play important role in the dissemination of antimicrobial drug resistance in health care settings. Therefore, continuous monitoring of resistance genes against these antibiotics in livestock is warranted.

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Conflict of interest: None.

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