RESEARCH ARTICLE

Status of MDR and Plasmid Profiling of ESBL Producing *E. coli* and *Klebsiella* spp. Isolated from Milk of Bovine in Gangetic Plain Zone of Uttar Pradesh

Vibha Yadav[1*], Rajesh Kumar Joshi,[2] Namita Joshi,[3] Satyavrat Singh,[4] Satyendra Kumar Maurya,[5] Dharam Prakash Shrivastva[6], Rakesh Kumar Gupta[6]

Abstract

Nowadays resistance to antibiotics of β-lactam groups is expanding rapidly and is one of the latest challenges faced by scientific community due to therapeutic failure. In the present study total 180 milk samples were collected from two district of Gangetic Plain Zone of Uttar Pradesh (India). Total 65 (36.11%) isolates were confirmed comprising 44 (24.4%) *E. coli* and 21 (11.67%) *Klebsiella* spp. by PCR analysis using uidA gene and 16S rRNA gene respectively, 35/180 (19.44%) isolates were confirmed as ESBL producers, constituting 12 (6.67%) *E. coli* and 23 (12.8%) *Klebsiella* spp. All ESBL positive isolates were subjected to antimicrobial resistance pattern using 17 antibiotics of 10 different classes. In this study all the isolates of *E. coli* and *Klebsiella* spp. were found 100% resistant to cefotaxime, cefpodoxime, ceftriazone and ampicillin. Both the isolates of *E. coli* and *Klebsiella* spp. revealed different susceptibility pattern to different non-β-lactam antibiotics except aminoglycosides class of antibiotics for which both the isolates were found 100% sensitive. The multi-drug resistance pattern was assessed by using MAR index. Out of total ESBL producers, 80.0% isolates were found MDR with 78.0% *E. coli* and 83.0% *Klebsiella* spp. Overall 12 resistance pattern were observed for *E. coli* ranging from 5 to 12 antibiotics and 09 for *Klebsiella* spp. ranging from 5 to 14 antibiotics. Plasmid profiling revealed total 71.42% isolates carrying plasmids with average molecular weight ranged between 1.0 Kb and > 10 Kb and number 1-4.

Key words: Bovine, *E. coli*, ESBLs, *Klebsiella* spp., MAR index, MDR, Plasmid.

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Introduction

Emergence of antimicrobial resistance (AMR) in animal husbandry is of utmost concern, especially among *Enterobacteriaceae,* it has been increasingly problematic and poses serious threat for both human and animal health as it has resulted in therapeutic failure (WHO, 2013). Among, *Enterobacteriaceae*, *E. coli* and *Klebsiella* spp. are major Extended-spectrum β-lactamase (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). ESBL encoding genes are mostly carried on plasmids, which can easily be transferred between isolates, bearing additional resistance determinants for other classes of antimicrobial agents, mainly fluoroquinolones, aminoglycosides and sulphonamides, which are attributed to the multidrug resistant (MDR) phenotype (Jiang *et al*., 2012). MDRs in *Enterobacteriaceae* are increasing day by day, leads to limited antimicrobial treatment options and major cause of morbidity and mortality worldwide (WHO, 2016). In Gram negative bacteria, most of the AMR genes are located on plasmids, which are horizontally transferred between close families of bacteria and also spread the AMR gene in the environment (Ansari *et al*., 2018). The plasmids also have ability to acquire novel gene through mobile genetic elements such as transposons or insertion sequences and

[1,2]Department of Veterinary Microbiology; College of Veterinary Science & Animal Husbandry, Replace with ANDUAT, Kumarganj, 224229, Ayodhya (U.P.).

[3]Department of Veterinary Public Health & Epidemiology; College of Veterinary Science & Animal Husbandry, Replace with ANDUAT, Kumarganj, 224229, Ayodhya (U.P.).

[4]Department of Veterinary Medicine; College of Veterinary Science & Animal Husbandry, Replace with ANDUAT, Kumarganj, 224229, Ayodhya (U.P.).

[5]Department of Veterinary Biochemistry and Physiology; College of Veterinary Science & Animal Husbandry, Replace with ANDUAT, Kumarganj, 224229, Ayodhya (U.P.).

[6]Department of Veterinary Pathology; College of Veterinary Science & Animal Husbandry, Replace with ANDUAT, Kumarganj, 224229, Ayodhya (U.P.).

Corresponding Author: Vibha Yadav, Department of Department of Veterinary Microbiology; College of Veterinary Science & Animal Husbandry, Replace with ANDUAT, Kumarganj, 224229, Ayodhya (U.P.). email: vibhavet2005@gmail.com

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their tendency to replicate in a wide range of hosts, make them perfect vectors for spread of AMR (Rozwandowicz *et al*., 2018)

There is limited information on MDR pattern and plasmid profiling of ESBL isolates of bovine in this area of study and also their possible role in development of resistance to other species or pathogens. Molecular characterization of plasmids and their nature in different bacterial host provide significant knowledge concerning to potential of spread of AMR genes, which is helpful for the researchers, field veterinarians and policy makers in monitoring of disease outbreaks and ascertain the evolution and spread of AMR genes among isolates in community setting. Keeping in view this fact, the present study was undertaken to determine the prevalence of MDR and plasmid profiling of ESBL producing isolates from bovine milk to demonstrate the relationship between AMR genes and plasmid with respect to molecular weight and number.

Materials and Methods

Sample Collection: In present study, total 180 milk samples of cattle and buffalo (120 apparently healthy and 60 clinical mastitic milk) were collected from 3 tehsils of Ayodhya district and 2 tehsils of Sultanpur district of U.P. (India) during the period from January, 2020 to March 2021. The research work was duly approved by Institutional Animal Ethics Committee (IAEC) as per letter no. IAEC/C.V/Sc./2019/34 dated 10-12-2019. Sampling was done randomly and consisted of 60 apparently healthy and 30 clinical mastitic milk samples of each cattle and buffalo equally from each of the tehsil. California Mastitis Test was used for screening of mastitic milk samples and approximately 5 mL of milk was collected in sterilized test tubes. All collected samples were transported immediately to Bacteriology Laboratory under cold chain for further processing.

Isolation and Identification: All 180 collected samples were inoculated in 2 mL nutrient broth and incubated at 37°C for 24 h. Further Isolation and identification of the colonies of isolates was carried out as per the method of Edward and Ewing (1972).

Molecular Characterization of *E. coli* and *Klebsiella* spp.: Molecular identification of all presumptively positive *E. coli* and *Klebsiella* spp. isolates was done by PCR amplification using species specific uidA and bacteria specific 16S rRNA genes as per method described by Anbazhagan *et al*. (2010) and Andersson *et al*. (2008) respectively. Genomic DNA extraction was done by using the snap-chill method described by Franco *et al*., 2008). Amplification was performed using thermal cycler (Bio-Rad, USA). The primer sequences and cycling conditions used are mentioned in Table 1.

Phenotypic Detection of ESBL Producing *E. coli* and *Klebsiella* spp.:

Screening of ESBL Producing Isolates: All the confirmed isolates of *E. coli* and *Klebsiella* spp. were subjected to ESBL screening using antibiotics of 3rd and 4th, generation cephalosporins viz., cefotaxime, cefpodoxime, ceftazidime, ceftriazone and monobactam *viz*., aztreonam having 30 µg conc. of disc (Hi-Media, India) using disc diffusion method described by Bauer's *et al*. (1966). The results were interpreted as per CLSI (2019) guidelines. The isolates showing reduced susceptibility to any one of these antibiotics were further subjected to confirmatory phenotypic tests.

Confirmation of ESBL Positive Isolates:

Double Disc Synergy Test (DDST): All screened isolates of *E. coli* and *Klebsiella* spp. for ESBL production were further confirmed by using ESBL kit 1 and kit 3 (Hi Media). The commercially available kit disc were placed 25 mm apart from each other on Muller Hinton Agar (MHA) plates (Hi-Media) inoculated with $1.5x10^8$ organism/mL and incubated at 37°C for 24 h. The results were interpreted as per CLSI guidelines (2019).

Minimum Inhibitory Concentration (MIC) ESBL-E Strip Test: This test was performed by placing E-strip on Mueller Hinton Agar paltes (MHA plates ,Hi-Media) inoculated with $1.5x10^8$ organism/mL and incubated at 37°C for 24 h. The results were interpreted as per CLSI guidelines (2019).

Study of Multi-Drug Resistance (MDR) Pattern: All phenotypically confirmed isolates of *E. coli* and *Klebsiella* spp. were examined for their resistance against 17 antibiotics of 10 different classes (Table 4). It was performed by agar disc diffusion method on MHA plates (HiMedia) infused with $1.5x10^8$ organism/mL and incubated at 37°C for 24 h. The results were interpreted as per CLSI guidelines (2019) and those showing resistance to at least one antibiotic of three or more classes were considered as MDR. This was assessed by calculating the MAR index of the isolates, as total number of

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 min at 95°C,35 cycles of 45 s at 95 °C,55s at 56°C, 1min at 72 °C,1 cycle of 7min at 72°C	Anbazhagan *et al.,* 2010
784F 1061R	F 5'AGGATTAGATACCCTGGTA3' R 5'CRRCACGAGCTGACGAC3'	265	1 cycle of 5 min at 95°C,35 cycles of 50 s at 95 °C,45 s at 54°C, 1min at 72 °C,1 cycle of 7 min at 72°C	Andersson *et al.,* 2008

antibiotics to which test isolates showed resistance divided by total number of antibiotics used to test the AMR.

Extraction and Profiling of Plasmid DNA: Plasmid DNA from phenotypically confirmed ESBL producing isolates was extracted using GeneJet plasmid Miniprep kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. The extracted plasmid DNA was stored at -20°C till further processing. Each extracted plasmid DNA sample (5 µL) mixed with 2 µL of loading dye (6X) and electrophoresis was done on 0.8% (w/v) agarose gel mixed 1µL ethidium bromide (conc. 5µg/mL) at 80-100V for 1 to 1.5 h using 1kb ladder. At the end of electrophoresis, the gel was visualized under UV trans-illuminator (EZ Gel Documentation system, Bio-Rad). Plasmid profile was analyzed for the presence, number and size of plasmids.

Results and Discussion

In this study, out of 120 apparently normal and 60 clinical mastitic milk samples processed for isolation and identification of *E. coli* and *Klebsiella* spp., total 33.33% isolates were presumed as *E. coli* and 13.33% as *Klebsiella* spp. on the basis of their morphology, growth on selective media and biochemical characteristics (Table 2). On molecular confirmation using PCR, 24.4% isolates were confirmed as *E. coli* and 11.67% as *Klebsiella* spp. (Table 2, Fig. 1, Fig. 2). These findings are in concordance with observations of various previous workers (Batabayal *et al*., 2018; Ibrahim *et al*., 2018; Geser *et al*., 2012).

The present study was also aimed to determine the occurrence of ESBL phenotypes among apparently healthy and clinical mastitic milk samples. Total 35 (19.44%) isolates were found ESBL positive comprising 05(8.33%) and 08(13.33%) in apparently normal milk and 11(36.7%) in each mastitic milk of cattle and buffaloes respectively (Table 3). These findings were in concordance with Yadav *et al*. (2019) and Batabyal *et al*. (2018). ESBL positive *E. coli* (12.8%) isolates were much higher as compared to ESBL positive *Klebsiella* spp. (6.67%), which is not surprising as *E. coli* are dominant gut flora of ruminants. Occurrence of ESBL producing isolates was found higher among mastitic milk samples as compared to normal milk sample isolates for both pathogens (Table 3), which may be attributed to irrational use of antibiotics for treating animals suffering with mastitis in this region, without performing antibiotic susceptibility test. In this study, it was also noticed that the frequency of ESBL producer was higher in *Klebsiella* spp. (12/21, 57.14%) than *E. coli* (23/44, 52.27%) which has been also reported by Prajapati *et al*. (2020).

Nowadays, antimicrobial resistance is becoming an ultimate threat for both human as well as animals and has received the attention of larger scientific community across the globe. In this study all the isolates of *E. coli* and *Klebsiella* spp. were found 100% resistant to cefotaxime, cefpodoxime, ceftriazone and ampicillin. Both isolates of *E. coli* and *Klebsiella* spp. revealed different susceptibility pattern to different non-β-lactam antibiotics except aminoglycosides for which both isolates were found 100% sensitive (Table 4). There are abundant evidences that

Table 2: Isolation of *E. coli* and *Klebsiella* spp. in normal and mastitic milk samples of bovine origin:

Samples (Source/Origin)		Presumptive positive isolates (Biochemical tests)			Confirmed positive isolates (PCR analysis)		
		E. coli	***Klebsiella* spp.**	**Total**	***E. coli***	***Klebsiella* spp.**	**Total**
Cattle	Normal milk (n=60)	16(26.7%)	7(11.67%)	23(38.33%)	10(16.7%)	6(10.00%)	16(26.7%)
	Mastitic milk (n=30)	19(63.3%)	5(16.67%)	24(80.0%)	14(46.7%)	5(16.67%)	19(63.3%)
Buffaloes	Normal milk (n=60)	11(18.33%)	5(8.33%)	16(26.67%)	9(15.0%)	4(6.67%)	13(21.7%)
	Mastitic milk (n=30)	14(46.7%)	7(23.33%)	21(70.0%)	11(36.7%)	6(20.00%)	17(56.7%)
Total	**N=180**	**60(33.33%)**	**24(13.33%)**	**84(46.7%)**	**44(24.4%)**	**21(11.67%)**	**65(36.11%)**

Table 3: Distribution of ESBL producing of *E. coli* and *Klebsiella* spp. Bovine milk samples

Samples (Source/Origin)		***E. coli* isolates**	**ESBL positive *E. coli***	***Klebsiella* spp. isolates**	**ESBL positive *Klebsiella* spp.**	**Total ESBL positive isolates**
Cattle	Normal milk (n=60)	10(16.7%)	3(5.0%)	6(10.00%)	2(3.33%)	5(8.33%)
	Mastitic milk (n=30)	14(46.7%)	8(26.7%)	5(16.67%)	3(10.0%)	11(36.7%)
Buffalo	Normal milk (n=60)	9(15.0%)	5(8.33%)	4(6.67%)	3(5.0%)	8(13.33%)
	Mastitic milk (n=30)	11(36.7%)	7(23.33%)	6(20.00%)	4(13.33%)	11(36.7%)
Total	**N=180**	**44(24.4%)**	**23(12.8%)**	**21(11.67%)**	**12(6.67%)**	**35(19.44%)**

authenticate the emergence of resistance against 3rd and 4th generation cephalosporins and ampicillin in India and abroad for both isolates of bovine milk origin (Batabyal *et al*., 2018; Ramasamy *et al*., 2021; Ibrahim *et al*., 2018; Badri *et al*., 2017). In this study, resistance to carbapenem antibiotics was also found although these antibiotics are not used elsewhere across the world for treatment of animals, which may be attributed to horizontal transfer of these resistance genes between human and animals in community settings. The frequency of carbapenem resistant was found more in *Klebsiella* spp. as compared to *E. coli* as these antibiotics are mostly used in human and *Klebsiella* spp. being most commonly implicated in outbreaks of nosocomial infections across the world.

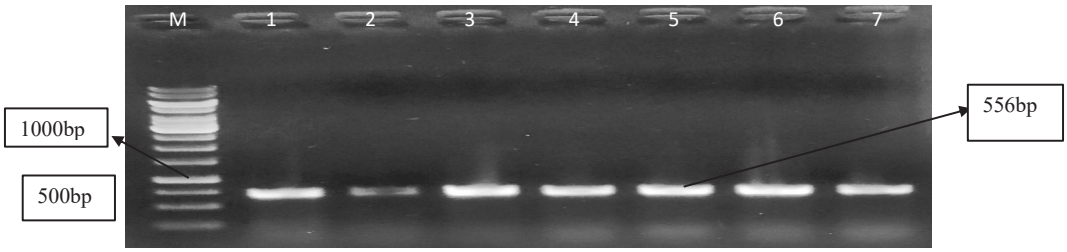

Fig. 1: PCR amplification of *uidA gene* (556bp)
M: 1Kb ladder, Lane 1, 2, 3, 4, 5, 6 and 7 positive for uidA gene (556bp).

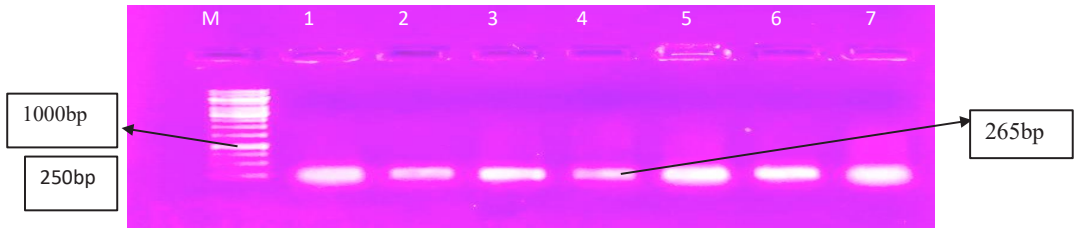

Fig. 2: PCR amplification of 16S rRNA gene (265)
M: 1Kb ladder, Lane 1, 2, 3, 4, 5, 6 and 7 positive for 16S rRNA (265)

Table 4: AMR pattern of ESBL positive *E. coli* and *Klebsiella* spp. isolate**s**

Group	**Antibiotics**	***E. coli* (n=23)**	***Klebsiella* spp. (n=12)**
		Resistance (%)	**Resistance (%)**
Aminogylcosides	Gentamicin (GEN)	0.0%	0.0%
	Amikacine (AK)	0.0%	0.0%
Carbapenems	Imepenem (IMP)	13.04%	33.33%
	Meropenem (MRP)	4.34%	16.7%
3rd and 4th generation Cephalosporins	Cefotaxime (CTX)	100%	100%
	Cefpodoxime(CPD)	100%	100%
	Ceftazidime (CAZ)	78.26%	75.0%
	Ceftriazone (CTR)	100%	100%
Monobactams	Aztreonam (AT)	30.43%	41.7%
2nd generation Cephalosporins	Cefoxitin (CX)	21.74%	25.0%
Penicillin	Ampicillin (AMP)	100%	100%
Quinolones	Enrofloxacin (EX)	26.1%	0.0%
	Ofloxacin (OF)	21.74%	16.7%
	Nalidixic acid (NA)	43.50%	41.7%
Tetracycline	Tetracycline(TE)	26.1%	25.0%
Amoxyclav	Amoxicillin/Clavulanic (AMC)	8.7%	41.7%
Chloramphenicol	Chloramphenicol (C)	0.0%	8.33%

Table 5: Distribution of plasmids in ESBL positive *E. coli* and *Klebsiella* spp. isolates among various sources

Samples (Source/Origin)		ESBL positive *E. coli*	Plasmid profile in *E. coli*			ESBL positive *Klebsiella* spp.	Plasmid profile in *Klebsiella* spp.		
			Positive isolates	No. of bands	Average size (kb)		Positive isolates	No. of bands	Average size (kb)
Cattle	Normal milk (n=60)	3	2	1	6.0	2	1	2	3 kb to > 10 kb
	Mastitic milk (n=30)	8	4	1-3	2.5 kb to > 10 kb	3	5	1-4	>10 kb
Buffaloes	Normal milk (n=60)	5	4	1-3	2.5 kb > 10 kb	3	1	1	>10 kb
	Mastitic milk (n=30)	7	6	1-2	8 kb > 10kb	4	2	3	1 kb to >10 kb
Total (N=180)		**23**	**16**	**1-3**	**2.5 kb to 10 kb**	**12**	**09**	**1-4**	**1kb >10 kb**

(* > sign of greater than, *< sign of lower than)

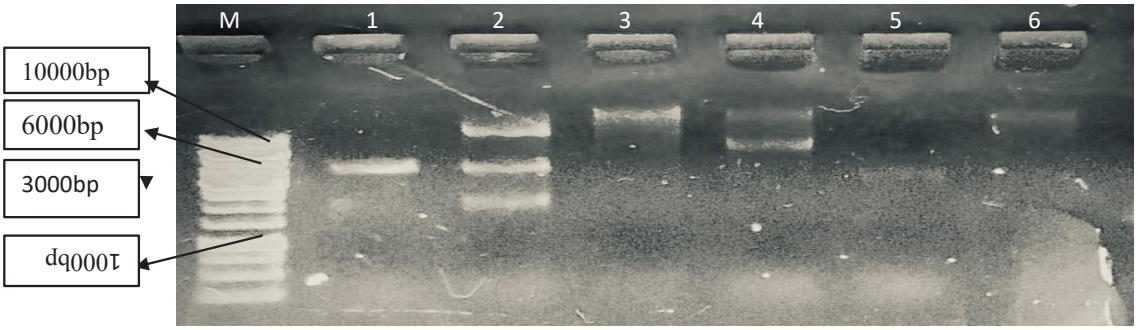

Fig. 3: Plasmid profile of ESBL positive *E. coli* isolates
M: 1Kb ladder (Thermo Scientific); lane, 1, 2, 3, 4 and 6 revealed plasmid bands

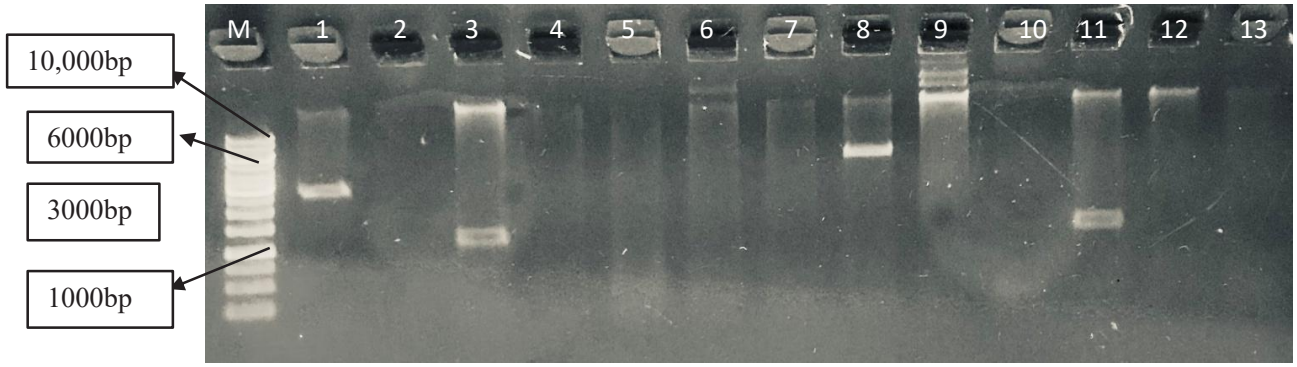

Fig. 4: Plasmid profile of ESBL positive *Klebsiella* spp. isolates
M: 1Kb ladder (Thermo Scientific); lane, 1, 3, 6, 7, 8, 9, 11 and 12 revealed plasmid bands

Multi-drug resistant (MDR) bacteria are cause of serious concern as they posses serious health complication by limiting treatment option to the practitioners. In this study MDR was assessed by calculating multiple antibiotic resistance (MAR) index of the isolates. Overall 12 resistance patterns were revealed by 18 *E. coli* isolates ranging from 5 to 12 antibiotics and 9 resistance patterns by 10 *Klebsiella* spp. isolates ranging from 5 to 14 antibiotics. MAR index for *E. coli* and *Klebsiella* spp. isolates were noticed in the range of 0.29-0.71 and 0.29-0.82 respectively. Total 80.0% ESBL positive isolates were found MDR which highlighted the potential threat by limiting the therapeutic options.

Plasmid profiling of ESBL positive *E. coli* and *Klebsiella* spp. was performed. Total (16/23) 69.56% isolates of *E. coli* revealed plasmid band in range of 1 to 3 with average plasmid size ranging from 2.5 to > 10 kb. Total 4 different plasmid profiling patterns were observed based on molecular weight and 3 on their numbers. One plasmid of >10 kb was found in 81.25% of isolates and maximum nine number of isolates, having 02 plasmid bands followed by five isolates with 01 plasmid band and two isolates with 03 plasmid bands (Table 5, Fig.3). Likewise 75.0% ESBL positive isolates of *Klebsiella* spp. revealed plasmid band in the range of 1 to 4 with average molecular weight 1 to > 10kb.

Total six different plasmid patterns were observed based on their molecular weight and four on their number. One plasmid of >10kb was found in all isolates and maximum four number of isolates having 03 plasmid bands followed by two isolates each having 04 and 02 plasmids and one isolate having only 01 plasmid band (Table 5, Fig. 4). The findings of this study were in conformity with the observations of many workers in India and abroad (Singh *et al*., 2020; Rawat *et al*., 2018; Motayo *et al*., 2013; Shrestha *et al*., 2020).

Conclusions

In this study most of the isolates showed resistance to 3rd and 4th generation cephalosporins and ampicillin, and showed MDR which is cause of concern for this area. Some isolates also showed resistance to carbapenems, even without its use in animal husbandry practices, which are not a good sign from public health point of view. Most of the isolates (90-100%) were found sensitive towards gentamicin, amikacin, enrofloxacine and chloramphenicol which could be alternative therapeutic option for this area. Presence of plasmid and one plasmid of more than 10 Kb in most of the isolates clearly indicates the horizontal spread of AMR genes in the environment and health care setting.

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