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Detection of Carbapenemase Genes (*bla*$_{-NDM}$, *bla*$_{-KPC}$, *bla*$_{-OXA-48}$) in *Escherichia coli* and *Klebsiella* species, Isolated from Milk Samples of Bovine in Eastern Plain Zone of Uttar Pradesh

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ABSTRACT

Background: Among enterobacteria *E. coli* and *Klebsiella* spp. are of great concern in health care settings, as these bacteria sometimes may contaminate the milk due to unhygienic practices and poor udder condition which have been associated with various illnesses. Therefore, this study aimed to detect the carbapenem resistant *E. coli* and *Klebsiella* spp. of bovine milk origin with regard to the risk of human transfer via the food chain in community.

Methods: Total 240 samples were collected from Ayodhya and Sultanpur districts of Eastern Plain Zone of Uttar Pradesh (India). Confirmation of *E. coli* and *Klebsiella* spp. isolates was done by using species specific uidA and 16S rRNA gene, respectively. Then, carbapenemase positive *E. coli* and *Klebsiella* spp. were confirmend by DDST, MBL E-strip test and PCR analysis by targeting (*bla*$_{-NDM}$, *bla*$_{-OXA-48}$ and *bla*$_{-KPC}$). Antibiogram of all carbapenemase positive isolates was performed against 20 antibiotics of 12 different classes.

Result: In the present study, total 74(30.83%) isolates were identified including 55(22.92%) *E. coli* and 19(7.92%) *Klebsiella* spp. by PCR, out of which 12(16.21%) isolates were confirmed as carbapenemase producers comprising 7(12.72%) *E. coli* and 5(26.31%) *Klebsiella* spp by DDST and E-strip. All carbapenemase positive *E. coli* were found 100% sensitive to polymyxin-B and chloramphenicol, while all *Klebsiella* spp. were 100% sensitive to amikacin and polymyxin-B. Resistance against imipenem, meropenem, cefotaxime, cefpodoxime, ceftazidime, ceftriazone, aztreonam and ampicillin ranged between 80.0%-100%. All carbapenemase positive isolates were found multidrug resistant. Carbapenemase genes *bla*-$_{NDM}$ and *bla*-$_{KPC}$ were detected in *E. coli* while *bla*-$_{OXA-48}$ and *bla*-$_{KPC}$ were detected in *Klebsiella* spp.

Key words: Carbapenemase, *E. coli*, *Klebsiella* spp., MBL-E test, Multidrug-resistance.

INTRODUCTION

Carbapenems are one of the most important groups of antimicrobials which are used as a last resort of antibiotics to combat the infection of multidrug resistant (MDR) pathogens. Unfortunately, the use of carbapenems has increased worldwide in past few years (Wang *et al.,* 2015), hence MDR and carbapenemase producing *Enterobacteriaceae* (CPE) have been increasing globally at a alarming rate. Resistance to carbapenem is generally mediated by various mechanisms such as loss of outer membrane porins, production of carbapenemase enzymes and over-expressed efflux pump. Majority of bacterial isolates that harbour these enzymes belong to *Enterobacteriacae,* because Gram negative bacteria producing ESBL enzyme led to higher carbepenem usage in human being which has resulted in the wider occurrence and spread of CPE (Muller *et al*., 2018) in environment. The spread of these bacteria into many species may occur though sewage and waste water. Among *Enterobacteriacae*, *E. coli* and *Klebsiella* spp. are main environmental pathogens, associated with various illnesses and acute bovine mastitis (Koovapra, 2015). Resistant genes of these antibiotics are frequently located on mobile genetic elements which can be horizontally transferred (Ansari *et al*., 2018) between the bacteria. CPE among livestock are of great public health concern because as these can be transferred

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to human and animal *via* contaminated food and water. Although very few works have been done on CPE in bovine in India and abroad, their occurrence in animal raises concern in particular to risk of transfer to human *via* food chain, through contact or environment.

Keeping these facts in view, the present study was undertaken to detect the carbapenem resistant genes in *E. coli* and *Klebsiella* spp., since, no in-depth study has been done on carbapenemase producing enterobacteria in cattle and buffaloes in Eastern Zone of Uttar Pradesh. It will help the researchers, field veterinarians and policy makers to develop appropriate control strategy for farmers of this region.

MATERIALS AND METHODS

Study area

The present study was carried out in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, 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 June 2020.

Sample collection

In present study, total 240 milk samples (160 normal and 80 mastitic milk) of cattle and buffaloes were collected from 5 tehsils of Ayodhya and 3 tehsils of Sultanpur district. Sampling consisted of 10 normal and 5 mastitic milk samples from each of the animal from total eight tehsils. California Mastitis Test was used for screening of mastitis. Approximately 5 ml normal and mastitic milk was collected into sterilized test tubes following strict aseptic conditions. All collected samples were transported to bacteriology laboratory of the department under a cold chain for further processing.

Isolation and identification

Samples were enriched with 2 ml nutrient broth and incubated for 24 hrs at 37°C. A loopful of inoculum was taken and directly streaked on MacConkey agar (MLA) plates added with 1 mg/L imipenem and incubated at 37°C for 24 hr. Colonies showing lactose fermenting characteristics were picked up and transferred to nutrient agar slant and incubated at 37°C for 24 hrs. Thereafter, cultures were streaked on Eosine Methylene Blue (EMB) agar plates and colonies showing specific characteristics were identified by the method of Cruickshank *et al.* (1975). Further identification of the isolates was done by various biochemical and sugar fermentation reaction as per the method of Edward and Ewing (1972).

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

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

Targeted gene	Prier 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

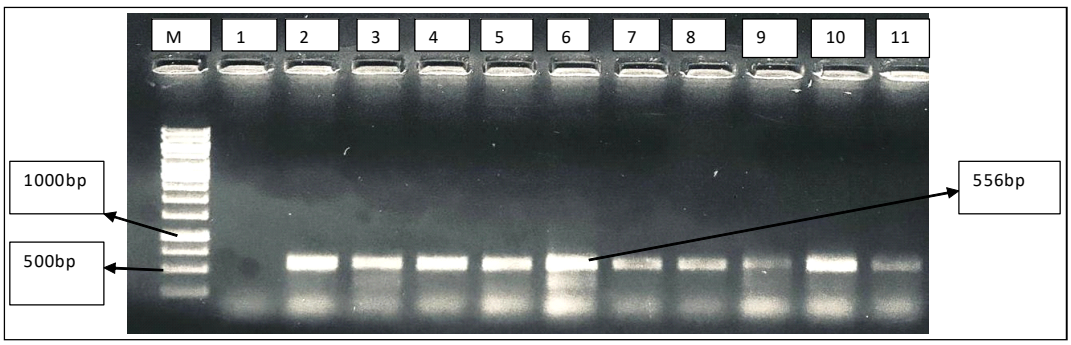


Fig 1: PCR amplification of uidA gene (556 bp).
M: 1Kb ladder, Lane 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 positive for uidA gene (556 bp), Lane 1 negative for uidA gene.

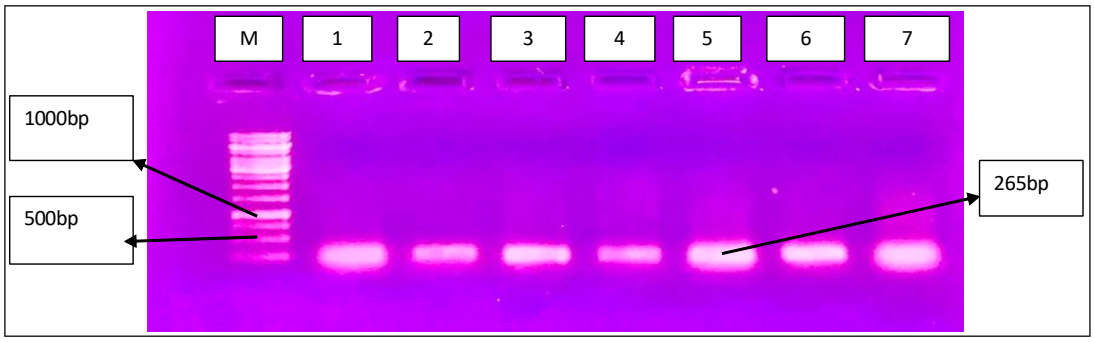


Fig 2: PCR amplification of 16S rRNA gene (265 bp).
M: 1 Kb ladder, Lane 1, 2, 3, 4, 5, 6 and 7 positive for 16S rRNA (265 bp).

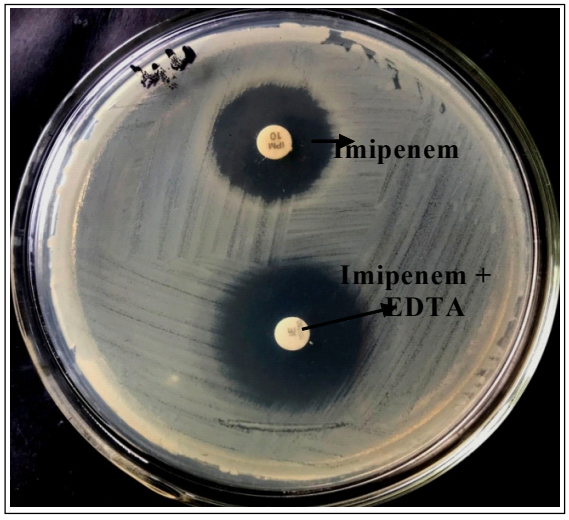


Fig 3: Double disc synergy test for confirmation of carbapenemase producing *E. coli* and *Klebsiella* spp.

was carried out in 25 µl volume that 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) 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). PCR amplicons were stored at 4°C until electrophoresis.

Sequencing of PCR amplicons

The *E. coli* and *Klebsiella* spp. PCR products (Fig 1, 2) were submitted to Sanger sequencing Biokart India Pvt Ltd, Bengaluru, India and sequences were subjected to Blast Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to confirm the identity of the isolates.

Screening of carbapenemase producing isolates

All confirmed isolates were subjected to carbapenemase screening using imipenem and meropenem having 10 µg conc. of disc (Hi-Media, India) by disk diffusion method (Bauer *et al*., 1966). The results were interpreted as per CLSI (2019) guidelines. The isolates showing reduced susceptibility to any one of these agents were further subjected to confirmatory phenotypic tests.

Confirmation of carbapenemase positive isolates

Two phenotypic methods were used for confirmation of carbapenemase production.

Double disc synergy test (DDST)

The test was performed by placing the commercially available imipenem disc (10 µg) and combination of imipenem + EDTA (10/750 µg) discs (HiMedia, India) at 25 mm apart on Muller Hinton agar (MHA) (HiMedia) plate inoculated with 1.5×10^8 organisms/ml and incubated at 37°C for 24 hrs (Fig 3). The results were interpreted as per CLSI guidelines (2019).

Minimum inhibitory concentration (MIC) MBL E-test

MBL E-strip test was done by placing strip on MHA plate inoculated with 1.5×10^8 organisms /ml and incubated at 37°C for 24 hrs. Results were interpreted as per CLSI guideline (2019) (Fig 4).

Detection of carbapenemase genes by PCR assay

Isolation of plasmid DNA

A single pure colony of each isolates was inoculated into 10 ml Luria-Bertani (LB) broth medium (HiMedia, India) and incubated at 37°C for 12-15 hrs in a shaking incubator. The pellets were prepared by centrifuging the bacterial suspension at 10,000 rpm for 3 min. Plasmid DNA was isolated using the GeneJet plasmid Miniprep kit (Cat No. #K0503, Thermo Scientific) as per the instruction of the manufacturers.

Detection of *bla*-$_{NDM}$, *bla*-$_{KPC}$ and *bla*-$_{OXA-48}$ genes

Carbapenemase gene detection was carried out in a total reaction volume of 25 µl as per method described by Mushi *et al*. (2014) for *bla*-$_{NDM}$ and Dallenne *et al*. (2010) for *bla*-$_{KPC}$ and *bla*-$_{OXA-48}$ genes. The primer sequence of targeted genes and amplicon sizes are listed in Table 2. 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 ethidium bromide 1 µl (5 µg/ml) in 60 ml and run slowly at 80-100V, 60-70 mA for 1 hrs and the gels were visualized using the UV illuminator (GeNei Bangalore, India). Ladder DNA of 1 kb (Thermo Scientific # SM 0311) was used as a marker for band interpretation.

Study of Multi-drug resistance (MDR) pattern

All phenotypically confirmed carbapenemase isolates of *E. coli* and *Klebsiella* spp. were checked for their multidrug resistance pattern using 20 antibiotics of 12 different classes. It was performed by agar disk diffusion test on MHA using following discs of HiMedia: Aminoglycosides *viz.* gentamicin (10 µg), amikacine (30 µg), polymyxin (300 unit), 3rd generations cephalosporins *viz.* cefotaxime (30 µg), cefpodoxime (30 µg), monobactams *viz.* aztreonam (30 µg), 2nd generation cephalosporins *viz.* cefoxitin (30 µg), fluoroquinolones *viz.* enrofloxacin (10 µg), ofloxacine (2 µg), nalidixic acid (30 µg), penicillins *viz.* ampicillin (25 µg), tetracyclines (30 µg), carbapenem *viz.* imipenem (10 µg), meropenem (10 µg), sulphonamide *viz.* trimethoprim (30 µg), co-tromoxazole (30 µg), amoxyclav (20/10 µg), chloramphenicol (30 µg). Isolates were classified as susceptible and resistant based upon interpretation criteria of CLSI (2019) and those showing resistance to at least one antibiotic in three or more classes were defined as MDR.

RESULTS AND DISCUSSION

In this study, total of 240 samples comprising 160 normal and 80 mastitic milk samples were processed for isolation and identification. On the basis of morphological, growth and biochemical characteristics, 29.58% and 9.16% isolates were presumed as *E. coli* and *Klebsiella* spp., respectively (Table 3). Thus, larger proportion of these was confirmed as (22.92%) *E. coli* while 7.92% as *Klebsiella* spp. through PCR amplification (Fig 2, 3 and Table 3). These findings were found in agreement with the reports of previous worker (Ibrahim *et al*., 2018; Geser *et al*., 2012). Comparatively higher isolation rate of *E coli* in this study can be attributed to high prevalence of *E. coli* in the GIT flora.

To confirm the identity of isolates, gene sequencing was done and uidA gene sequence data indicated that *E. coli*

Table 2: Detail of primers used for molecular characterization of carbapenemase genes in isolates of *E. coli* and *Klebsiella* spp.

Genes	Primers pair	Product size (bp)	References
bla-$_{NDM}$	F-5′GGTTTGGCGATCTGGTTTTC 3′ R-5′CGGAATGGCTCATCACGATC 3′	521	Mushi *et al*., 2014
bla-$_{KPC}$ variant 1-5	F-5′CATTCAAGGGCTTTCTTGCTGC 3′ R-5′ACGACGGCATAGTCATTTGC 3′	538	Dallenne *et al*., 2010
bla-$_{OXA-48}$	F-5′GCTTGATCGCCCTCGAT 3′ R-5′GATTTGCTCCGTGGCCGAAA 3′	281	Dallenne *et al*., 2010

Table 3: Isolation rate of *E. coli* and *Klebsiella* spp. in normal and mastitic milk samples of cattle and buffaloes.

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=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%)
Buffalo	Normal milk (n=80)	14 (17.5%)	4 (5.0%)	18 (22.5%)	11 (13.75%)	3 (3.75%)	14 (17.5%)
	Mastitic milk (n=40)	17 (42.5%)	8 (20.0%)	25 (62.5%)	14 (35.0%)	7 (17.5%)	21 (52.5%)
Total	N=240	71 (29.58%)	22 (9.16%)	93 (38.75%)	55 (22.92%)	19 (7.92%)	74 (30.83%)

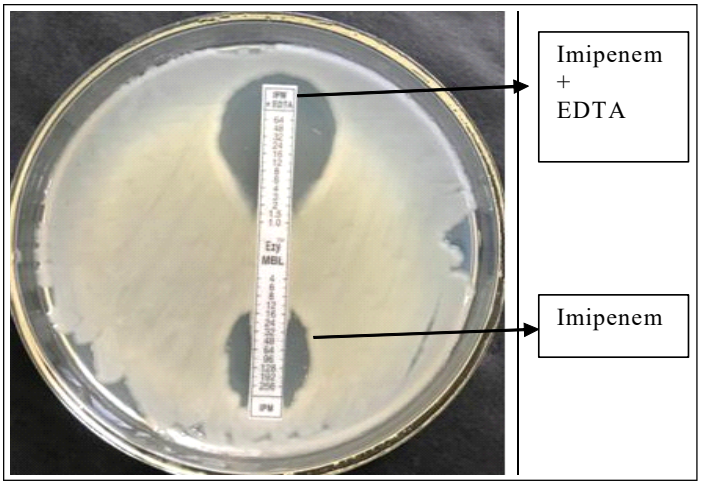


Fig 4: MIC MBL E- strip test for confirmation of carbapenemase producing *E. coli*. and *Klebsiella* spp.

isolates with Accession No: MW353603 and Accession No: MW353604 possessed great similarities (99.82%) to *E. coli* strain K-12 (LR881938.1), *E. coli* ST18 strain (CP060709.1), *E. coli* O68:H12 strain (CP061758.1) and *E. coli* strain EC93 (CP061329.1). *Klebsiella* isolates with Accession No: MW346043 and Accession No: MW346044 were identified as *Klebsiella pneumoniae* strain K1 and *Klebsiella pneumoniae* strain K3, respectively. These isolates possessed 100% similarities to *Klebsiella pneumoniae* strain NU-CRE047 (CP025037.1), *Klebsiella pneumoniae* strain G17SC 16S (KX610833.1) and *Klebsiella pneumoniae* strain WP5-S18-ESBL-06 (AP022157.1) on gene sequencing of 16S rRNA.

To study the occurrence of carbapenemase producers in milk, total 74 confirmed isolates were selected. On preliminary screening, 21.62% isolates were presumed as carbapenemase producers, out of which 17.56% isolates were found positive using DDST and 16.21% using MBL-E-strip test (Fig 5). There was little difference in the sensitivity of both of the test used for detection of carbapenemase producers and this observation corroborated with the findings of Gupta *et al*. (2013) and Bora *et al*. (2014). The finding of present study revealed higher frequency of carbapenemase producers in *Klebsiella* spp. (26.31%) than in *E. coli* (12.72%), which has been reported as major carbapenemase producer in previous studies also (Gupta *et al*., 2013 and Bora *et al*., 2014). The occurrence of carbapenemase producers was 10.0% (5.0% *E. coli* and 5.0% *Klebsiella* spp.) in cattle's mastitic milk, while 20.0% (12.5% *E. coli* and 7.5% *Klebsiella* spp.) in buffaloes' mastitic milk. The overall prevalence of carbapenemase producers was 5.0% with 2.91% *E. coli* and 2.08% *Klebsiella* spp. None of the carbapenemase producers was detected in normal milk (Table 4). There is scanty information on isolation of carbapenemase producers from milk both in India and abroad. In India, Ghatak *et al*. (2013) have reported very low percentage of carbapenemase positive *E. coli* in mastitic milk from NEH region while Diab *et al*. (2017) reported 1.6% *Klebsiellae* in raw milk samples from Lebanon. Similar to our finding, low percentage of carbapenemase positive *E. coli* has also been reported by Braun *et al*. (2016), Webb *et al*. (2016) and Nirupama *et al*. (2018) from faecal sample of different species. However, some of the workers have reported higher prevalence of carbapenemase producers among faecal samples ranging between 21.64%- 29.03% from different parts of India (Gupta *et al*., 2019; Murugan *et al*., 2019; Pruthvishree *et al*., 2017). These differences in findings of various co-workers may be due to variations in source, type of samples, animal husbandry practices in geographical locations.

Genotypic analysis of 12 phenotypically confirmed carbapenem resistant isolates (07 *E. coli* and 05 *Klebsiella* spp.) was done by targeting carbapenem genes *viz.* *bla*-$_{NDM}$, *bla*-$_{KPC}$ and *bla*-$_{OXA-48}$ (Fig 7, 8, 9). The overall gene distribution study showed 42.85% occurrence of *bla*-$_{NDM}$ and *bla*-$_{KPC}$ in *E. coli* isolates while 20.0% of *bla*-$_{OXA-48}$ and *bla*-$_{KPC}$ in *Klebsiella* spp. *E. coli* isolates of cattle harboured both *bla*-$_{NDM}$

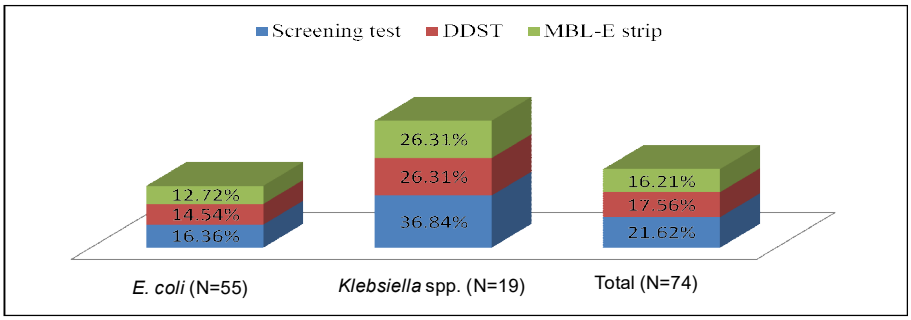


Fig 5: Distribution of carbapenem positive *E. coli* and *Klebsiella* spp. isolates according to screening and phenotypic confirmation tests (DDST and MBL-E strip).

Table 4: Occurrence of carbapenemase producing of *E. coli* and *Klebsiella* spp. among various sources.

Samples (Source/ Origin)		*E. coli* isolates	Carbapenem -ase positive *E. coli*	*Klebsiella* spp. isolates	Carbapene -mase positive *Klebsiella* spp.	Total isolates	Total Carbapene -mase positive isolates
Cattle	Normal milk (n=80)	12 (15.0%)	Nil	5 (6.25%)	Nil	17 (21.25%)	Nil
	Mastitic milk (n=40)	18 (45.0%)	2 (5.0%)	4 (10.0%)	2 (5.0%)	22 (55.0%)	4 (10.0%)
Buffalo	Normal milk (n=80)	11 (13.75%)	Nil	3 (3.75%)	Nil	14 (17.5%)	Nil
	Mastitic milk (n=40)	14 (35.0%)	5 (12.5%)	7 (17.5%)	3 (7.5%)	21 (52.5%)	8 (20.0%)
Total	N=240	55 (22.91%)	7 (2.91%)	19 (7.91%)	5 (2.08%)	74 (30.8%)	12 (5.0%)

and *bla*-$_{KPC}$ gene (14.28% each) whereas *Klebsiella* spp. harboured only *bla*$_{-OXA-48}$ gene (20.0%) in mastitic milk. In case of Buffalo, *E. coli* isolates exhibited *bla*-$_{NDM}$ and *bla*-$_{KPC}$ gene (28.57% each), while *Klebsiella* spp. isolates revealed only *bla*-$_{KPC}$ gene (20.0%) in mastitic milk (Table 5). It was notable in this study that *bla*-$_{KPC}$ gene was present in both *E. coli* and *Klebsiella* spp. isolates and *bla*-$_{OXA-48}$ gene was present only in one isolates of *Klebsiella* spp. Although occurrence of these genes in animals is very low but it is very significant from public health point of view, as these are increasingly being reported from nosocomial infection in which *Klebsiella* spp. is most commonly implicated. To the best of our knowledge, this is the first report of *bla*-$_{KPC}$ and *bla*-$_{OXA-48}$ gene detection in mastitic milk of bovine from India. Earlier to our study, only Ghatak *et al*. (2013) have reported *bla*-$_{NDM}$ gene in mastitic milk. The occurrence of such genes in milk samples of bovine may be presumed due to horizontal transfer by unclean hands of workers, water supplied for washing of udder and utensils or poor environmental condition. These carbapenemase genes have also been reported in previous studies like *bla*-$_{NDM-1}$ and *bla*-$_{OXA-48}$ genes in piglets (Pruthvishree *et al*., 2017; Nirupama *et al*., 2018), *bla*-$_{VIM}$ in calves (Murugan *et al*., 2019), *bla*-$_{OXA-48}$ in cattle faeces (Braun *et al*., 2016) and in milk (Diab *et al*., 2017)

Table 5: Distribution of carbapenemase genes according to various sources and organisms.

Samples (Source/ Origin)		Carbapenem resistant *E. coli* (n=07)			carbapenemase producing *Klebsiella* spp. (n=05)		
		bla-$_{NDM}$	*bla*-$_{OXA-48}$	*bla*-$_{KPC}$	*bla*-$_{NDM}$	*bla*-$_{OXA-48}$	*bla*-$_{KPC}$
Cattle	Normal milk	Nil	Nil	Nil	Nil	Nil	Nil
	Mastitic milk	1/7 (14.28%)	Nil	1/7 (14.28%)	Nil	1/5 (20.0%)	Nil
Buffaloes	Normal milk	Nil	Nil	Nil	Nil	Nil	Nil
	Mastitic milk	2/7 (28.57%)	Nil	2/7 (28.57%)	Nil	Nil	1/5 (20.0%)
Total		3/7 (42.85%)	Nil	3/7 (42.85%)	Nil	1/5 (20.0%)	1/5 (20.0%)

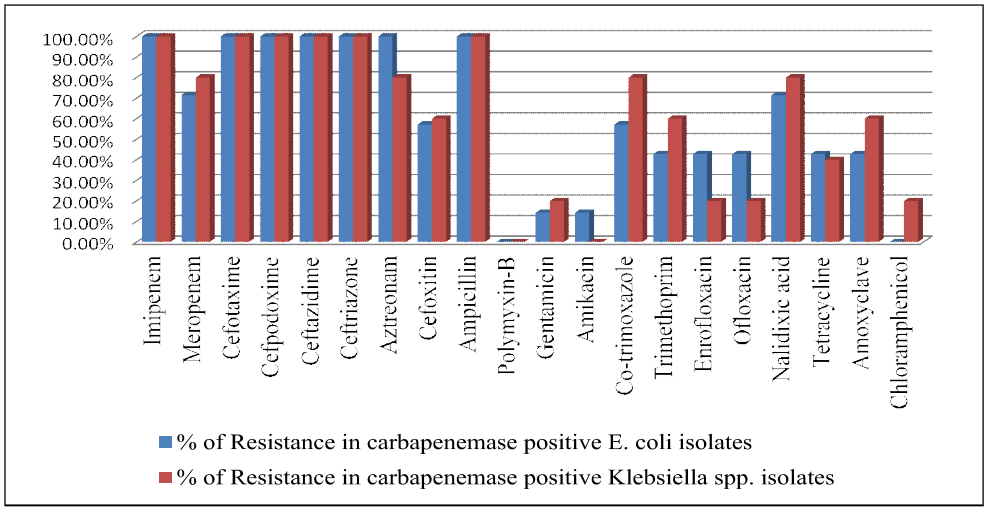


Fig 6: AMR pattern of carbapenemase positive *E. coli* and *Klebsiella* spp. isolates of bovine origin.

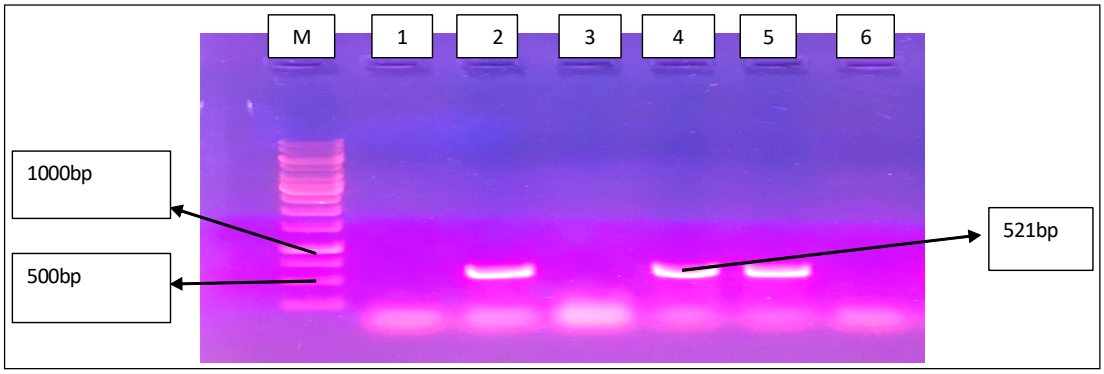


Fig 7: PCR amplification of *bla*-NDM gene (521 bp).
M: 1 Kb ladder, Lane 2, 4 and 5 positive for *bla*-$_{NDM}$ gene (521 bp), Lane 1 and 6 negative for *bla*-$_{NDM}$ gene.

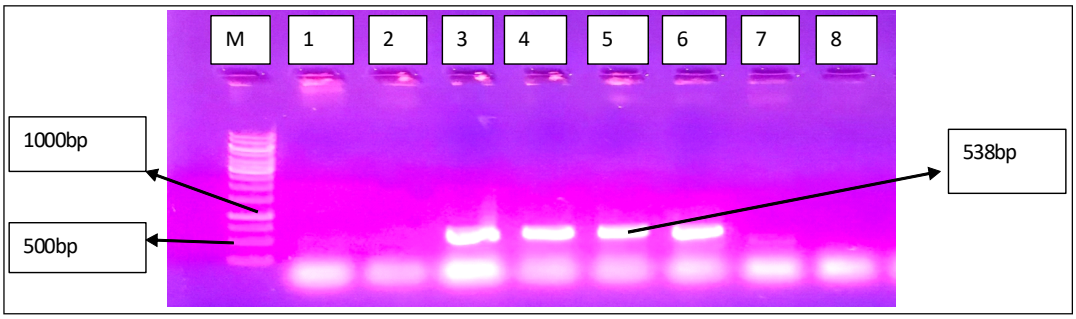


Fig 8: PCR amplification of *bla*-KPC gene (538 bp).
M: 1 Kb ladder, Lane 3, 4, 5 and 6 positive for *bla*-$_{KPC}$ gene (538 bp), Lane 1, 2,7 and 8 negative for *bla*-$_{KPC}$ gene.

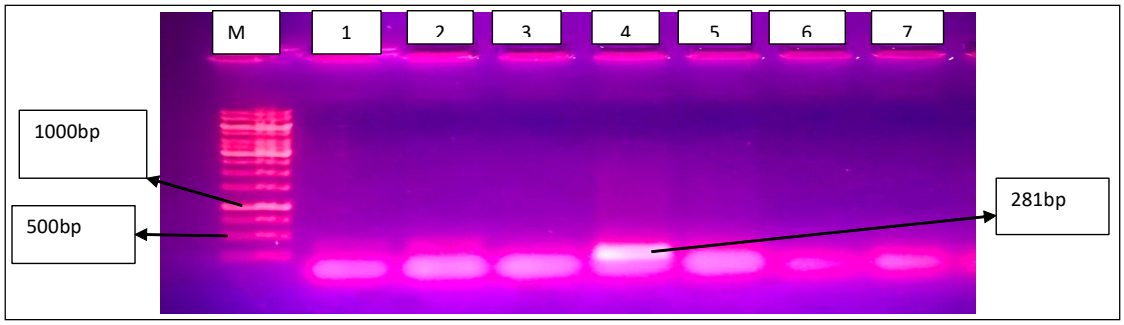


Fig 9: PCR amplification of *bla*-OXA-48 gene (281 bp).
M: 1 Kb ladder, Lane 4 positive for *bla*-$_{OXA-48}$ gene (281 bp), Lane 1, 2, 3 4, 5, 6 and 7 negative for *bla*-$_{OXA-48}$ gene.

from India and abroad. The detection of these genes in milk sample isolates is a matter of serious concern, because it is very easier for such isolates to enter the food chain leading to serious food-borne illnesses.

Antimicrobial resistance is currently a serious global problem that has received the attention of larger scientific community. Antimicrobial susceptibility testing (AST) of carbapenemase positive isolates revealed that all isolates of *E. coli* and *Klebsiella* spp. were (80%-100%) resistant to imipenem, meropenem cefotaxime, cefpodoxime, ceftazidime, ceftriazone, aztreonam and ampicillin. The plausible factors for high degree of resistance to these antibiotics may be due to persistent antibiotic pressure or acquired from environmental sources or farm workers. Susceptibility pattern of these isolates differed with different classes of non-β-lactam antibiotics except polymyxin-B for which both isolates were found 100% susceptible (Fig 6). *E. coli* isolates were found 85.0% to 100% sensitive against gentamicin and chloramphenicol, respectively while *Klebsiella* spp. was found 80% to 100% sensitive against chloramphenicol and amikacin. There is abundant evidence to corroborate the emergence of resistance against 3rd generation cephalosporins and ampicillin in India and abroad for both *E. coli* and *Klebsiella* spp. isolated from milk of bovine origin (Batabyal *et al*., 2018; Ghatak *et al*., 2019; Badri *et al*., 2017; Ibrahim *et al*., 2018). In this study all carbapenemase producing isolates were found to be multi-drug resistant (MDR) *i.e*., resistant to three or more classes of antimicrobials. This finding was in concordance with the finding of Bora *et al*. (2014). The occurrence of MDR in this study highlighted a potential threat by limiting the therapeutic options.

CONCLUSION

It is noteworthy from the present findings that carbapenems are not used in animal husbandry practices in this area; even then resistance in animal isolates has been observed which may be attributed to horizontal transfer of resistance gene between human and animal in community setting. Horizontal transfer of such pathogen or genes from one species to another may easily occur in highly populous country like India. Most of the isolates were resistant to 3rd and 4th generation cephalosporins and showed MDR which is cause of concern about *Enterobacteriaceae*. Therefore, a specific study on rational use of antibiotics and continuous monitoring for resistance gene against these antibiotics in livestock is warranted. Recovery of carbapenemase producers is very worrisome to human health in this area.

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REFERENCES

Anbazhagan, D., Kathirvalu, G.G., Mansor, M., Yan, G.O.S., Yusof, M.Y. and Sckaran, S.D. (2010). Multiplex PCR assays for the detection of *Enterobacteriaeceae* in clinical samples. African Journal of Microbiology Research. 4(11): 1186-1191.

Andersson, A.F., Lindberg, M., Jakobsson, H., Backhed, F., Nyren, P. and Engstrand, L. (2008). Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One: https://doi.org/10.1371/journal.pone.0002836.

Ansari, M., Munir, T., Sadd, N. (2018). Phenotypic identification, frequency distribution and antibiogram of carbapenemase producing *Enterobacteriaceae* in clinical isolates. Journal Call Physicians Surg Pak. 28: 274-278.

Badri, A.M., Ibrahim, T.I., Mohamed, S.G., Garbi, M.I., Kabbashi, A.S. and Arbab, M.H. (2017). Prevalence of ESBL producing *E. coli* and *K. pneumoniae* isolated from raw milk samples in Al Jazirah State, Sudan. Molecular Biology: Open Access. 7(1). DOI: 10.4172/2168-9547.1000201.

Batabyal, K., Banerjee, A., Pal, S., Dey, S., Joardar, S.N., Samanta, I., Isore, D.P. and Singh, A.D. (2018). Detection, characterization and antibiogram of ESBL *E. coli* isolated from bovine milk samples in West Bengal, India. Veterinary World. 11(10): 1423-1427.

Bauer, A.W., Kirby, W.M., Sherris, J.S. and Turck, M. (1996). Antibiotic susceptibility testing by standardized single disc method. American Journal of Clinical Pathology. 45: 493-496.

Bora, A., Sanjana, R. Jha, B.K., Mahaseth. S.N. and Pokhrel, K. (2014). Incidence of metallo-β-lactamase producing clinical isolates of *E. coli* and *K. pneumoniae* in Central Nepal. BMC Research Note. 7: 557. http://www.biomedcentral.com/1756-0500/7/557.

Braun, S.D., Ahmed, M.F.E., El-Adawy, H., Hotzel, H., Engelmann, I., Daniel W.D., Monecke, S. and Ehricht, R. (2016). Surveillance of ESBL producing *E. coli* in dairy cattle farms in the Nile Delta, Egypt. Frontiers in Microbiology. 7: 1020. https://doi.org/10.3389/fmicb.2016.01020.

Clinical and Laboratory Standards Institute (2019). Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Ninth Informational Supplement. CLSI Document M100-S29. Wayne, PA: Clinical and Laboratory Standards Institute.

Cruickshank, R., Duguid, J.P., Marmion, B.P. and Ewing, W.H. (1975). Identification of *Enterobacteriaceae* 3rd ed. Burges Publishing Co. Atlanta, Georgia, U.S.A. 152-154.

Dallenne, C., Costa, D.A., Decre, D., Favier, C. and Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in *Enterobacteriaceae*. Journal of Antimicrobial Chemotherapy. 65(3): 490-495.

Diab, M., Hamze, M., Bonnet, R., Saras, E., Madec, J. and Haenni, M. (2017). OXA-48 and CTX-M-15 ESBL in raw milk in Lebanon: Epidemic spread of dominant *K. pneumoniae* clones. Journal of Medical Microbiology. 66: 1688-1691.

Edward, P.R. and Ewing, W.H. (1972). Identification of *Enterobacteriaceae* (3rd edn.). Burges Publicity Co. Minneapolis, Minnesota. 55: 415.

Franco, S., Murphy, M.M., Li, G., Borjeson, T., Boboila, C. and Alt, F.W. (2008). DNA-PKcs and joining phase of Immunoglobulin heavy chain class switch recombination. Journal of Experimental Medicine. 205: 557-564.

Geser, N., Stephan, R. and Hachler, H. (2012). Occurance and characteristics of ESBL producing *Enterobacteriaceae* in food producing animals, minced meat and raw milk. BMC Veterinary Research. 21-19.

Ghatak, S., Singha, A., Sen, A., Guha, C., Ahuja, A., Bhattacharjee, U., Das, S., Pradhan, N. R., Puro, K., Jana,C., Dey, T.K., Prashantkumar, K.L., Das. A., Shakuntala, I., Biswas, U. and Jana, R.S. (2013). Detection of *bla*-$_{NDM}$ β-lactamase and ESBL genes in *E. coli* isolated from mastitic milk samples. Transboundary and Emerging Diseases. 60: 385-389.

Gupta, V., Bansal, N., Singla, N. and Chander, J. (2013). Occurrence and phenotypic detection of class A carbapenemases among *E. coli* and *K. pneumoniae* blood isolates at a tertiary care center. Journal of Microbiology, Immunology and Infections. 46: 104-108.

Gupta, S., Abhishek, Shrivastav, S. and Verma, A.K. (2019). Isolation, identification, molecular characterization and antibiogram of *E. coli* isolates from neonatal calves. International Journal of Current Microbiology and Applied Sciences. https://doi.org/10.20546/ijcmas.2019.806.238 8(6): 1996-2007.

Ibrahim, E.I., Sayed, F.H., Ashraf, M., Abd, E.I., Wahab, S.A.K. and Helmy, A.T. (2018). Prevalence of ESBL producing *Enterobacteriaceae* isolated from bovine mastitis milk. Alexandria Journal of Veterinary Sciences. 58(1): 102-108.

Koovapra, S. (2015). Characterization of ESBL Producing *Klebsiella* spp. of Bovine Mastitis Origin. Thesis M. V. Sc. Deemed University, ICAR-IVRI, Izatnagar, India. 13-14.

Muller, A. *et al.* (2018). Higher third-generation cephalosporine prescription proportion is associated with lower probability of reducing carbapenem use: A nationwide retrospective study. Antimicrobial Resistance and Infection Control. 7: 1-10.

Murugan, M.S., Sinha D.K., Vinodh, O.R., Yadav, A.K., Pruthvishree, B.S., Vadhana, P., Nirupama, K.R., Bhardwaj, M., Singh, B.R. (2019). Epidemiology of carbapenem resistant *E. coli* and first report of *bla*-$_{VIM}$ carbapenemase genes in calves from India. Epidemiology and Infection. 147(59): 1-5.

Mushi, M.F., Mshana, S.E., Imirzalioglu, C, Bwanga, F. (2014). Carbapenemase genes among multidrug resistant gram negative clinical isolates from a tertiary hospital in Mwanza, Tanzania. BioMed Research International. 303104.

Nirupama, *et al.* (2018). Molecular characterization of *bla*-$_{OXA-48}$ carbapenemase, ESBL and Shiga-toxin producing *E. coli* isolated from farm piglets in India. Journal of Global Antimicrobial Resistance. 13: 201-205.

Pruthvishree, B.S., Vinodh Kumar, O.R., Sinha, D.K., Malik, Y.P.S., Dubal, Z.B., Desingu, P.A., Shivakumar, M., Krishnaswamy, N. and Singh, B.R. (2017). Spatial molecular epidemiology of carbapenem-resistant and New Delhi metallo-β-lactamase (*bla*-$_{NDM}$)-producing *E. coli* in the piglets of organized farms in India. Journal of Applied Microbiology. 122: 1537-1546.

Wang, J.T., Wu, U.I., Lauderdale, T.L., Chen, M.C., Li, S.Y., Hsu, L.Y. and Chang, S.C. (2015). Carbapenem Non-susceptible *Enterobacteriaceae* in Taiwan. 10: e0121668.

Webb, H.E., Bugarel, M., Bakker, H.C., Nightingale, K.K., Granier, S.A., Scott, H.M. and Loneragan, G.H. (2016). Carbapenem resistant bacteria recovered from faeces of dairy cattle in the high plains region of the USA. PLoS ONE. 11(1): e0147363.