RESEARCH ARTICLE

Antimicrobial Resistance and Detection of Extended Spectrum β-Lactamase Producing Genes in *Klebsiella* Isolated from Buffalo Milk and Farm Environment

Prashant Prajapati, Daljeet Kour Chhabra*, Rakhi Gangil, Ravi Sikrodia, Joycee Jogi, Rakesh Sharda

Abstract

Klebsiella is an emerging pathogen of one health importance. It is ubiquitously distributed in environment as well as associated with humans and animals infections. Three hundred samples were collected for bacteriological isolation and identification of *Klebsiella* from buffalo mastitic milk and farm environment. All *Klebsiella* isolates were tested for susceptibility to 15 antibiotics belonging to different classes by disc diffusion method. Extended spectrum *beta* lactam (ESBL) *Klebsiella* and ESBL genes were also detected phenotypically by combined disc diffusion test and PCR, respectively. *Klebsiella* spp. were isolated from 15 (5.00%) out of 300 samples. Prevalence of both *Klebsiella* spp. and *K. pneumoniae* in buffaloes and farm environment was 4.66 and 5.33 %, respectively. All the *Klebsiella* isolates were simultaneously resistant towards 2 to 8 antibiotics. Eight isolates were found to be multidrug resistant (MDR). Two ESBL genes, *viz., bla* TEM and *bla* SHV were detected among 15 *Klebsiella* isolates and the prevalence was found to be 26.66 and 53.33 %, respectively. None of the bacterial isolates were positive for *bla* DHAM and *bla* CTX-M gene. It was recorded that multiple drug resistance (MDR) strains of *Klebsiella* and *K. pneumoniae* were prevalent in buffaloes and farm environment.

Key Words: Antibiotic, ARGs, ESBL, *Klebsiella,* MDR.

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Introduction

Klebsiella can cause serious infections in mammals besides bovine mastitis and other infections in livestock (Ammar *et al*., 2021). *K. pneumoniae* are opportunistic pathogen mostly prevalent in organic matter, such as animal house bedding of moist soils and manure. *Klebsiella* mastitis management has become serious problem even in herds with inorganic bedding and good environment (Munoz *et al.,* 2006). *Klebsiella* species belongs to the ESKAPE group of pathogens capable of 'escaping' the biocidal action of antibiotics and mutually representing new paradigms in pathogenesis, transmission and resistance (Omoya and Ajayi, 2016). The swift rise of antimicrobial resistance amongst pathogens generates a serious thought about the potential post-antibiotic era, threatening present and future medical advances (Carattoli, 2008). The hyper-virulent and beta-lactam drug-resistant strains of *K. pneumoniae* are capable of causing fatal, life-threatening infections both in animals and humans (Shon *et al.,* 2013; Lee *et al.*, 2017). Therefore, considering the above facts present research pursuit was undertaken with the objective of determining antibiotic resistance and detection of some ESBL genes in *Klebsiella* isolates of mastitis milk and farm environment.

Materials and Methods

The work was carried out in the Department of Microbiology, Veterinary College, Mhow (M.P., India). Total three hundred samples from buffaloes (mastitic milk, faeces) and farm environment (drinking water, bedding material, floor and soil sample) were collected from organized and unorganized buffalo farms.

Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Mhow-453441, M.P., India

Corresponding Author: Dr. Daljeet Kour Chhabra, Department of Veterinary Microbiology, College of Veterinary Science and AH, NDVASU, Mhow-453441, M.P., India. e-mail: daljeetchhabra@yahoo.com, drdaljeet@gmail.com

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Isolation, Identification and ABST of Pathogens

The samples were first inoculated in BHI broth and incubated aerobically at 37°C for 24 h, followed by inoculation on basic, differential and selective media and Gram staining for the presumptive identification of bacterial isolates as *Klebsiella* and *K. pneumoniae* by colonial and microscopic morphology followed by biochemical tests (Barrow and Feltham, 1993) and PCR using primers for genus specific gene *gyrA* and species specific gene *K. pneumoniae 16S-23S ITS* (Younis *et al.,* 2017; Prajapati *et al*., 2025).

The isolates of *Klebsiella* were tested for *in vitro* sensitivity towards 15 antibacterial drugs using disc diffusion method. Broth culture of isolates matched with 0.5 McFarland opacity was used for the test.

Phenotypic Detection and PCR for ESBL Genes

Phenotypic detection of ESBL was performed by combined disc diffusion test. Disc containing 30 µg of cefotaxime and disc containing a combination of cefotaxime plus clavulanic acid (30/10 µg) (HiMedia, Mumbai, India) were placed independently, approx. 30 mm apart. After an overnight incubation period, isolates were considered to be ESBL positive if the inhibitory zone surrounding one of the combination discs was at least 5 mm greater than the corresponding cephalosporin disc (Dash *et al*., 2022).

The PCR was standardized for the detection of four ESBL genes (*bla* TEM, *bla* SHV, *bla* DHAM and *bla* CTX-M) in *Klebsiella* using published primers (Table 1).

Extraction of DNA and Thermocyclic Condition for ESBL Genes

The *Klebsiella* isolates were cultivated by inoculating in Brain heart infusion broth (Hi Media) and incubating at 37°C for 12-18 h (overnight grown culture). DNA was extracted with the kit (GeneElute Bacterial Genomic DNA Kit). PCR was performed for identification of four ESBL genes in *Klebsiella* isolates. The details of thermocyclic conditions used are given in Table 2. The amplified products were electrophoresed in 1.5% agarose gel and visualized in gel documentation system.

Results and Discussion

Isolation and Identification of *Klebsiella* spp.

Out of the 300 samples collected for the bacteriological isolation and identification of *Klebsiella* and *Klebsiella pneumoniae* using phenotypic and genotypic methods, 15 samples (5%) were found to be positive for *Klebsiella* (Table 4). Prevalence of both *Klebsiella* spp. and *K. pneumoniae* in buffaloes and farm environment was 4.66 and 5.33 %, respectively. The cultural characteristics and results of biochemical tests are in agreement with the standard book reference and other reports (Barrow and Feltham, 1993; Arya *et al.,* 2020; Ammar *et al*., 2021). All 15 *Klebsiella* spp. isolates produced a desired product of 441 bp size of gene *gyrA* and 130 bp of gene *16 S-23 ITS* in PCR, which has been published earlier (Prajapati *et al*., 2025). The same genes were also targeted by the Younis *et al.* (2017) and Cheng *et al.* (2021) for the molecular detection of *Klebsiella* and *K. pneumoniae.*

Antimicrobial Resistance Pattern in *Klebsiella* Isolates

All *Klebsiella* isolates were tested for susceptibility to 15 antibiotics belonging to different classes and sensitivity of individual isolates to various drugs was interpreted according to the manufacturer's (HiMedia, CLSI) instructions. The highest resistance was attributed towards fosfomycin (100.00%), tigecycline (93.33%), gentamicin (66.66%), ciprofloxacin and amikacin (53.33% each), chloramphenicol (33.33%), cefazolin (26.66%), ertapenem (20.00%), tetracycline

Table 1: Details of primers used for molecular characterization of ESBL genes

Target gene	Primer Sequence	Product size	Reference
bla TEM	F: TTGGGTGCACGAGTGGGTTA R: TAATTGTTGCCGGGAAGCTA	506 bp	Gundran *et al.* (2020)
bla SHV	F:TCGGGCCGCGTAGGCATGAT R:AGCAGGGCGACAATCCCGCG	628 bp	Gundran *et al.* (2020)
bla DHAM	F:AACTTTCACAGGTGTGCTGGGT R: CCGTACGCATACTGGCTTTGC	405 bp	Van *et al.* (2008)
bla CTX-M	F: GTGCAGTACCAGTAAAGTTATGG R: CGCAATATCATTGGTGGTGCC	538 bp	Adesoji *et al.* (2015)

Table 2: Thermocyclic conditions for identification of ESBL genes

Genes	Initial Denaturation		Denaturation		Annealing		Extension		Final extension	
			Total No. of cycles (35)							
	Temp. (°C)	Duration	Temp. (°C)	Duration	Temp. (°C)	Duration	Temp. (°C)	Duration	Temp. (°C)	Duration
bla SHV	95	3 min	94	60 sec	64	30 sec	72	60 sec	72	7 min
bla TEM	94	3 min	94	60 sec	56	30 sec	72	60 sec	72	7 min
bla DHAM	95	15 min	94	30 sec	54	30 sec	72	60 sec	72	10 min
bla CTX-M	95	60 esc	96	30 sec	52	30 sec	72	30 sec	72	10 min

(13.33%), ampicillin/sulbactum, cefotaxime/clavulanic acid and ofloxacin (6.66% each). None of isolate was resistant to amoxyclav, aztreonam and ticarcillin/clavulanic acid.

Aly *et al.* (2014) also recommended that ABST should be invariably done to treat *Klebsiella* infections as there may be a change in drug sensitivity due to long use of specific antibiotics. The antibiotics used in our study were also reported by Webb *et al*. (2016), Badri *et al*. (2017), Effah *et al.* (2020), Cheng *et al*. (2021), Ammar *et al*. (2021) and Kashefieh *et al*. (2021). Extended spectrum beta lactam (ESBL) antibiotics have widely been used for treatment of serious Gram-negative infections. These include penicillins, cephalosporins, cephamycins, carbapenems and monobactam group of antibiotics. In the present study the sensitivity towards ESBL antibiotics ranged from 6.66 to 86.66 %.

Resistance observed in our study against ampicillin/sulbactum was 6.66% which is low as compared to the finding of 26.6 % and 53.2 % by Cheng *et al.* (2021) and Kashefieh *et al*. (2021), respectively. No resistance was observed in case of amoxyclav. Ertapenem (a carbapenem group of antibiotic) showed 20.00% resistance towards *Klebsiella* isolates in the present research, which is very close with the findings of 24 % resistance by Webb *et al.* (2016). In the present study, the resistance towards aztreonam was seen to be 0 %, on the contrary, Koovapra *et al.* (2016) reported resistance of 100% against aztreonam. In current study, cefotaxime/clavulanic acid showed no sensitivity to *Klebsiella* isolates, which is similar to the findings of 100% resistance reported by Effah *et al.* (2020). On the contrary, a very lower rate of resistance, *i.e.,* 4.8% to cephalosporin was recorded by Bedekelabou *et al.* (2020). In a report from India, 80-90% of *Klebsiella* spp. isolates were resistant to third generation cephalosporins (CDDEP, 2015).

Table 3: Multiple drug resistance in *Klebsiella* strains isolated from buffaloes and farm environment

S. No.	No. of antibiotics	No. of resistant isolates	Resistant isolates % (n=15)
1.	2	1	6.66
2.	3	5	33.33
3.	4	1	6.66
4.	5	2	13.33
5.	6	2	13.33
6.	7	3	20.00
7.	8	1	6.66
		Total 15	

Simultaneous and Multiple Drug Resistance

All 15 isolates were simultaneously resistant towards 2 to 8 antibiotics, but none of the isolate was found to be resistant to all the 15 antibiotics (Table 3). Highest percent of resistant isolates (33.33%) was against total 3 antibiotics. The MDR in *Klebsiella* in samples from buffaloes were also reported by Osman *et al*. (2014) and Yadav *et al*. (2021). The variations in prevalence of MDR *K. pneumoniae* isolates in diverse studies may be attributed to the dissimilarity in clinical conditions of patients and animals, the samples involved in the survey, the antibiotics used and the application of the infection control measures (Ammar *et al*., 2021).

Phenotypic Detection of ESBL

The *Klebsiella* isolates were confirmed for ESBL production phenotypically by combined disc diffusion test. The inhibitory zone around cefotaxime disc was 10-12 mm, while the zone size around cefotaxime/clavulanic acid discs recorded were 22 mm to 30 mm for different isolates which confirms ESBL production by *Klebsiella* isolates by phenotypic method. Manchanda *et al*. (2005) and Elhassan *et al*. (2016) also reported phenotypic detection of ESBL in *Klebsiella* isolates. In the present study phenotypic method was found sensitive to detect ESBL production.

Detection of ESBL Genes by PCR in *Klebsiella*

DNA extracted from 15 isolates was subjected to PCR for detection of four ESBL genes using published primers. An amplicon size of 506 bp (Gundran *et al.,* 2020), was taken as positive for *bla* TEM gene. Out of 15 isolates, 4 (26.66%) were positive for *bla* TEM gene (Fig. 1). A desired product of 628 bp of *bla* SHV (Gundran *et al.,* 2020) in PCR was produced by 8 (53.33%), strains (Fig. 2). An amplicon size of 405 bp (Van *et al*., 2008) was considered positive for *bla* DHAM gene and an amplicon size of 538 bp (Adesoji *et al.,* 2015) was considered positive for *bla* CTX-M. Out of 15 isolates, none of the isolate was positive for *bla* CTX-M and *bla* DHAM genes.

For Gram-negative bacteria, the production of β-lactamase enzymes is the main resistance mechanism to β-lactam antibiotics. In *Klebsiella* isolates out of four only two ESBL genes were recovered in all source of samples. Maximum 2 genes were detected in one sample. The gene

Table 4: Prevalence of ESBL genes in *Klebsiella* isolates

S. No.	Sources of sample	No. of *Klebsiella* isolates (N=15)	Prevalence of ESBL gene in *Klebsiella* isolates, number and percentage				Total no. of gene detected in source
			***bla* SHV**	***bla* TEM**	***bla* DHAM**	***bla* CTX-M**	
1.	Mastitic milk	4	0 (0.00)	2 (50.00)	0	0	1
2.	Faeces	3	2 (66.6)	1 (33.3)	0	0	2
3.	Bedding & Floor	2	2 (100)	0	0	0	1
4.	Drinking water	5	3 (60.00)	1 (20.00)	0	0	2
5.	Soil sample	1	1 (100)	0	0	0	1
	Total isolates	**15**	**8 (53.33%)**	**4 (26.66%)**	**0 (0.00%)**	**0 (0.00%)**	

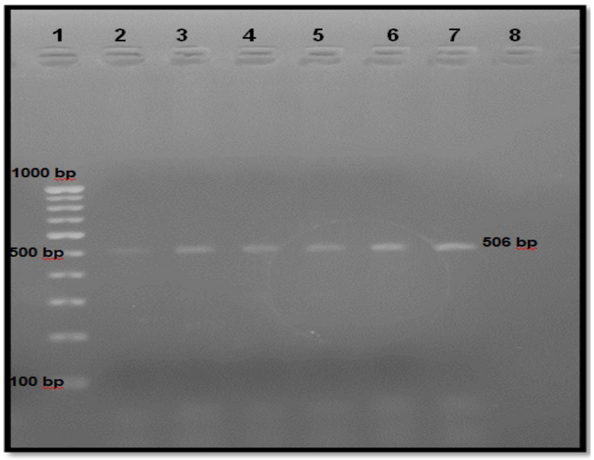

Fig. 1: Agarose gel electrophoresis showing amplified product (506 bp) of *bla* TEM gene. Lane 1: Ladder (100 bp), Lane 2: Positive control, Lane 3 to 7: Samples, Lane 8: Negative control.

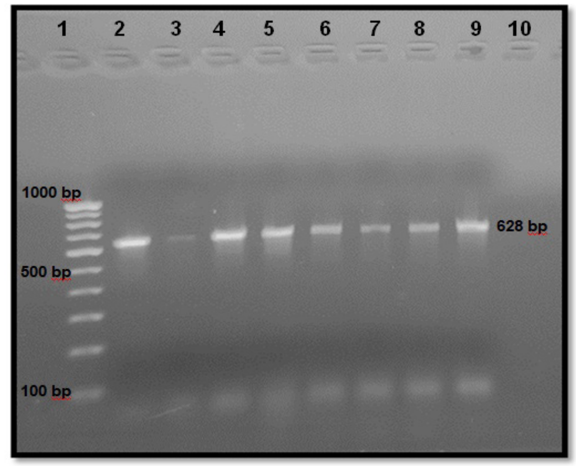

Fig. 2: Agarose gel electrophoresis showing amplified product (628 bp) of bla SHV gene. Lane 1: Ladder (100 bp), Lane 2: Positive control, Lane 3 to 9: Samples, Lane 10: Negative control

bla SHV was detected in 8 isolates and gene *bla* TEM in 4 isolate. The gene *bla* TEM was detected in all sources, except bedding material and floor, while the gene *bla* SHV was detected in all sources, except mastitic milk (Table 4). Bandyopadhyay *et al*. (2018) reported 3 genes in *Klebsiella* from buffalo milk, *i.e., bla* CTX-M gene in all the 12 isolates, whereas *bla* TEM and *bla* SHV were detected in 2 and 1 isolate(s), respectively.

In the present study only two ESBL genes, *viz., bla* TEM and *bla* SHV were detected among 15 *Klebsiella* isolates and the prevalence was found to be 26.66 and 53.33 %, respectively (Table 4). SHV was the predominant ESBL gene detected in the present investigation. The prevalence of *bla* SHV and *bla* TEM was reported as 42.5 % and 87.5%, respectively, by Enferad and Mahdavi (2020). But they also detected *bla* CTX-M gene. On the contrary, Koovapra *et al*. (2016) observed a lower prevalence (13%) of *bla* SHV. Similarly Badri *et al*. (2017) found only 23% and 16% *Klebsiella* spp. isolates possessing *bla* SHV and *bla* TEM. Ferreira *et al*. (2019) found *K. pneumoniae* strains isolated to be ESBL producers and carried *bla* TEM (100%) and *bla* SHV (96%).

According to Koovapra *et al*. (2016), Badri *et al*. (2017) and Enferad and Mahdavi (2020) the prevalence of *bla* CTX-M gene was 82.6%, 61% and 62.5% which is in contrast to zero percent prevalence found in present study. The presence of *bla* DHAM gene in *Klebsiella* isolates was also zero percent. But Japoni-Nejad *et al*. (2014) reported *bla* DHAM in 5.2% *K. pneumoniae* strains.

The prevalence of ESBL genes varies, between different geographical regions. *K. pneumoniae* can develop resistance and acquire other genes that contribute to the survival of the pathogen in different environments. Systemic treatments result in the excretion of antimicrobial residue through animals' feces, urine and milk. Antimicrobial residue contributes to pathogen selection in the environment and, by transferring genetic material; new bacteria can acquire resistance genes. Additionally, microorganisms producing resistance enzymes can be found naturally in soil, which may facilitates the acquisition of resistance genes by *Klebsiella* (Hammad *et al.,* 2008; Nobrega *et al*., 2013).

Conclusions

The current findings revealed that multdrug resistance (MDR) strains of *Klebsiella* and *K. pneumoniae* were prevalent in buffalo milk and farm environment. The evidence of the presence of MDR strains indicates injudicious use of antibiotics and emphasizes the need for their epidemiological monitoring. To preserve the efficacy of extended-spectrum cephalosporins and other antibiotics for the treatment of problematic cases of bovine mastitis or other clinical conditions, it is essential that culture and sensitivity testing always be performed, especially for recurrent infections. ARGs such as *bla* SHV and *bla* TEM, detected in these drug resistant *Klebsiella* strains may be responsible in inducing multiple drug resistance. The detection of ESBLs in dairy animals and herds could be a major concern for both public and animal health.

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