

Original Research Article

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Occurrence, Phenotypic and Molecular Characterization of Extended-Spectrum β -Lactamase-Producing and Multidrug-Resistant *Klebsiella pneumoniae* from Bovine Mastitis and Dairy-Farm-Associated Samples

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ABSTRACT

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Klebsiella pneumoniae is an important opportunistic pathogen associated with bovine mastitis and may serve as a reservoir of antimicrobial resistance determinants within dairy production systems. The present study investigated the occurrence of *K. pneumoniae* and characterized extended-spectrum β -lactamase (ESBL) production and multidrug resistance among isolates recovered from bovine mastitis and dairy farm-associated samples. A total of 584 samples were collected from 30 dairy farms, comprising 494 milk samples and 90 farm-associated samples, including hand swabs, Cowshed swabs and sewage. Bacterial isolates were identified by cultural, morphological and biochemical characteristics and confirmed by *16S rRNA* gene amplification and sequencing. Antimicrobial susceptibility was determined by the Kirby–Bauer disc diffusion method according to CLSI guidelines. ESBL production was evaluated phenotypically by the double-disc synergy test (DDST) and MIC-based E-test. ESBL-associated *blaCTX-M*, *blaSHV* and *blaTEM* genes were detected by PCR. Twenty *K. pneumoniae* isolates were recovered, of which 12 (60.0%) originated from mastitic milk and eight (40.0%) from farm-associated samples. Eight isolates (40.0%) were resistant to third-generation cephalosporins and seven (35.0%) were confirmed as ESBL producers. All ESBL isolates carried *blaCTX-M*, while *blaSHV* and *blaTEM* were detected in three (42.9%) and two (28.6%) isolates, respectively. Thirteen isolates (65.0%) were multidrug resistant, including all seven ESBL producers. The findings demonstrate the occurrence of ESBL-producing MDR *K. pneumoniae* in bovine mastitis and associated dairy farm environments, highlighting the importance of antimicrobial surveillance and prudent antimicrobial use in dairy production systems.

Introduction

Bovine mastitis is one of the major infectious diseases affecting dairy cattle and is associated with reduced milk production, increased treatment costs and compromised milk quality (Bradley, 2002; Halasa *et al.*, 2007). Both clinical and subclinical mastitis contribute substantially to production losses, with subclinical infections often remaining undetected without specific diagnostic testing (Ruegg, 2017). A wide range of bacterial pathogens are associated with bovine mastitis, among which *Klebsiella pneumoniae* is an important environmental pathogen capable of causing intramammary infections and severe clinical disease (Munoz *et al.*, 2007).

The increasing occurrence of antimicrobial-resistant bacteria in livestock is an important veterinary and public-health concern. Antimicrobial exposure can select resistant organisms, while mobile genetic elements facilitate dissemination of resistance determinants among bacterial populations (Aarestrup, 2015; Martínez *et al.*, 2015). Dairy farms provide several potential interfaces for the persistence and circulation of antimicrobial-resistant organisms through animals, handlers, farm environments and waste.

Klebsiella pneumoniae is particularly important because it can acquire and disseminate resistance determinants encoding extended-spectrum β -lactamases (ESBLs). ESBL enzymes hydrolyse many penicillins and cephalosporins and are frequently associated with plasmid-mediated resistance determinants (Paterson and Bonomo, 2005; Bush and Bradford, 2016). Among ESBL-associated genes, *bla*CTX-M, *bla*SHV and *bla*TEM are widely reported in *K. pneumoniae* from animal, food and human sources (Cantón *et al.*, 2012; D'Andrea *et al.*, 2013). The presence of multidrug-resistant (MDR) ESBL-producing *K. pneumoniae* in dairy-associated samples is therefore relevant to antimicrobial treatment, animal health and One Health surveillance.

Although antimicrobial resistance among bovine mastitis pathogens has been investigated in several regions, information on the phenotypic and molecular characteristics of ESBL-producing *K. pneumoniae* recovered simultaneously from mastitic milk and associated dairy farm samples remains limited. Therefore, the present study was undertaken to investigate the occurrence of *K. pneumoniae* in bovine mastitis and dairy farm-associated samples and to characterize the isolates with respect to antimicrobial

resistance, ESBL production and selected ESBL-associated genes.

Materials and Methods

Study design and sample size

A cross-sectional study was conducted involving 30 dairy farms. A total of 584 samples were examined, comprising 494 milk samples and 90 dairy farm-associated samples. The milk samples included samples from clinically, subclinical and from apparently healthy/non-mastitic animals. The dairy farm-associated samples comprised 30 hand swabs, 30 Cowshed swabs and 30 sewage samples, with one sample of each type collected from each of the 30 dairy farms.

The required milk sample size was calculated using the formula:

$$n = 4pq/L^2$$

Where p = expected prevalence (50%), $q = 100 - p$, and L = allowable error (4.5%). The calculated sample size was 493.83 and was rounded to 494 milk samples (Snedecor and Cochran, 1967).

Collection of milk and farm-associated samples

Milk samples were collected from lactating cows with clinical mastitis, subclinical mastitis and apparently non-mastitic quarters. Clinical mastitis was assessed based on clinical examination and abnormal milk characteristics. Subclinical mastitis was screened using the California Mastitis Test as described by Ruegg and Reinemann (2002). Approximately 15 mL of milk was collected aseptically into sterile containers. Farm-associated samples comprised 30 hand swabs, 30 Cowshed swabs and 30 sewage samples. Swabs were collected by unidirectional swabbing of the relevant surfaces and transferred aseptically into sterile tubes. All samples were appropriately labelled, maintained under chilled conditions and transported to the laboratory for bacteriological examination.

Isolation and identification of *Klebsiella pneumoniae*

Samples were initially enriched in Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 h. Following

enrichment, cultures were streaked onto BHI agar and examined for colony morphology and staining characteristics. Gram-negative, catalase-positive isolates showing mucoid colony morphology were further cultured on MacConkey lactose agar and incubated at 37°C for 24 h.

Large, mucoid, lactose-fermenting colonies suggestive of *Klebsiella* spp. were purified and subjected to Gram staining and biochemical characterization using a commercial biochemical identification system. Isolates exhibiting characteristics consistent with *K. pneumoniae* were retained for molecular confirmation and antimicrobial susceptibility testing (Markey *et al.*, 2013).

Genomic DNA extraction by hot–cold lysis

Genomic DNA was extracted from pure bacterial cultures using the hot–cold lysis method. Briefly, 4–5 well-isolated colonies were suspended in sterile distilled water or normal saline in a sterile microcentrifuge tube to obtain a homogeneous suspension. The suspension was heated at 100°C for 10 min and immediately transferred to ice. The lysate was subsequently centrifuged to sediment cellular debris, and the supernatant containing bacterial DNA was carefully transferred to a sterile tube and stored at –20°C until PCR analysis (Brisse *et al.*, 2014).

Molecular confirmation by 16S rRNA gene amplification

Molecular confirmation of the bacterial isolates was performed by PCR amplification of the 16S rRNA gene. The PCR reaction was carried out in a final volume of 25 µL containing 12.5 µL of 2× PCR master mix, 8.0 µL nuclease-free water, 1.25 µL each of forward and reverse primers and 2.0 µL of template DNA. The 16S rRNA gene was amplified using the primer pair 27F and 1492R as described by Agung *et al.*, (2019).

The PCR cycling consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 52°C for 1 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on 1% agarose gel. The expected amplicon size was approximately 1465 bp. PCR products selected for sequencing were outsourced for Sanger sequencing. Obtained sequences were analysed using BioEdit and

compared with sequences available in the NCBI database using sequence alignment and BLAST analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar. Bacterial suspensions were prepared from fresh cultures and tested against the selected antimicrobial agents. The diameter of zones of inhibition was measured after incubation and isolates were categorized as susceptible, intermediate or resistant according to the interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI, 2020).

The resistance profiles were used to identify isolates resistant to third-generation cephalosporins and to determine multidrug-resistant phenotypes.

Definition of multidrug resistance

An isolate was considered multidrug resistant when it demonstrated acquired non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories, following the internationally accepted definition proposed by Magiorakos *et al.*, (2012).

Phenotypic detection of ESBL production

Isolates showing resistance to third-generation cephalosporins were subjected to phenotypic ESBL confirmation using the double-disc synergy test (DDST) and MIC-based E-test.

Double-disc synergy test

For DDST, cefotaxime (30 µg) and cefotaxime–clavulanic acid (30/10 µg) discs were used. The discs were placed 24 mm apart on Mueller–Hinton agar inoculated with the test isolate and incubated at 37°C for 16–24 h. An increase of ≥5 mm in the inhibition zone diameter around the cefotaxime–clavulanic acid disc compared with cefotaxime alone was interpreted as evidence of ESBL production according to the applied interpretive criteria.

MIC-based ESBL detection

An ESBL detection E-test strip containing the antibiotic combination with and without clavulanic acid was placed

on Mueller–Hinton agar inoculated with the test isolate and incubated at 37°C for 16–18 h. An MIC ratio (MIC of antibiotic alone/MIC of antibiotic plus clavulanic acid) of ≥ 8 was interpreted as ESBL production.

An isolate was considered phenotypically ESBL positive when it fulfilled the established criteria in the phenotypic confirmation procedure.

PCR detection of ESBL-associated genes

Phenotypically confirmed ESBL-producing *K. pneumoniae* isolates were screened by PCR for the ESBL-associated genes *blaCTX-M*, *blaSHV* and *blaTEM*. PCR was performed in a final volume of 25 μ L containing 12.5 μ L of 2 $\times$ PCR master mix, nuclease-free water, gene-specific forward and reverse primers and template DNA.

The primer sequences and amplification conditions were based on published protocols (Kar *et al.*, 2015). PCR products were separated by agarose gel electrophoresis and visualized using a gel documentation system. Positive and negative controls were included during PCR amplification.

The expected amplicon sizes were 585 bp for *blaCTX-M*, 393 bp for *blaSHV* and 686 bp for *blaTEM*.

Data analysis

The occurrence of *K. pneumoniae*, ESBL production, third-generation-cephalosporin resistance and MDR phenotypes was expressed as percentages. Gene detection results were expressed as the proportion of positive isolates among phenotypically confirmed ESBL-producing *K. pneumoniae*.

Results and Discussion

Distribution of samples

A total of 584 samples were examined from 30 dairy farms. Of these, 494 were milk samples and 90 were farm-associated samples. The milk samples comprised 37 clinical mastitis samples, 87 subclinical mastitis samples and 370 non-mastitic samples. The farm-associated samples consisted of 30 hand swabs, 30 Cowshed swabs and 30 sewage samples (Table 1).

Occurrence of *Klebsiella pneumoniae*

Twenty *K. pneumoniae* isolates were recovered from the 584 samples examined, corresponding to an overall recovery rate of 3.42%. Twelve isolates (60.0%) were obtained from mastitic milk, whereas eight isolates (40.0%) originated from other dairy farm-associated samples (Table 2).

Among the 124 mastitis-associated milk samples, *K. pneumoniae* was recovered from 12 samples, giving a recovery rate of 9.68%.

Among the 90 farm-associated samples, eight *K. pneumoniae* isolates were recovered (8.89%).

Third-generation-cephalosporin resistance and ESBL production

Of the 20 *K. pneumoniae* isolates, eight (40.0%) showed resistance to third-generation cephalosporins. Phenotypic ESBL testing by DDST and MIC-based E-test confirmed seven isolates (35.0% of all *K. pneumoniae* isolates) as ESBL producers. Thus, seven of the eight third-generation-cephalosporin-resistant isolates (87.5%) were confirmed as ESBL producers (Table 3).

The eight third-generation-cephalosporin-resistant isolates comprised four from clinical mastitis, two from subclinical mastitis, one from cowshed swabs and one from sewage. No resistant isolate was recovered from hand swabs. Of the seven ESBL-producing isolates, four were from clinical mastitis, two from subclinical mastitis and one from sewage.

Molecular detection of ESBL-associated genes

All seven phenotypically confirmed ESBL-producing *K. pneumoniae* isolates carried *blaCTX-M*, giving a positivity rate of 100%. The *blaSHV* gene was detected in three isolates (42.9%), while *blaTEM* was detected in two isolates (28.6%) (Table 4).

Source-wise analysis showed that *blaCTX-M* was detected in all seven ESBL-producing isolates. The *blaSHV* gene was detected in one clinical mastitis isolate, one subclinical mastitis isolate and one sewage isolate. The *blaTEM* gene was detected in one clinical mastitis isolate and one sewage isolate.

Multidrug resistance

Thirteen of the 20 *K. pneumoniae* isolates (65.0%) fulfilled the definition of multidrug resistance. All seven ESBL-producing isolates were also MDR. Therefore, ESBL-producing MDR isolates accounted for 35.0% of all *K. pneumoniae* isolates and 53.85% of the MDR isolates (Table 5).

The 13 MDR isolates comprised five from clinical mastitis, two from subclinical mastitis, one from hand swabs, one from cowshed swabs and four from sewage.

The present study demonstrated the occurrence of *K. pneumoniae* in bovine mastitis as well as in dairy farm-associated samples. Twenty isolates were recovered from 584 samples, with 60% originating from mastitic milk and 40% from associated farm samples.

The recovery of *K. pneumoniae* from mastitic milk is consistent with its recognized role as an environmental mastitis pathogen. *K. pneumoniae* has previously been implicated in bovine mastitis outbreaks and has been associated with both environmental sources and intramammary infection (Munoz *et al.*, 2007).

The recovery of *K. pneumoniae* from farm-associated samples is also relevant because environmental reservoirs may contribute to continued exposure of animals to opportunistic pathogens. Farm environments and sewage can provide conditions supporting bacterial persistence and dissemination.

However, the present study was not designed to establish transmission pathways; therefore, recovery of the organism from different sample types should be interpreted as an indication of occurrence rather than direct evidence of transmission.

An important finding was that 40.0% of the isolates were resistant to third-generation cephalosporins, and seven of the eight resistant isolates were phenotypically confirmed as ESBL producers. The high proportion of ESBL production among third-generation-cephalosporin-resistant isolates is consistent with the established association between resistance to extended-spectrum cephalosporins and ESBL-mediated hydrolysis of these antimicrobial agents (Paterson and Bonomo, 2005). ESBL-producing *K. pneumoniae* has also been reported from bovine milk, demonstrating that livestock-

associated isolates can harbour important β -lactam resistance determinants (Koovapra *et al.*, 2016).

The molecular analysis demonstrated complete detection of *blaCTX-M* among the seven phenotypically confirmed ESBL isolates. In contrast, *blaSHV* and *blaTEM* were detected in 42.9% and 28.6% of isolates, respectively. The predominance of *blaCTX-M* agrees with reports describing CTX-M enzymes as major contributors to the global dissemination of ESBL-mediated resistance (Cantón *et al.*, 2012; D'Andrea *et al.*, 2013). Koovapra *et al.*, (2016) similarly reported *blaCTX-M*, *blaSHV* and *blaTEM* among ESBL-producing *K. pneumoniae* isolated from bovine milk in India.

The coexistence of multiple ESBL-associated genes in some isolates indicates that resistance may involve more than one β -lactamase determinant. Such genetic combinations are important because resistance genes are frequently associated with mobile genetic elements capable of facilitating their dissemination between bacterial populations (Carattoli, 2009; Wyres and Holt, 2018). Nevertheless, the present study focused on detection of selected genes and did not investigate plasmid structure, gene localization or horizontal gene transfer.

MDR was detected in 65.0% of the *K. pneumoniae* isolates. Importantly, all seven ESBL-producing isolates were MDR. The simultaneous presence of ESBL production and MDR reduces the number of potentially effective antimicrobial options and may complicate therapeutic management. Similar associations between ESBL production and multidrug resistance have been described in *K. pneumoniae* from animal-associated sources (Koovapra *et al.*, 2016; Xu *et al.*, 2022).

The detection of an ESBL-producing isolate in sewage is particularly relevant from a One Health perspective because farm waste may provide an environmental reservoir for antimicrobial-resistant organisms. However, the present study did not establish the direction of movement of resistant organisms between cattle and the environment.

Further investigations incorporating whole-genome sequencing, plasmid characterization and longitudinal sampling would be required to determine epidemiological relationships among isolates.

Table.1 Distribution of samples examined in the study

Sample category	Sample type	Number	Percentage of total (%)
Milk	Clinical mastitis	37	6.34
Milk	Subclinical mastitis	87	14.90
Milk	Non-mastitic/negative	370	63.36
Farm-associated	Hand swabs	30	5.14
Farm-associated	Cowshed swabs	30	5.14
Farm-associated	Sewage	30	5.14
Total		584	100.00

Among the 494 milk samples, clinical and subclinical mastitis accounted for 7.49% and 17.61%, respectively.

Table.2 Distribution of *Klebsiella pneumoniae* isolates according to sample source

Source	Sample type	Number of isolates	Percentage (%)
Mastitic milk	Clinical mastitis	9	45.0
	Subclinical mastitis	3	15.0
Subtotal – mastitic milk		12	60.0
Other farm-associated samples	Hand swabs	1	5.0
	Cowshed swabs	2	10.0
	Sewage samples	5	25.0
Subtotal – farm-associated samples		8	40.0
Total		20	100.0

Percentage calculated with respect to the total number of *K. pneumoniae* isolates ($n = 20$).

Table.3 Source-wise distribution of third-generation-cephalosporin-resistant and phenotypic ESBL-producing *Klebsiella pneumoniae* isolates

Source	Sample type	Third-generation-cephalosporin-resistant isolates	Percentage (%)	Phenotypic ESBL producers	Percentage (%)
Mastitic milk	Clinical mastitis	4	50.0	4	57.1
	Subclinical mastitis	2	25.0	2	28.6
Other farm-associated samples	Hand swabs	0	0.0	0	0.0
	Cowshed swabs	1	12.5	0	0.0
	Sewage samples	1	12.5	1	14.3
Total		8	100.0	7	100.0

Percentages for third-generation-cephalosporin resistance are calculated with respect to the total number of resistant isolates ($n = 8$), whereas percentages for phenotypic ESBL producers are calculated with respect to the total number of ESBL-producing isolates ($n = 7$).

Table.4 Source-wise distribution of phenotypic ESBL-producing *Klebsiella pneumoniae* and associated ESBL genes

Source	Sample type	Phenotypic ESBL producers, n (%)	<i>blaCTX-M</i> , n (%)	<i>blaSHV</i> , n (%)	<i>blaTEM</i> , n (%)
Mastitic milk	Clinical mastitis	4 (57.1)	4 (57.1)	1 (14.3)	1 (14.3)
	Subclinical mastitis	2 (28.6)	2 (28.6)	1 (14.3)	0 (0.0)
Other farm-associated samples	Hand swabs	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Cowshed swabs	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Sewage samples	1 (14.3)	1 (14.3)	1 (14.3)	1 (14.3)
Total		7 (100.0)	7 (100.0)	3 (42.9)	2 (28.6)

Percentages are calculated with respect to the total number of phenotypic ESBL-producing isolates ($n = 7$). Gene categories are not mutually exclusive.

Table.5 Source-wise distribution of multidrug-resistant *Klebsiella pneumoniae* isolates

Source	Sample type	MDR isolates, n	Percentage (%)
Mastitic milk	Clinical mastitis	5	38.5
	Subclinical mastitis	2	15.4
Other farm-associated samples	Hand swabs	1	7.7
	Cowshed swabs	1	7.7
	Sewage samples	4	30.8
Total		13	100.0

Percentages are calculated with respect to the total number of MDR *K. pneumoniae* isolates ($n = 13$).

Overall, the findings demonstrate that bovine mastitis and associated dairy farm environments can harbour *K. pneumoniae* exhibiting antimicrobial resistance phenotypes and ESBL-associated genes. Routine antimicrobial susceptibility testing, prudent antimicrobial use, improved mastitis control and environmental hygiene are important components of resistance-management strategies in dairy production systems.

In conclusion, the study identified *K. pneumoniae* in bovine mastitis and dairy farm-associated samples, with 20 isolates recovered from 584 samples. Eight isolates were resistant to third-generation cephalosporins, seven were phenotypically confirmed as ESBL producers and 13 were multidrug resistant. *blaCTX-M* was detected in all phenotypically confirmed ESBL isolates, while *blaSHV* and *blaTEM* were detected less frequently. All ESBL-producing isolates were multidrug resistant. These findings emphasize the importance of integrated antimicrobial-resistance surveillance, appropriate antimicrobial use and strengthened mastitis and farm-environment management practices to reduce the persistence of resistant *K. pneumoniae* in dairy production systems.

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Conflict of Interest

The authors declare that they have no conflicts of interest related to the research, authorship, or publication of this manuscript.

Ethical Approval and Informed Consent

The study was conducted in accordance with the applicable institutional and ethical guidelines for the collection and analysis of bovine and dairy-farm-associated samples. Appropriate permission was obtained for sample collection from the participating dairy farms.

No personally identifiable information from farm owners or other individuals was collected or reported.

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Data Availability

The data generated and analyzed during the present study are available from the corresponding author upon reasonable request, subject to applicable institutional and confidentiality requirements.

Authors Contributions

Sreeramdas Srikanth: Methodology, investigation, data collection, data curation, formal analysis, interpretation of results, visualization, literature review, and writing of the original manuscript. Arfan Ali: Conceptualization, development of the research idea, study design, supervision, scientific guidance, and critical review and revision of the manuscript. Chandrami Goswami: Provided inputs to the research work; contributed to manuscript writing, review, and revision. Durlav P. Bora: Provided valuable scientific insights and contributed to supervision of the research work. Pankaj Deka: Contributed to the methodology and experimental design.

Arijit Shome: Contributed to software-related work and standardization of the research procedures. Lukumoni Buragohain: Contributed to software-related work and standardization of the research procedures. Sophia M. Gogoi: Contributed to standardization of SOPs and research procedures. Nagendra N. Barman: Contributed to project administration and funding acquisition. Kanyaga Parameshwari: Contributed to methodology, resources, and data curation.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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