RESEARCH ARTICLE

Emergence of ESBL-Producing *Klebsiella oxytoca* in Bovine Mastitic Milk: A Study in Gujarat, India

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Abstract

The present study reports the first detection of extended spectrum β-lactamase (ESBL) producing *Klebsiella oxytoca*, an opportunistic pathogen, from bovine milk samples collected from milk-shed regions of Gujarat. The study included a total of 270 bovine milk samples comprising of 175 fresh milk sample from apparently healthy dairy animals and 95 mastitic milk samples. A total of five *K. oxytoca* isolates were identified based on results of cultural, biochemical and molecular techniques, all from mastitic milk samples only, yielding an overall prevalence of 5.26 % (5/95). None of the milk sample from healthy animals was found positive for the presence of *Klebsiella oxytoca.* Phenotypic characterization revealed that 80 % (4/5) isolates were ESBL producing *Klebsiella oxytoca* based on both results of Combination Disc Diffusion test (CDDT) and HiCrome ESBL agar culture. Further, ESBL producing genes, *viz.*, bla_{TEM} and bla_{SHV} were found in one and four isolates, respectively, whereas virulent gene *wab*G was found in all five isolates. None of the *K. oxytoca* isolates was found positive for the presence of ESBL producing gene $bla_{CTX-M-1}$ and virulent gene *kfu*BC.

Key words: Bovine milk, ESBL producing genes, *Klebsiella oxytoca*, Virulence genes.

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Introduction

India emerged as the leading milk producing country, with a total milk production of 221.06 million tonnes in the year 2021-22. Among the states in India, Gujarat ranked fourth in terms of milk production, accounting for a share of 7.56 % (BAHS, 2023). Conversely, mastitis is still a significant problem for milk production and a barrier to achieving targeted milk production of 266.5 million tonnes by the year 2030 (Solanki *et al.,* 2022). The development of antimicrobial resistance among mastitis pathogens poses a significant obstacle to effective mastitis management. This is particularly evident in the case of Gram-negative bacteria, such as *Klebsiella* spp., which are often implicated in bovine mastitis. *Klebsiella* spp., is recognized as an opportunistic pathogen that contributes to environmental mastitis and is responsible for approximately 2 to 9% of clinical bovine mastitis cases (Masse *et al.,* 2020). The World Health Organization (WHO) has designated *Klebsiella* spp. as a "critical mastitis" pathogen in order to address the challenges posed by antimicrobial resistance. Two most common *Klebsiella* spp. involved in bovine mastitis are *K. pneumoniae* followed by *K. oxytoca* (Ahmed and Shimamoto, 2011). *Klebsiella oxytoca* is Gram-negative, rod-shaped, facultative anaerobic, non-spore forming, indole positive, lactose-fermenting, encapsulated, non-motile coliform. Numerous virulence-associated genes play crucial roles in the virulence mechanisms of *Klebsiella* spp., such as *wab*G, a gene associated with endotoxin production, and *kfu*BC, a gene involved in the acquisition of iron. The *wab*G gene is primarily responsible for the biosynthesis of outer membrane core lipopolysaccharide, and alterations in this gene have been linked to decreased colonization and virulence potential in *Klebsiella* spp. (Izquierdo *et al.,* 2003). While extensive research has focused on the detection of *wab*G and *kfu*BC genes in *Klebsiella pneumoniae*, limited information is currently available regarding the presence of these genes in *Klebsiella oxytoca*.

ESBLs, or extended spectrum β-lactamases, are enzymes that facilitate the hydrolysis of penicillins and cephalosporins, thereby conferring resistance to Gram-negative enteric bacteria belonging to the Enterobacteriaceae family including *Klebsiella* species. The worldwide spread of ESBLs represents a significant challenge, especially among key pathogens of the Enterobacteriaceae family (Prajapati *et al*., 2020). The primary mechanism behind resistance to β-lactam class antimicrobial drugs in bacterial pathogens is

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the acquisition of the ESBL gene and subsequent production of related enzymes. When compared to *K. pneumoniae*, *K. oxytoca* has not garnered as much attention while referring to possession of ESBL producing genes (Tsuka *et al.,* 2021). The genes responsible for the production of ESBLs are widely recognized and can be primarily categorized into three types: bla_{CTX-M}, bla_{TEM}, and bla_{SHV}. These types exhibit variations in their predominant genotypes across different geographic regions (Yang *et al.,* 2018). Considering the limited availability of data regarding the presence of virulence genes and ESBL-producing genes in *Klebsiella oxytoca*, particularly in isolates from bovine raw milk and mastitic milk, the objective of this study was to investigate the prevalence of these genes. The study aimed to assess both the phenotypic and molecular characteristics of *Klebsiella oxytoca* strains isolated from bovine milk samples, focusing on their virulence gene profiles and their ability to produce ESBLs.

Materials and Methods

A total of 270 milk samples were collected for the study. These samples consisted of fresh raw milk obtained from apparently healthy animals, with 175 samples (108 from cattle and 67 from buffalo), and 95 mastitic milk samples collected from animals suffering from clinical mastitis (62 from cattle and 33 from buffalo). Thus, the samples were aseptically collected in sterilized vials from a population of 170 dairy cows and 100 buffaloes from the Banaskantha milk-shed areas of north Gujarat, India.

Milk samples were placed on MacConkey agar (MCA) plates and incubated at 37°C for 24 h. Pure lactose fermenting cultures were identified by rose pink, dome-shaped, mucoid colonies on the MCA. These cultures were streaked on Eosin Methylene Blue (EMB) agar plates and incubated overnight at 37°C. The resulting colonies on the EMB agar were dark and mucoid, but lacked a metallic sheen. To perform biochemical identification of the isolates, the catalase test, oxidase test, IMViC (indole, methyl red, Voges Proskauer, citrate utilization) test, triple sugar iron (TSI) test, urease test, motility test and capsule staining were conducted as per standard methods (Quinn *et al.,* 2011). For molecular confirmation, all the recovered isolates underwent amplification of the *pehX* gene using *K. oxytoca* species-specific primers (Table 1).

All the PCR-confirmed isolates of *K. oxytoca* were subjected to phenotypic confirmation for ESBL production using Combination Disc Diffusion test (CDDT) as per Carter *et al.* (2000) and HiCrome ESBL agar method. The luxurious growth with bluish green colonies on HiCrome ESBL agar was indicator of ESBL production by *K. oxytoca*. Molecular confirmation of ESBL producing *K. oxytoca* isolates was carried out by detecting three genes, *viz.*, $bla_{CTX-M-1}$, bla_{TEM}, and bla_{SHV} (Dallenne *et al.,* 2010). The investigation of two crucial virulence genes, *wab*G and *kfu*BC, was conducted using PCR with their respective specific primers (Table 1).

Results and Discussion

Among the total of 270 milk samples analysed, a total of five strains of *K. oxytoca* were successfully isolated from 95 mastitic samples (Table 2). Thus, prevalence of *K. oxytoca* in mastitic cases was 5.26 %. In contrast, none of the 175 samples of raw fresh milk yielded any isolate of *K. oxytoca*, as determined by employing cultural and biochemical criteria. All five isolates of *K. oxytoca* exhibited positive catalase activity, negative oxidase activity, and demonstrated the IMViC pattern of + - + +. The TSI test revealed acid production in both the slant and butt regions, leading to a yellow coloration in the slant accompanied by gas production. In the motility test, the isolates exhibited growth exclusively along the inoculation line, indicating their non-motile nature. Under microscopic observation during capsule staining, capsules were distinctly visible in all of the isolates.

Table 1: Specific primers used for amplification of the target genes

Target gene	Primer Sequence (5' to 3')	Annealing temp. (°C)	Amplicon size	References
*peh*X	F: GATACGGAGTATGCCTTTACGGTG R: TAGCCTTTATCAAGCGGATACTGG	55	343 bp	Kovtunovych *et al.* (2003)
*kfu*BC	F: GAA GTG ACG CTG TTT CTG GC R: TTT CGT GTG GCC AGT GAC TC	59	797bp	Brisse *et al.* (2009)
*wab*G	F: CGG ACT GGC AGA TCC ATA TC R: ACC ATC GGC CAT TTG ATA GA	54	683bp	
$bla_{CTX-M-1}$	F: TTA GGA ATG ATG CCG CTG CA R: CGA TAT CGT TGG TGG TAC CAT	60	688bp	Dallenne *et al.* (2010)
bla_{TEM}	F: CAT TTC CGT GTC GCC CTT ATT C R: CGT TCA TCC ATA GTT GCC TGA C	60	800bp	
bla_{SHV}	F: AGC CGC TTG AGC AAA TTA AAC R: ATC CCG CAG ATA AAT CAC CAC	60	713bp	

Table 2: Prevalence of *Klebsiella oxytoca* in dairy cattle and buffaloes milk samples from Banaskantha district of Gujarat

Type of sample	Animal	No. of samples tested	No. of samples found positive
Raw fresh milk	Dairy cattle	108	0 (0 %)
	Buffaloes	67	0 (0 %)
	Total	175	0 (0 %)
Mastitic milk	Dairy cattle	62	3 (4.84 %)
	Buffaloes	33	2 (6.06 %)
	Total	95	5 (5.26 %)

The current study presents a noteworthy finding, indicating that only mastitic milk samples showed the presence of *K. oxytoca*, while none of the raw milk samples from apparently healthy animals tested positive for the bacterium. These results suggest that fresh raw milk in the study area may generally be considered safe in terms of potential zoonotic transmission of *K. oxytoca*. However, it is crucial to note that this finding is preliminary, and further investigation involving a larger sample size from the study region is necessary to draw a definitive conclusion. Conversely, a study from Kolkata, India, reported a 7% prevalence of *K. oxytoca* in marketed raw milk, based on biochemical tests (Saini *et al*., 2020), while Korashy *et al*. (2006) reported a 6.6% prevalence in cows' raw milk from Egypt. In another study from Egypt, 12.12% prevalence of *K. oxytoca* in mastitis cow cases was found and 50% of isolates (6.6%) were identified as multi-drug resistant strains (Ahmed and Shimamoto, 2011). Generally, it is observed that the prevalence of *K. oxytoca* in clinical mastitis cases is lower than that of *K. pneumoniae* (Masse *et al.,* 2020; Tsuka *et al.,* 2021). However, there are reports suggesting that in cows with clinical mastitis, the prevalence of *K. pneumoniae* and *K. oxytoca* may be equal (Gao *et al.,* 2019).

After obtaining the results from cultural and biochemical analyses, all five isolates underwent molecular confirmation. As a result, a 343 bp amplicon was successfully amplified from the genomic DNA of each isolate, indicating the presence of the *pehX* gene specific to *K. oxytoca* species (Fig. 1).

Fig. 1: PCR amplification of *peh*X gene for *K. Oxytoca.* L: 100 bp DNA marker, P: Positive control, Lane 10-12: Positive isolates, N: Negative control

Molecular detection of virulence genes revealed presence of *wabG* gene in all five isolates, whereas *kfuBC* gene was not detected in any of five isolates. In the present study, we detected the *wabG* virulence gene, which plays a crucial role in endotoxin biosynthesis. Virulence genes associated with endotoxin production are known to be significant contributors to the pathogenesis of *Klebsiella* species (Wu *et al.,* 2022). While there were no previous reports of *wabG* gene detection in *K. oxytoca*, its importance is increasingly recognized due to its higher detection rate in *K. pneumoniae* isolates in various studies. For instance, Cheng *et al.* (2018) reported a 91% detection rate in cattle respiratory infections, Zhang *et al.* (2018) found it in 77.4% of food samples, and Wu *et al.* (2022) identified it in over 85% of dairy cattle samples. The wide distribution of this virulence gene with high frequency in diverse samples related to dairy cattle and food products indicates not only its pathogenic significance but also poses a significant risk to public health.

Among the five isolates of *K. oxytoca* tested, four (80.0%) were identified as ESBL producers using both the HiCrome ESBL agar method and the combination disc diffusion test. Moreover, in the analysis of ESBL producing genes, the majority of isolates, specifically four, tested positive for bla_{SHV} genes, with one of these isolates also showing positivity for bla_{TEM}, however, none of the isolates were found to be positive for $bla_{CTX\text{-}M\text{-}1}$ gene (Table 3).

One isolate was phenotypically determined to be a non-ESBL producer using both the HiCrome ESBL agar method and CDDT. However, upon further analysis, this isolate was found to carry two ESBL-producing genes, namely bla_{SHV} and bla_{TEM}. In line to our finding, Diagbouga *et al.* (2016) also reported a case of *K. oxytoca* isolate that possessed the bla_{TEM} gene but did not test positive phenotypically in the double disk test synergy. Therefore, while both methods used in present study were equally sensitive in phenotypic detection of ESBL producers in *K. oxytoca*, but they could not correlate with ESBL-producing genes responsible for ESBL production. This highlights the need for genotypic characterization to rule out the possibility of biochemical mechanisms of resistance unrelated to enzyme involvement.

There are meagre reports available on the detection of ESBL producing *K. oxytoca* in veterinary species. Bobbadi *et al.* (2019) reported presence of $bla_{CTX\text{-}M\text{-}2}$ gene in *K. oxytoca* isolated from pork sample in Andhra Pradesh, India. In contrast, they could not detect other ESBL producing genes specifically, bla_{SHV} and bla_{TEM} in pork sample. Similarly, Mechaala *et al.* (2021) found an isolate of *K. oxytoca* from buttermilk but could not detect presence of any ESBL producing gene. In another study reported from Japan, *K. oxytoca* isolates from bovine mastitis showed presence of $bla_{CTX\text{-}M\text{-}2}$ gene. This gene is not much studied in the *K. oxytoca* isolated from veterinary species (Tsuka *et al.,* 2021). ESBL producing genes, *viz.*, bla_{TEM-1}, bla_{OXA-30}, $bla_{CTX-M-15}$, bla_{SHV-1}, and bla_{SHV-12}, were also identified in *K. oxytoca* isolates from bovine mastitis cases in Egypt (Ahmed and

Table 3: Detection of ESBL producing genes in *K. oxytoca isolated* from mastitic milk samples (out of five confirmed isolates).

Type of sample	Number of samples found positive by		Number of ESBL genes		
	CDDT	ESBL agar	bla_{TEM}	bla_{SHV}	$bla_{CTX\text{-}M\text{-}1}$
Mastitic milk	4 (80.00 %)	4 (80.00 %)	1 (20.00 %)	4 (80.00 %)	0 (0.00 %)

Shimamoto, 2011). The present study revealed the presence of ESBL-producing *K. oxytoca* in the milk of infected cows, harbouring resistance genes. This finding indicates that these infections could potentially serve as a route for the transmission of these genes, not only within animal populations but also to human populations. Furthermore, the continued presence of these pathogens in the udder or milk leads to the ineffectiveness of the treatment for intra-mammary infection.

Conclusion

This report presents the prima facie findings on the characterization and detection of virulence and ESBL-producing genes in *K. oxytoca* isolated from bovine mastitic milk in Banaskantha district of Gujarat State, India. The presence of *K. oxytoca* was observed only in mastitic milk samples and not in the raw milk samples. Notably, the virulence genes *wabG* and the ESBL-producing genes bla_{SHV} and bla_{TEM} were detected in the isolates. This report highlights the significance of ESBL-producing *Klebsiella oxytoca* in bovine mastitic cases, indicating the need for further large-scale studies to establish the epidemiology of this pathogen in the region.

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