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Genetic characterization of virulence and extended spectrum β-lactamase producing genes of *Klebsiella pneumoniae* isolated from bovine milk

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Abstract

Mastitis associated *Klebsiella pneumoniae* species were isolated from bovine milk to characterize virulence genes (*wabG* and *kfuBC*) and extended spectrum β-lactamase (ESBL) genes (*bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$). A total number of 325 bovine milk samples (195 raw and 130 mastitic milk specimens) collected from Banaskantha, a milk-shed district of Gujarat, India, were included in the study. A total number of 27 *K. pneumoniae* isolates were recovered, consisting of 17 (62.96%) isolates from raw milk and 10 (37.03%) isolates from mastitic milk samples, giving an overall prevalence of 8.31%. Antibiotic sensitivity patterns revealed that 20 out of 27 isolates were found to be multi-drug resistant. Based on combination disc diffusion test and HiCrome ESBL agar method, 20 (74.07%) and 25 (92.59%) isolates were detected as ESBL producers, respectively. Among virulence genes studied, presence of *wabG* (25/27; 92.59%) was higher than *kfuBC* (5/27; 18.51%). Beta-lactamase genes *viz.,* *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M-1}$ were detected in 23/27 (85.18%), 3/27 (11.11%) and 2/27 (7.40%) of isolates, respectively; while, none of the isolates was found to be positive for *bla*$_{CTX-M-9}$ and *bla*$_{OXA-1}$ genes. Outcome of the study provided an insight into virulence genes and ESBL producing *K. pneumoniae* isolated from bovine milk samples in India.

Introduction

India ranks first in milk production, accounting for nearly 23.00% of world production.[1] However, mastitis remains to be a major challenge in milk production globally and India is not an exception. Resistance acquired by mastitis pathogens to conventional anti-microbial drugs is a great barrier in successful management of mastitis. Gram-negative bacteria, including *Klebsiella* spp., are one of the common pathogens causing bovine mastitis. *Klebsiella pneumoniae* is considered as an opportunistic pathogen for causing environmental mastitis and found to be involved in 2.00 - 9.00 % of clinical bovine mastitis cases.[2] It is listed as a critical mastitis pathogen by World Health Organization to tackle anti-microbial resistance problems. *Klebsiella pneumoniae* is a Gram-negative, facultative anaerobic, rod-shaped, non-spore forming, catalase-positive, oxidase-negative, lactose-fermenting, encapsulated and non-motile coliform, and is a common environmental agent of clinical and sub-clinical bovine mastitis decreasing the quality of milk and milk products.[3,4] Mastitis caused by *K. pneumoniae* is more severe due to its poor response to antibiotic therapy, toxic shock and animal deaths.[5] Many virulence genes are involved in the virulence process of *Klebsiella* spp, such as, *wabG*, an endotoxin-related gene, and *kfuBC*, the iron acquisition system-related gene. The virulence-related function of *wabG* gene is regarded to the biosynthesis of outer membrane core lipopolysaccharide and thus, contributes to virulence of *K. pneumoniae*.[6] The *kfu* as a putative pathogenic gene plays role in an iron uptake system. Iron is a crucial element for bacterial survival and *K. pneumoniae* synthesizes small iron-scavenging particles termed as siderophores permitting it to evade from the host.[7] However, the presence of these genes in mastitis-causing *K. pneumoniae* has rarely been studied.[8]

Extended spectrum β-lactamases (ESBLs) catalyse the hydrolysis of penicillins and cephalosporins and provide

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resistance to the Gram-negative enteric bacteria of the *Enterobacteriaceae* family. The dissemination of ESBLs is a global problem particularly in sentinel members of the *Enterobacteriaceae*.[9] The ESBLs are rapidly spreading worldwide which are frequently encountered among clinical isolates of *K. pneumoniae* in human and animals.[10] Thus, resistance of bacterial pathogens to β-lactam class of anti-microbial drugs is mainly due to acquiring the ESBL gene and producing related enzymes. The various genes responsible for acquiring resistance by *K. pneumoniae* are located in transferable genetic elements transferrable to other bacteria. Such resistant *K. pneumoniae* species have been isolated from range of food items including meat, vegetables and sea-food;[11] however, studies in milk samples are limited. The well-known genes for production of ESBLs can be majorly divided into three types *viz*., $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} types and their predominant genotypes vary across different geographic regions.[12] However, knowledge and ESBL producing *K. pneumoniae* species present in bovine raw milk and mastitic milk are still limited particularly in India and there is a dearth of information about their prevalence.[13,14] The aim of this study, therefore, was to assess the prevalence of virulence genes and ESBL producing *K. pneumoniae* from bovine milk samples as well as the phenotypic and molecular characterization.

Materials and Methods

Sample collection. A total number of 325 fresh milk samples comprising both raw milk from apparently healthy animals (n = 195; cow: 117, buffalo: 78) and mastitic milk from animals suffering from clinical mastitis (n = 130; cow: 83, buffalo: 47) from a total of 200 dairy cows and 125 buffaloes were collected aseptically in sterilized vials and immediately sent to the laboratory for culture. The study period was ranged from June 2019 to November 2020 and study region was Banaskantha, milk-shed district of Gujarat state, India, with the bovine population of more than 1.60 million.

Isolation and identification of *Klebsiella* spp. Milk samples were inoculated on MacConkey agar (MCA) plates (HiMedia Laboratories, Mumbai, India) and incubated at 37.00 ˚C for 24 hr. Pure lactose fermenting cultures characterised by rose pink, dome-shaped and mucoid colonies obtained on MCA were further streaked on eosin methylene blue (EMB) agar plates and incubated over-night at 37.00 ˚C. Dark mucoid colonies without metallic sheen on EMB were picked up for overnight incubation at 37.00 ˚C on HiCrome *Klebsiella* selective agar (HiMedia Laboratories) for selective isolation of *Klebsiella* spp. Magenta coloured colonies were identified as *Klebsiella* spp. Till further characterization using biochemical tests, isolated pure cultures were preserved in brain heart infusion broth (HiMedia Laboratories) at 4.00 ˚C.

Biochemical identification of *Klebsiella* spp. isolates. Biochemical identification of the isolates was done by performing catalase, oxidase, IMViC (*i.e.*, indole, methyl red, Voges Proskauer and citrate utilization), triple sugar iron and urease tests, as well as motility and capsule staining as *per* standard methods.[15]

Molecular confirmation of recovered isolates. All the recovered isolates were subjected to molecular confirmation using genus-specific *gyrA* (for *Klebsiella* spp.) as well as species-specific *rpoB* (for *K. pneumoniae*) genes amplification. Genomic DNA was extracted from isolates as *per* the method described by Younis *et al*.[16] The polymerase chain reaction (PCR) protocol for amplifying the *16S rRNA* gene was standardised using *Klebsiella* genus-specific and *K. pneumoniae* species-specific primers (Table 1). The PCR amplification was carried out in a total volume of 25.00 µL with 2.00 µL DNA template, 12.50 µL 2.00X PCR Master Mix (Qiagen, Germany), 8.50 µL nuclease free water (Qiagen, Germany) and 1.00 µL of each primer. The PCR protocol was carried out in Mastercycler Nexus Gradient (Eppendorf Hauppauge, USA) included (i) initial denaturation at 95.00 ˚C for 15 min., (ii) followed by 35 cycles of denaturation at 95.00 ˚C for 1 min, annealing at 55.00 ˚C for 1 min, extension at 72.00 ˚C for 2 min, and (iii) a final extension at 72.00 ˚C for 10 min. The PCR products were separated on 1.50% agarose gel including 1.00% of ethidium bromide and visualised under Gel Documentation System (DNR Bio-Imaging Systems Ltd, Jerusalem, Israel).

Antibiotic sensitivity patterns of *K. pneumoniae* isolates. The antibiotic sensitivity patterns of recovered isolates of *K. pneumoniae,* picked up from HiCrome *Klebsiella* selective agar were determined using a disc diffusion test.[17] Drug monodiscs (HiMedia Laboratories) *viz.,* ampicillin (10.00 µg), amoxicillin/clavulanic acid (20.00/10.00 µg), ampicillin/sulbactam (10.00/10.00 µg), ceftriaxone (30.00 µg), cefoperazone (75.00 µg), imipenem (10.00 µg), gentamicin (10.00 µg), amikacin (30.00 µg), tetracycline (30.00 µg), ciprofloxacin (5.00 µg), levofloxacin (5.00 µg) and chloramphenicol (30.00 µg) were used in the present antibiogram study. Resultant zones of inhibition were graded as sensitive, intermediate or resistant according to zone size interpretative chart provided along with monodiscs.

Molecular detection of virulence genes. Two important virulence genes, *wabG* and *kfuBC*, were investigated by PCR using respective specific primers (Table 1). The PCR program was comprised of initial denaturation at 94.00 ˚C for 5 min, 35 cycles of denaturation at 94.00 ˚C for 30 sec, annealing at 59.00 ˚C (for *kfuBC*) or 57.00 ˚C (for *wabG*) for 1 min, extension at 72.00 ˚C for 90 sec and then, a final elongation at 72.00 ˚C for 10 min. The PCR products were separated and visualised.

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

Target gene	Primer sequences (5′ to 3′)	Amplicon size (bp)	References
gyrA	F: CGC GTA CTA TAC GCC ATG AAC GTA R: ACC GTT GAT CAC TTC GGT CAG G	441	18
rpoB	F: CAA CGG TGT GGT TAC TGA CG R: TCT ACG AAG TGG CCG TTT TC	108	19
kfuBC	F: GAA GTG ACG CTG TTT CTG GC R: TTT CGT GTG GCC AGT GAC TC	797	20
wabG	F: CGG ACT GGC AGA TCC ATA TC R: ACC ATC GGC CAT TTG ATA GA	683	
***bla*$_{\text{CTX-M-1}}$**	F: TTA GGA ATG ATG CCG CTG CA R: CGA TAT CGT TGG TGG TAC CAT	688	21
***bla*$_{\text{CTX-M-9}}$**	F: TCA AGC CTG CCG ATC TGG T R: TGA TTC TCG CCG CTG AAG	561	
***bla*$_{\text{OXA-1}}$**	F: GGC ACC AGA TTC AAC TTT CAA G R: GAC CCC AAG TTT CCT GTA AGT G	564	
***bla*$_{\text{TEM}}$**	F: CAT TTC CGT GTC GCC CTT ATT C R: CGT TCA TCC ATA GTT GCC TGA C	800	
***bla*$_{\text{SHV}}$**	F: AGC CGC TTG AGC AAA TTA AAC R: ATC CCG CAG ATA AAT CAC CAC	713	

Phenotypic identification of ESBL producing *K. pneumoniae.* All the confirmed isolates of *K. pneumoniae* were subjected to phenotypic confirmation for ESBL production using following two methods:

Combination disc diffusion test (CDDT). In this experiment, zones of inhibition for the antibiotic discs of ceftazidime (30.00 µg) and cefotaxime (30.00 µg) were compared to the corresponding discs with ceftazidime/ clavulanic acid (30.00/10.00 µg) and cefotaxime/ clavulanic acid (30.00/10.00 µg), respectively. After overnight incubation at 37.00 ˚C, an increase of 50.00% or 5.00 mm or more in the zone of inhibition in disc containing the antibiotic drug along with clavulanic acid compared to the disc containing the drug alone was considered indicative of ESBL production.[22]

HiCrome ESBL agar method. In this experiment, zone of inhibition using HiCrome ESBL agar (HiMedia Laboratories), chromogenic screening medium for the selective isolation of ESBL producing organisms, was used for phenotypic characterisation of isolates. The luxurious growth with bluish green colonies was considered to be positive indicating ESBL production by *K. pneumoniae*.

Molecular confirmation of ESBL producing *K. pneumoniae.* Molecular confirmation of ESBL production by *K. pneumoniae* isolates was carried out by detecting genes *viz., bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{OXA-1}}$. The PCR was carried out in Mastercycler Nexus Gradient (Eppendorf) in following steps: Initial denaturation at 94.00 ˚C for 10 min followed by 30 cycles of denaturation at 94.00 ˚C for 40 sec, annealing at 60.00 ˚C for 40 sec, extension at 72.00 ˚C for 1 min and a final extension at 72.00 ˚C for 7 min. The PCR products were separated and visualised.

Statistical analysis. The prevalence results of *K. pneumoniae* obtained for the raw milk and mastitic milk samples were analyzed statistically using Pearson's chi-square method (SPSS version 20.0; IBM Corp., Armonk, USA). For test of significance, the *p* value was considered to be 0.05 or less. Similarly, prevalence results obtained for cattle and buffaloes in the present study were analyzed statistically using the same test.

Results

Out of 325 milk samples, 27 isolates of *Klebsiella* spp. were recovered based on cultural characteristics of bacterial growth on MCA, and EMB and HiCrome *Klebsiella* selective agars (Table 2). All of 27 presumptive *Klebsiella* spp. isolates were catalase-positive and oxidase-negative, and showed typical IMViC pattern (- - + +) of urease-positive. The triple sugar iron test showed acid production in slant and butt resulting in a yellow colour slant with gas production. All the isolates grew only along the line of inoculation in motility test, indicating non-motile organisms. In capsule staining, capsules were clearly observed under a microscope (Nikon, Tokyo, Japan) in all the isolates. All of 27 isolates were successfully amplified 441 bp amplicon as well as 108 bp amplicon (Fig. 1) from the genomic DNA, confirming both genus and species as *K. pneumoniae*. Number of confirmed isolates was 17/195 in raw milk and 10/130 in mastitic milk samples.

Table 2. Incidence of *Klebsiella* spp. in dairy cattle and buffaloes milk samples from the study region.

Type of sample	Animals	Tested samples	Positive samples
Raw milk	Dairy cattle	117	12 (10.25%)
	Buffaloes	78	5 (6.41%)
	Total	195	17 (8.71%)
Mastitic milk	Dairy cattle	83	6 (7.22%)
	Buffaloes	47	4 (8.51%)
	Total	130	10 (7.69%)

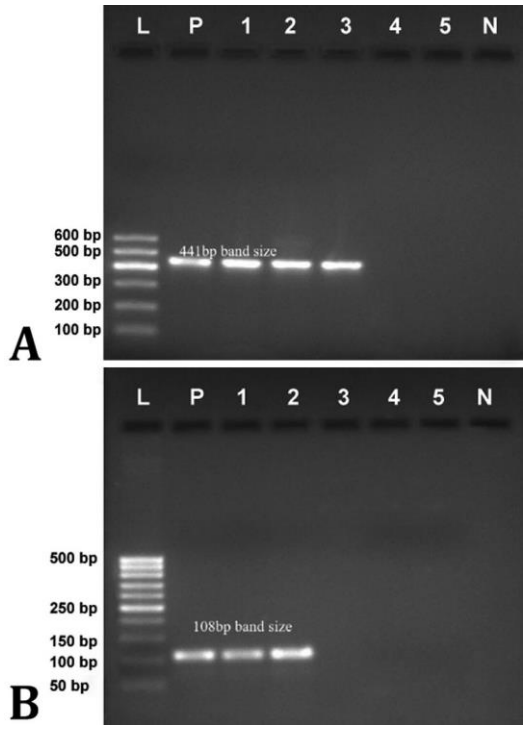

Fig. 1. Polymerase chain reaction amplification of **A)** *gyrA* (441 bp) gene of *Klebsiella* spp., Lanes 1-3: Positive isolates; Lanes 4-5: Blank; Lane P: Positive control; Lane N: Negative control; Lane L: 100 bp DNA marker; **B)** *rpoB* (108 bp) gene of *K. pneumonia*, Lanes 1-2: Positive isolates; Lanes 3-5: Blank; Lane P: Positive control; Lane N: Negative control; Lane L: 50 bp DNA marker.

Graphical representations of antibiotic sensitivity patterns of 12 anti-microbial drugs against 27 *K. pneumoniae* isolates are presented in Figure 2. All of 27 *K. pneumoniae* isolates were resistant to amoxicillin/clavulanic acid (100%) followed by ampicillin (92.59%), ceftriaxone (66.67%) and amikacin (48.15%). The highest sensitivity was recorded for levofloxacin (85.19%) followed by chloramphenicol and tetracycline (77.78%) each, ampicillin/sulbactam (74.08%), imipenem and gentamicin (55.56%) each, cefoperazone (48.15%) and ciprofloxacin (44.45%). The *wabG* genes were detected in 25/27 (92.59%) isolates; whereas, *kfuBC* gene was found in 5/27 (18.51%) isolates (Fig. 3). All five isolates positive for *kfuBC*, were also found to be positive for *wabG* genes. However, in 20 isolates, only *wabG* genes were detected.

Out of 27 isolates of *K. pneumoniae*, 25 isolates (92.59%) and 20 isolates (74.07%) were found to be ESBL producers determined by HiCrome ESBL agar method and CDDT, respectively. Further, results for the detection of ESBL producing genes showed that only 2/27 (7.40%) isolates were found to be positive for $bla_{\text{CTX-M-1}}$ and 3/27 (11.11%) isolates were found to be positive for bla_{TEM}; while, maximum isolates, *i.e.*, 23/27 (85.18%) were found to be positive for bla_{SHV} genes (Fig. 4). No isolate was found to be positive for $bla_{\text{CTX-M-9}}$ and $bla_{\text{OXA-1}}$ genes. Detection of ESBL producing genes in *K. pneumoniae* isolated from bovine raw and mastitic milk samples is compared in Table 3.

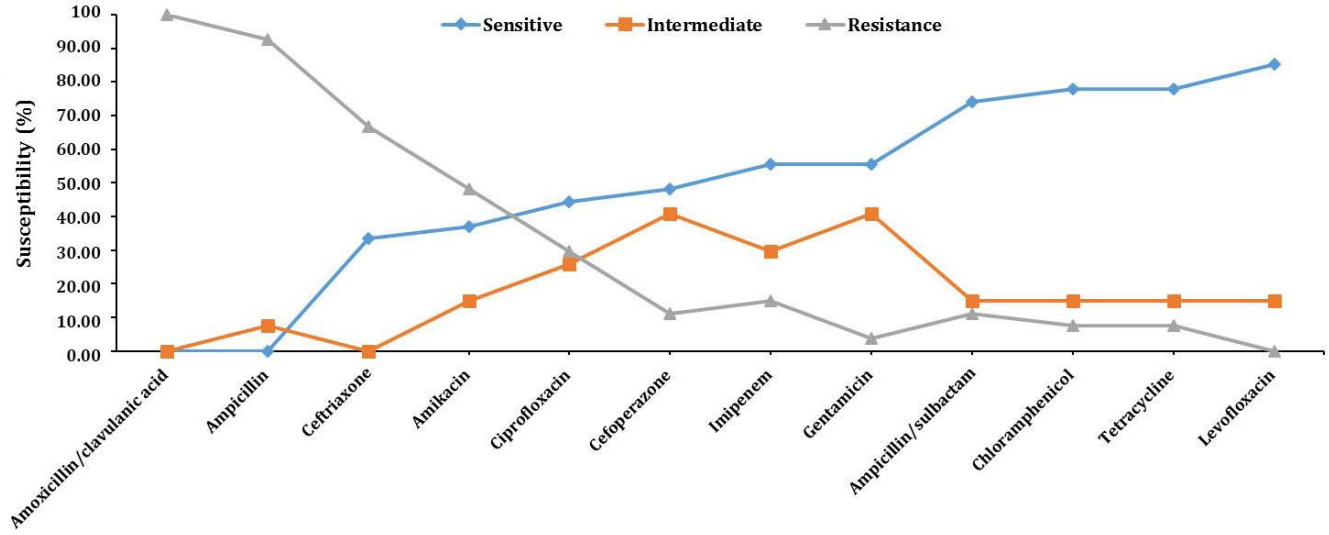

Fig. 2. Antibiogram of *Klebsiella pneumoniae* isolates (n = 27).

Table 3. Detection of extended spectrum β-lactamase (ESBL) producing genes in *Klebsiella pneumoniae* isolated from bovine milk samples.

Type of sample	Number of positive samples		Number of ESBL genes		
	Combination disc diffusion test	ESBL agar	bla_{TEM}	bla_{SHV}	$bla_{\text{CTX-M-1}}$
Raw milk	12	16	1	14	0
Mastitic milk	8	9	2	9	2
Total	20 (74.07%)	25 (92.59%)	3 (11.11%)	23 (85.18%)	2 (7.40%)

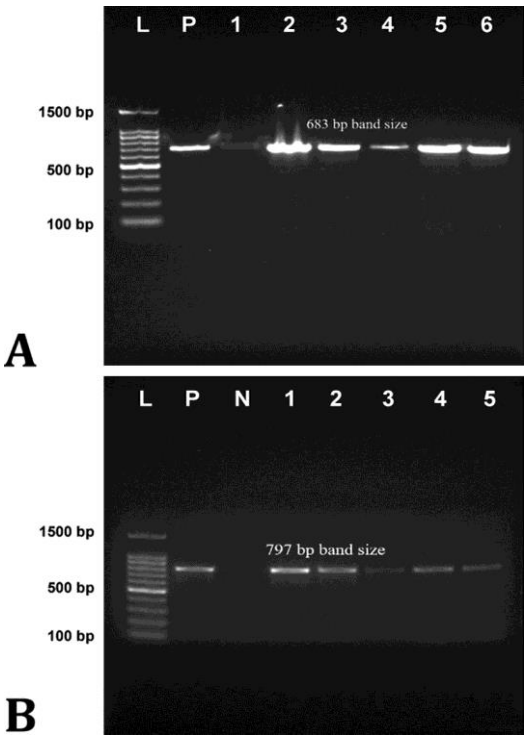

Fig. 3. Polymerase chain reaction amplification of **A)** *wabG* (683bp) gene, Lanes 1 - 6: Positive isolates; Lane P: Positive control; Lane L: 100 bp plus DNA marker; **B)** *kfuBC* (797 bp) gene, Lanes 1 - 5: Positive isolates; Lane P: Positive control; Lane N: Negative control; Lane L: 100 bp plus DNA marker.

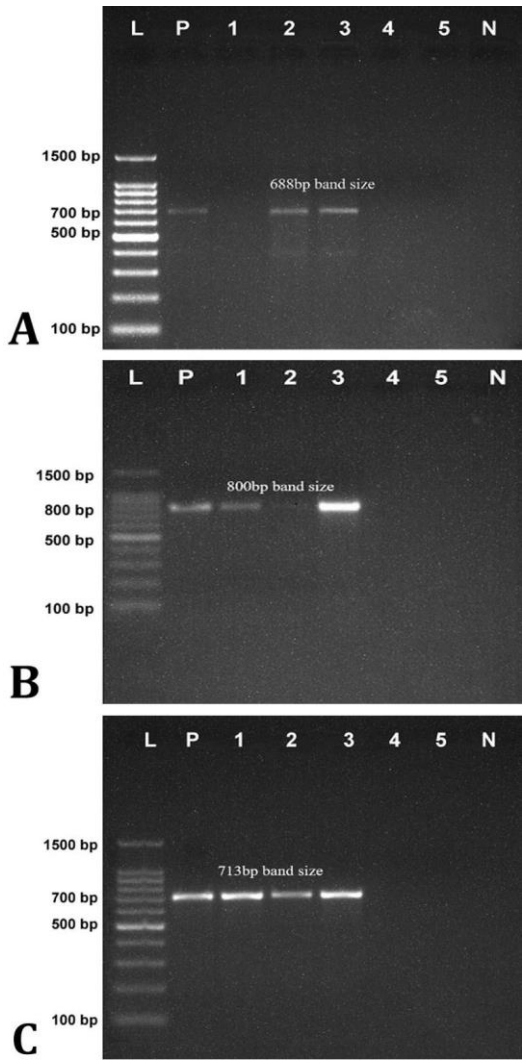

Fig. 4. Polymerase chain reaction amplification of **A)** *bla*$_{\text{CTX-M-1}}$ (688 bp) gene, Lanes 2 - 3: Positive isolates; Lanes 1, 4 and 5: Negative isolates; Lane P: Positive control; Lane N: Negative control; Lane L: 100 bp plus DNA marker; **B)** *bla*$_{\text{TEM}}$ (800 bp) gene, Lanes 1- 3: Positive isolates; Lane P: Positive control; Lanes 4 - 5: Negative isolates; Lane N: Negative control; Lane L: 100 bp plus DNA marker; **C)** *bla*$_{\text{SHV}}$ (713 bp) gene, Lanes 1 - 3: Positive isolates; Lane P: Positive control; Lanes 2 and 5: Negative isolates; Lane N: Negative control; Lane L: 100 bp plus DNA marker.

Discussion

Klebsiella pneumoniae forms one of the important pathogens transmitted through milk and milk products[23] which may cause urinary tract infections, pneumonia, septicaemia and pyogenic liver abscesses in human.[24]

In this study, 17/195 raw milk (8.71%) and 10/130 mastitic milk (7.69%) samples were detected positive for *K. pneumoniae*. This was an interesting finding indicating that not only mastitic milk but also raw milk from apparently healthy animal could be a potential source of zoonotic transmission of *K. pneumoniae*. Thus, the issue related to practice of raw milk consumption in many parts of south-east Asia needs to be properly addressed in the view of public health significance. A similar kind of prevalence, *i.e.,* 9.60 and 5.40% in raw milk were recorded for cows and buffaloes in and around Cairo, Egypt, respectively.[25] However, Enferad and Mahdavi[26] reported high presence (40.00%) of *K. pneumoniae* in raw cow milk samples collected from north-east of Iran. Recent studies by Yang *et al.*[27] and Wu *et al.*[28] showed comparable isolation rates of *K. pneumoniae* in raw and mastitic cow milk samples as 7.83 and 25.36%, respectively. Badri *et al.*[29] also detected higher prevalence of 51.42% (36/70) in raw milk samples of cows in Sudan. In the present study, there was no significant difference in the prevalence of *K. pneumoniae* in cow and buffalo, for both raw and clinical mastitic milk samples. The prevalence of *K. pneumoniae* reported in clinical mastitic milk samples from cow and buffalo was 14.60 and 4.20%, respectively,[25] the corresponding values for the present study were 7.22 and 8.51%, respectively, showing variation in prevalence in different geographical areas. The variation in the prevalence could be attributed to the nature of the local livestock rearing conditions like soil, vegetation, water[30] and practices which may affect the environment; certain type of bedding material affects the multiplication of *K. pneumoniae,* as fecal shedding of this organism is the major source of contamination.[3]

The PCR-mediated amplification of genus-specific and species-specific *16S rRNA* gene is a sensitive, specific and rapid method for the confirmation of *K. pneumoniae* and effectively distinguishes this pathogen from the closely related bacteria.[31] DNA gyrase, comprising two A subunits and two B subunits, is encoded by the *gyrA* and *gyrB* genes. The *gyrA* encodes the A subunit of DNA gyrase which is the primary target of fluoroquinolones in *Klebsiella*. This gene is utilized to detect the *Klebsiella* genus.[32] Members of the *K. pneumoniae* species complex have been differentiated using *gyrA* sequences assigning them as phylogroups within *K. pneumoniae*.[33] In a broader context, the gene tree constructed based on *rpoB* aligns more effectively with the established classification of *Klebsiella* compared to the trees based on *16S rRNA* gene. This underscores the importance of *rpoB* as a potent tool for identifying *K. pneumoniae* isolates.[34] Therefore, PCR methods targeting *gyrA* and *rpoB* genes were used to detect *Klebsiella* spp. and *K. pneumoniae* pathogens in the present investigation. The same method of genotypic confirmation using the similar primers was also reported by Koovapra *et al*.[13] and Razmyar and Zamani.[35] The PCR outcomes confirmed the results of cultural, staining and biochemical tests employed in the present study.

In order to prevent infection in animals, veterinarians are more concerned about anti-microbial resistance in Gram-negative opportunistic pathogens including *K. pneumoniae* since these bacteria are more likely to share resistance determinants with one another.[36] The anti-biogram of present study graded aminopenicillins like amoxicillin/clavulanic acid and ampicillin to be the most resistant anti-bacterial agents. Similarly, Chehabi *et al*.[37] and Yang *et al*.[27] also reported high resistance against ampicillin. The highest sensitivity was recorded for levo-floxacin (85.19%), followed by chloramphenicol (77.78%). These results also correlated with use/misuse level of these anti-bacterial agents in dairy animals of the study area, *i.e.,* amino-penicillin was used mostly in mastitic cases and levofloxacin and chloramphenicol were not used in food producing animals. The findings of this research would certainly help large animal veterinarians to select the proper antibiotics to overcome the multi-drug resistant problem of the bacteria in the study region.

Two virulence genes of *K. pneumoniae* detected in the present study *viz., wabG* and *kfuBC* were involved in endotoxin biosynthesis and iron acquisition system for bacterial evasion, respectively. Virulence genes associated with endotoxin production and iron uptake may play important roles in the pathogenesis of *K. pneumoniae*.[28] In the present study, detection of *wabG* gene was more common (92.59%) than that of *kfuBC* gene (18.51%). The 74.07% of confirmed isolates of *K. pneumoniae* only had *wabG* and not *kfuBC*. The importance of *wabG* gene may be appreciated by its higher detection rate in *K. pneumoniae* isolates in many studies like in cattle respiratory infection (91.00%) by Cheng *et al*.,[38] in food samples (77.40%) by Zhang *et al*.,[39] and in dairy set-up including cows (over 85.00%) by Wu *et al*.[28] The wide distribution of virulence gene in high frequency among variety of samples related to dairy cattle and food products signifies not only its pathogenic value but also a greater risk to public health.

Remya *et al.*[40] have suggested that *K. pneumoniae* that is pathogenic in nature has a variety of virulence genes; however, an invasive infection of *K. pneumoniae* carrying even a single virulence gene may result in poor clinical outcome. In the same study, frequency of virulence genes varied in different clinical samples like blood, urine, exudates and respiratory secretions. In the present study, the samples were raw and mastitic milk samples and it might be one of the reasons for low frequency of *kfuBC* gene. Urine isolates of *K. pneumoniae* had been found to have high frequency (81.80%) of *kfuBC* gene;[7] whereas, in food samples including those from animal-origin like meat, the frequency of *kfuBC* gene in isolates of *K. pneumoniae* was reported to be 29.00% in China.[39] In contrast to our study, mastitis milk samples from Egypt showed high frequency (77.78%) of *kfuBC* gene; however, only 9 samples were screened for presence of virulence genes out of 45 recovered isolates of *K. pneumoniae* in that study.[25] In another study from China, Cheng *et al*.[41] also found high frequency of *kfu* genes in clinical (54.26%) as well as sub-clinical (72.72%) mastitic milk samples. They stated that there was no difference in the distribution of virulence genes across isolates from occurrences of mild, moderate and severe clinical mastitis, suggesting that virulence genes had a small influence in mastitis severity.

The phenotypic conventional methods employed to detect ESBLs included HiCrome ESBL agar method and CDDT. In this study, out of 27 isolates, 25 were detected as ESBL producers by agar method; whereas, 20 were detected as ESBL producers by CDDT, yielding a prevalence of 92.59 and 74.07%, respectively. These results were consistent with the CDDT finding (72.20%) of Badri *et al*.;[29] however, very low incidence rate of 10.90% was recorded by CDDT in another study.[13] Thus, HiCrome ESBL agar was found more sensitive than CDDT to detect ESBLs producers. However, both methods were not able to distinguish between the specific enzymes responsible for ESBL production, necessitating the genotypic characterization of ESBL producing *K. pneumoniae*. Furthermore, molecular detection of ESBL producing genes would be an essential epidemiological tool to characterize patterns of anti-microbial resistance as well as to exclude the possibility of biochemical mechanisms of resistance other than involvement of enzymes. In the present study, 23 out of 27 isolates (85.18%) were found to have at least one of three resistance genes, *i.e., bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$; whereas, no isolate was found to be positive for two resistance genes *viz., bla*$_{\text{CTX-M-9}}$ and *bla*$_{\text{OXA-1}}$. Among raw and mastitic milk isolates, 14/17 (82.35%) and 9/10 (90.00%) isolates

were found to be positive for at least one of the three resistant genes, respectively, with *bla*$_{SHV}$ as the most predominant gene (85.18%). Similarly, Locatelli *et al*.[42] and Sudarwanto *et al*.[43] reported 100% prevalence of *bla*$_{SHV}$ gene in *K. pneumoniae* isolates from bovine milk samples. In contrast, Koovapra *et al*.[13] reported *bla*$_{CTX-M}$ as a predominant gene (82.60%).

High frequencies of resistance genes in ESBL producing *K. pneumoniae* species from both raw and mastitic milk samples signified that these pathogens might act as a potential source of these genes transfer within and between the human and animal populations. Moreover, persistence of such pathogens in udder or milk results into the failure of therapeutic regimen of intra-mammary infection. Total 3/27 (11.11%) isolates were found to harbour two ESBL genes simultaneously, *i.e.*, *bla*$_{SHV}$ and *bla*$_{TEM}$ (n = 2) and *bla*$_{SHV}$ and *bla*$_{CTX-M-1}$ (n = 1); whereas, only one isolate (3.70%) harboured all three genes. Since no conclusive pattern was found between the ESBL producing isolates of *K. pneumoniae* and the virulence genes *viz*., *wabG* and *kfuBC* detected in those isolates, association between the virulence genes and ESBL producing *K. pneumoniae* could not be established in the present study.

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Conflict of interest

The authors declare no competing interest.

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