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PREVALENCE AND MOLECULAR CHARACTERIZATION OF BLACTX-M-1, BLATEM, AND BLASHV-1 ESBL-PRODUCING KLEBSIELLA PNEUMONIAE IN BOVINE MASTITIS

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ABSTRACT

Background: Bovine mastitis is one of the most significant diseases affecting dairy animals worldwide, leading to considerable economic losses due to reduced milk production and treatment costs. This study aimed to determine the prevalence of ESBL-producing *Klebsiella pneumoniae* isolated from mastitic milk samples and to detect the presence of β-lactamase genes (blaTEM, blaSHV-1, and blaCTX-M-1) in isolates from District Mardan, Khyber Pakhtunkhwa, Pakistan.

Methods: *Klebsiella pneumoniae* was isolated through culture and identified using standard biochemical tests (lactose fermentation test, urease, Indole ring and Simmon Citrate), followed by confirmation via PCR. A total of 46 multidrug-resistant (MDR) *K. pneumoniae* isolates were subjected to antimicrobial susceptibility testing. From these, 6 randomly selected isolates were further analyzed for molecular detection of β-lactamase genes. PCR analysis revealed the presence of blaCTX-M-1, blaTEM, and blaSHV-1 genes, and subsequent sequencing confirmed these resistance determinants.

Results: No significant difference in prevalence was observed between species (p=0.47). Among these isolates, 29.9% (46/154) were confirmed as Extended-Spectrum beta-Lactamase (ESBL) producers. Molecular characterization of representative isolates via PCR detected blaTEM, blaSHV-1, and blaCTX-M-1 in equal proportions (33.3% each). Antibiogram analysis revealed high resistance to cefotaxime (100%), amoxicillin (86.9%), and ceftriaxone (82.6%), though resistance to chloramphenicol and amoxicillin–clavulanic acid remained low. Logistic regression identified previous antibiotic usage as a significant risk factor (AOR = 2.27, p = 0.037$), indicating that a history of treatment significantly increases the likelihood of harboring ESBL-producing *K. pneumoniae*.

Conclusion: The present study demonstrates the presence of ESBL-producing *Klebsiella pneumoniae* harboring blaCTX-M-1, blaTEM, and blaSHV-1genes in mastitic milk samples from dairy animals in Mardan. Molecular analysis revealed mutation in bla TEM gene at D10 E at sample 9; E3K at 20 and 34; F131and F22Y at 41; and D10E at samples 47 respectively, while a single nucleotide mutation was observed at bla SHV-1 in sample 9 at M42L. The high prevalence of antimicrobial resistance highlights the urgent need for rational antibiotic use, improved mastitis management practices, and continuous molecular surveillance of antimicrobial resistance in dairy farms to minimize the spread of ESBL-producing pathogens.

KEYWORDS: *Klebsiella pneumoniae*, mastitis, ESBL, blaCTX-M-1, blaTEM, blaSHV-1, antimicrobial resistance, dairy cattle.

INTRODUCTION

Mastitis is one of the most prevalent and costly infectious diseases affecting dairy animals worldwide. It causes significant loss to the dairy industry through reduced milk production, increased veterinary costs, discarded milk, and premature culling of affected animals (1, 2). The disease can occur in both clinical and subclinical forms and is commonly caused by a wide range of bacterial pathogens. Among these pathogens, members of the family Enterobacteriaceae, particularly *Klebsiella pneumoniae*, have emerged as important environmental mastitis-causing agents in dairy herds (3).

In recent years, the emergence and rapid spread of antimicrobial resistance among mastitis pathogens have become a major concern for both veterinary and public health sectors. One of the most important mechanisms of resistance in Gram-negative bacteria is the production of extended-spectrum β-lactamases (ESBLs) (4). These enzymes are capable of hydrolyzing a wide range of β-lactam antibiotics, including penicillins, third-generation cephalosporins, and monobactams, thereby reducing the effectiveness of commonly used antimicrobial agents.

ESBL-producing bacteria are particularly challenging because they often carry additional resistance genes, resulting in multidrug-resistant strains that limit therapeutic options (5-8).

Among the ESBL genes, blaCTX-M-1, blaTEM, and bla-SHV-1 are the most frequently detected determinants of antimicrobial resistance. Several molecular studies have shown that blaCTX-M-1genes are the most prevalent, with detection rates ranging from 45% to 65%, followed by blaTEM (30–50%) and blaSHV-1(20–40%) among ESBL-producing *K. pneumoniae* isolates. The increasing presence of these resistance genes in dairy-associated pathogens highlights the potential risk of antimicrobial resistance transmission between animals, the environment, and humans through the food chain (9).

The occurrence of ESBL-producing *Klebsiella pneumoniae* in dairy animals suffering from mastitis has been increasingly reported in several countries. The misuse and overuse of antibiotics in veterinary practice, particularly for the treatment of mastitis, are considered major cause of antimicrobial resistance. In developing countries, including Pakistan, antibiotics are frequently used without proper veterinary supervision, which may accelerate the emergence and spread of resistant bacterial strains in dairy farms (10, 11). Despite the growing concern regarding antimicrobial resistance, limited information is available on the molecular epidemiology of ESBL-producing *K. pneumoniae* in bovine mastitis cases in many regions of Pakistan, particularly in the Khyber Pakhtunkhwa province. Mardan district is an important livestock-producing area in Khyber Pakhtunkhwa, where dairy farming plays a significant role in the livelihood of rural communities. However, systematic investigations on the prevalence of ESBL-producing bacteria and the distribution of resistance genes among mastitis pathogens in this region remain scarce. Understanding the molecular mechanisms of antimicrobial resistance in mastitis-associated pathogens is essential for improving disease management strategies and guiding rational antimicrobial use in dairy production systems. Therefore, the present study was conducted to determine the prevalence of ESBL-producing *Klebsiella pneumoniae* isolated from mastitic milk samples of cows and buffaloes and to detect molecular identification of their resistance genes related to bovine mastitis in dairy farms at District Mardan, Pakistan**.**

MATERIALS AND METHODS

Study Area

The present study was conducted in District Mardan, located in the Khyber Pakhtunkhwa (KPK) Province of Pakistan.

Study Population

The study population consisted of lactating dairy cows and buffaloes located in and around District Mardan.

Sample Size and Sampling Procedure

Klebsiella pneumoniae isolates were obtained through culture and identified using standard biochemical tests (lactose fermentation test, urease test, Indole ring and Simmon Citrate), followed by confirmation via PCR. Total of 46 multidrug-resistant (MDR) *K. pneumoniae* isolates were subjected to antimicrobial susceptibility testing. From these, 6 randomly selected isolates were further analyzed for molecular detection of β-lactamase genes. PCR results revealed the presence of blaCTX-M-1, blaTEM, and blaSHV-1 genes. Subsequent sequencing of these genes confirmed their identity and demonstrated the presence of ESBL-associated resistance determinants in the analyzed isolates.

Detection of Clinical and Subclinical Mastitis

Clinical mastitis was identified through physical examination of the udder and milk, whereas subclinical mastitis was detected using the California Mastitis Test (CMT) as described by Deka *et al,.* (2020).

Bacterial Culture and Biochemical Identification

Samples were cultured on Muller Hinton Agar (MHA) plates and incubated at 37°C for 24 hours for bacterial growth. Preliminary identification of bacterial isolates was performed based on colony morphology, pigmentation, hemolytic characteristics, Gram staining, bacterial morphology, and cellular arrangement. Further identification was carried out by subculturing isolates on MacConkey agar (Merck, Darmstadt, Germany) for selective growth. Identification of *K. pneumoniae* was confirmed through biochemical tests including lactose fermentation (positive), urease test (positive), indole test (negative), and Simmons citrate test (positive).

Triple Sugar Iron (TSI) Test

For biochemical confirmation, isolates were cultured on MacConkey agar and incubated overnight at 37°C. A well-isolated colony was inoculated into Triple Sugar Iron (TSI) agar slants using the stab and streak method and incubated at 37°C for 24 hours. Results were interpreted based on gas production, hydrogen sulfide (H_2S) production, and color changes in the butt and slant of the agar medium.

RapID™ ONE System Identification

The RapID™ ONE System (Remel, Kansas, USA) was used as a rapid micro-method for bacterial identification. ESBL-producing isolates were subcultured on MacConkey agar plates and incubated at 37°C for 24 hours. Bacterial growth was suspended in RapID™ inoculation fluid until a turbidity equivalent to 2 McFarland standard was achieved. The suspension was inoculated into RapID™ ONE panels according to the manufacturer's instructions and incubated aerobically at 37°C for 4 hours. After incubation, RapID™ reagents were added to the cavities, and reactions were recorded. Identification results were determined using the ERIC® (Electronic RapID Compendium) system.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of all identified *K.pneumoniae* isolates was performed using the Kirby–Bauer disk diffusion method on Muller Hinton Agar (MHA) following Clinical and Laboratory Standards Institute (CLSI) guidelines (2017). Bacterial colonies were inoculated into peptone water and incubated for 4–6 hours at 37°C. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard. The inoculum was evenly spread onto MHA plates, and antibiotic discs were placed aseptically on the surface of the medium. The plates were incubated at 37°C for 24 hours, and zones of inhibition were measured. Antibiotics tested included gentamicin, ciprofloxacin, norfloxacin, enrofloxacin, cefotaxime, cefotaxime–clavulanic acid, tobramycin, cephradine, ceftriaxone, amoxicillin, erythromycin, kanamycin, doxycycline, chloramphenicol, lincomycin, and amoxicillin–clavulanic acid. The results were interpreted as sensitive, intermediate, or resistant according to CLSI guidelines.

Identification of Multidrug Resistance

Multidrug resistance (MDR) was defined as resistance to at least three different classes of antibiotics. MDR isolates were identified by analyzing the antibiotic resistance profiles of each bacterial isolate.

Multiple Antibiotic Resistance (MAR) Index

The Multiple Antibiotic Resistance (MAR) index was calculated using the formula:

MAR = a / b where a represents the number of antibiotics to which the isolate showed resistance, and b represents the total number of antibiotics tested. A MAR index greater than 0.2 indicates that the bacterial isolates originated from environments with high antibiotic exposure.

Phenotypic Detection of ESBL-Producing Isolates

Phenotypic detection of ESBL production was carried out using the Combination Disk Diffusion Test according to CLSI (2017) guidelines. Cefotaxime (30 µg) and cefotaxime combined with clavulanic acid (30/10 µg) discs were placed on Muller Hinton agar plates inoculated with bacterial cultures. Plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured. Isolates were considered ESBL-positive when the inhibition zone around the combination disc was ≥5 mm larger than that of cefotaxime alone.

DNA Extraction

Genomic DNA was extracted using the Phenol–Chloroform extraction method described by Sambrook (1989). A single colony from MacConkey agar was inoculated into 3 mL of Tryptic Soy Broth (TSB) and incubated overnight at 37°C. The culture was centrifuged at 10,000 rpm for 10 minutes, and the pellet was resuspended in Tris buffer. The cells were lysed using Sodium Dodecyl Sulfate (SDS) and incubated at 65°C for two hours. The lysate was treated with phenol–chloroform–isoamyl alcohol (25:25:1) and centrifuged. The aqueous phase was collected, and DNA was precipitated using isopropanol, washed with 70% ethanol, air-dried, and resuspended in Tris buffer. DNA quality was assessed using 1% agarose gel electrophoresis, and samples were stored at −20°C for use as a PCR template.

Data Management and Statistical Analysis

Data obtained from farmers and laboratory analyses were entered into Microsoft Excel 2013. Relevant risk factors associated with the emergence of multidrug-resistant *K. pneumoniae* were compiled and exported to the Statistical Package for Social Sciences (SPSS) for analysis. Descriptive statistics including frequencies and percentages were used for categorical variables, while mean ± standard deviation was used for continuous variables. Logistic regression analysis was conducted to evaluate associations between subclinical mastitis, risk factors, daily milk production, and CMT results. Additionally, an independent sample t-test was used to compare the mean levels of between CMT-positive and CMT-negative groups. A p-value less than 0.05 was considered statistically significant.

RESULTS

The data indicate that *Klebsiella spp.* were isolated from a total of 154 cases, representing 9.74% of all samples analyzed, with a slightly higher occurrence in buffalo (80 isolates, 51.9%) compared to cows (74 isolates, 48.1%). This suggests that while the distribution between the two animal groups is relatively balanced, buffalo may have a marginally greater susceptibility or exposure to *Klebsiella* infections in the studied population.

Table 2. Prevalence of bacterial strains isolated from mastitis milk samples

Bacterial Isolate	Cows (n)	Cows (%)	Buffalo (n)	Buffalo (%)	Total (n)	Total (%)
Klebsiella spp.	74	48.1	80	51.9	154	9.74

These primer sets enabled the specific amplification and molecular identification of ESBL-associated resistance genes in the selected *K. pneumoniae* isolates.

Table 1. Oligonucleotide primers used for detection of β-lactamase genes

Gene	Primer Sequence (5′–3′)	TM (°C)	GeneBank Accession	Expected Amplicon Size
blaCTX-M1	F:ATGTGCAGYACCAGTAA R: ACCAGAAYVAGCGGBGC	55°C	DQ303459	585 bp

blaSHV-1	F: AAGCGAAAGCCAGCTGTCG R: TTCGCTCCAGCTGTTCGTC	55°C	EF125011	178 bp
blaTEM	F: TCCGCTCATGAGACAATAACC R:ATAATACCGCACCACATAGCAG	58°C	AB282997	296 bp

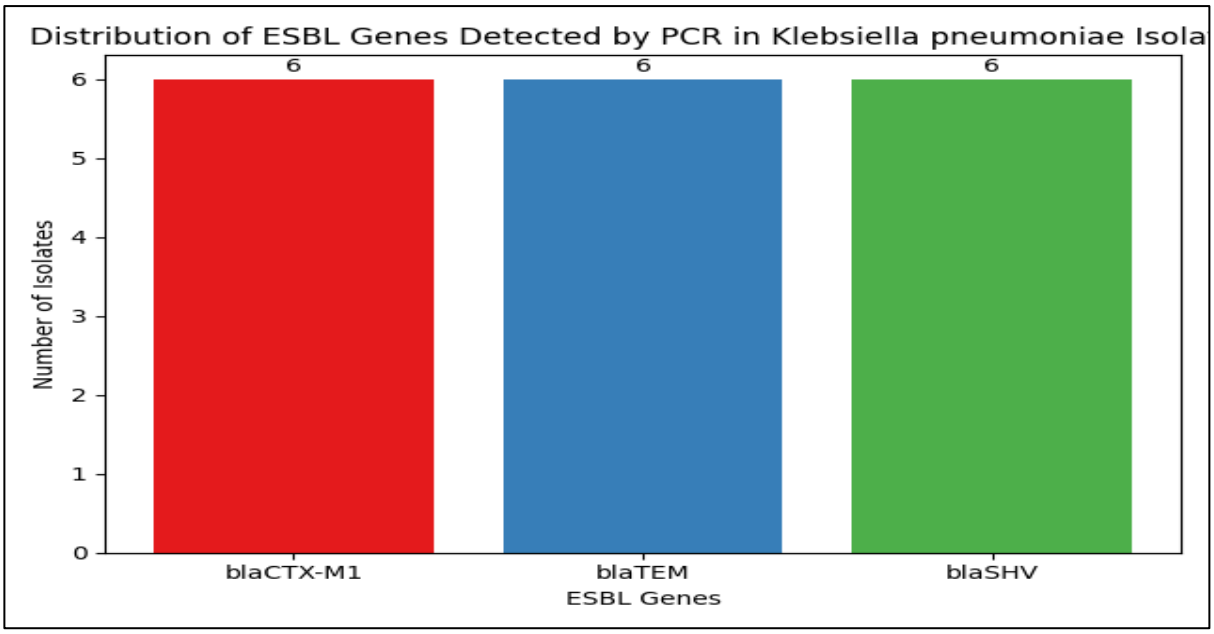

Figure 1. Frequency of ESBL-producing genes among *Klebsiella pneumoniae* isolates.

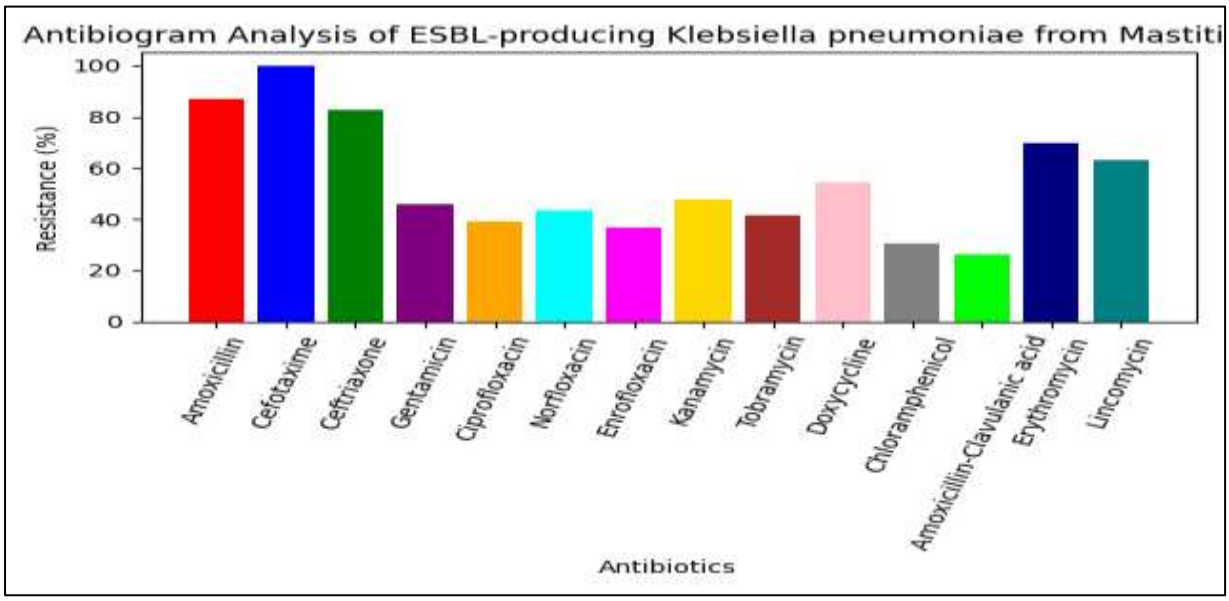

Figure 2. Antibiogram analysis of tested antibiotics against ESBL-producing *Klebsiella pneumoniae* isolated from mastitis milk.

Among the isolates 46 (29.9%) were confirmed as MDR *K. Pneumoniae* strain comprising 21 isolates from cows and 25 from buffaloes, and this difference was also not statistically significant (p = 0.69). For molecular characterization, 12 representative MDR isolates were selected for molecular analysis. The sequences results revealed that bla CTX-M-1 was most prevalent gene detected in 6 isolates (33.3%), followed by blaTEM in 6 isolates (33.3%) and blaSHV-1in 6 isolates (33.3%). For molecular characterization, 12 representative MDR isolates were selected for molecular analysis. These findings indicate the presence of multiple ESBL-associated resistance genes among *K. pneumoniae* isolates obtained from mastitis cases in the study area.

Table 3. Distribution of *Klebsiella pneumoniae*, ESBL-producing isolates, and detection of β-lactamase genes

Parameter	Cows (n)	Buffaloes (n)	Total (n)	Percentage (%)	P value
Total milk samples examined	884	698	1582	100	–
Klebsiella pneumoniae isolates	74	80	154	9.74	0.47
ESBL-producing *K. pneumoniae*	21	25	46	29.9 (of Klebsiella isolates)	0.69
Isolates selected for PCR analysis	–	–	12	–	–
Detection of β-lactamase genes (PCR)					
blaCTX-M-1	–	–	6	33.3	-
blaTEM	–	–	6	33.3	-
blaSHV-1	–	–	6	33.3	-

The analysis showed that animal species was not significantly associated with ESBL occurrence, as buffaloes had slightly higher odds of ESBL positivity compared with cows (AOR = 1.18, 95% CI: 0.60–2.31, p = 0.63). Similarly, breed type did not show a significant effect, where exotic breeds (Holstein Friesian/Nili Ravi) had nearly similar odds compared with local breeds (Achai/Azakhel/non-descriptive) (AOR = 1.07, 95% CI: 0.54–2.13, p = 0.84). Regarding the stage of lactation, animals in early lactation showed moderately higher odds of ESBL infection (AOR = 1.42, 95% CI: 0.68–2.96, p = 0.34) compared with mid-lactation animals, while late lactation showed slightly lower odds (AOR = 0.92, 95% CI: 0.40–2.10, p = 0.85), although these associations were not statistically significant. Likewise, parity was not significantly associated with ESBL production, as animals with parity ≥3 had slightly higher odds than those with parity ≤ 2 (AOR = 1.36, 95% CI: 0.68–2.74, p = 0.39). However, previous antibiotic usage showed a statistically significant association with ESBL-producing isolates (AOR = 2.27, 95% CI: 1.05–4.90, p = 0.037), indicating that animals with a history of antibiotic treatment were more than twice as likely to harbor ESBL-producing *K. pneumoniae*. The milking system also showed no significant relationship with ESBL occurrence, although animals milked by hand had slightly higher odds compared with machine milking (AOR = 1.52, 95% CI: 0.73–3.16, p = 0.26). Overall, the findings suggest that antibiotic usage history was the most important risk factor associated with ESBL production in *K. pneumoniae* isolates in this study.

Table 4. Multivariable logistic regression analysis of factors associated with ESBL-producing *K. pneumoniae*

Variable	Category	ESBL Positive (n)	ESBL Negative (n)	Adjusted Odds Ratio (AOR)	95% CI	p-value
Animal species	Cow	21	53	1.00 (Reference)	–	–
	Buffalo	25	55	1.18	0.60 – 2.31	0.63
Breed type	Local (Achai/Azakhel/ND)	18	44	1.00 (Reference)	–	–
	Exotic (Holstein Friesian / Nili Ravi)	28	64	1.07	0.54 – 2.13	0.84
Stage of lactation	Early	20	36	1.42	0.68 – 2.96	0.34
	Mid	15	40	1.00 (Reference)	–	–
	Late	11	32	0.92	0.40 – 2.10	0.85
Parity	≤ 2	17	48	1.00 (Reference)	–	–
	≥ 3	29	60	1.36	0.68 – 2.74	0.39
Antibiotic usage history	No	12	48	1.00 (Reference)	–	–
	Yes	34	60	2.27	1.05 – 4.90	**0.037***
Milking system	Hand milking	30	68	1.52	0.73 – 3.16	0.26
	Machine milking	16	40	1.00 (Reference)	–	–

The oligonucleotide primers used for the molecular detection of β-lactamase genes in *K. pneumoniae* isolates were designed to specifically amplify the blaCTX-M-1, blaSHV-1, and blaTEM genes**,** which are commonly associated with extended-spectrum β-lactamase (ESBL) production. The blaCTX-M-1 gene was amplified using primers with sequences ATGTGCAGYACCAGTAA (forward**)** and ACCAGAAYVAGCGGBGC (reverse) at an annealing temperature of 55°C**,** producing an expected amplicon size of 585 bp, corresponding to the GeneBank accession number DQ303459. Similarly, the blaSHV-1 gene was detected using primers AAGCGAAAGCCAGCTGTCG (forward) and TTCGCTCCAGCTGTTCGTC (reverse) with an annealing temperature of 55°C, yielding a 178 bp PCR product associated with GeneBank accession EF125011. A single mutation M42L (Methionine to Leucine) was identified in the SHV-1 gene of Sample 9, indicating comparatively limited variability in SHV among the analyzed isolates. The blaTEM gene was amplified using primers TCCGCTCATGAGACAATAACC (forward) and ATAATACCGCACCACATAGCAG (reverse**)** at an annealing temperature of 58°C, producing an expected 296 bp amplicon corresponding to GeneBank accession AB282997. Sequence analysis of the TEM gene revealed multiple amino-acid substitutions among the studied isolates. These included E3K (Glutamic acid to Lysine) mutations in Samples 20 and 34, D10E (Aspartic acid to Glutamic acid) mutations in Samples 9 and 47, and signal peptide mutations P20R (Proline to Arginine) in Sample 9 and F13I (Phenylalanine to Isoleucine) and F22Y (Phenylalanine to Tyrosine) in Sample 41. These substitutions were identified through sequence alignment and comparison with reference TEM sequences and were primarily located within the signal peptide and N-terminal regions, which may influence protein processing and enzyme expression. Molecular analysis shows mutation in bla TEM gene and bla SHV-1, as shown in figure.

Figure 1bla TEM: sample 9 shows mutation

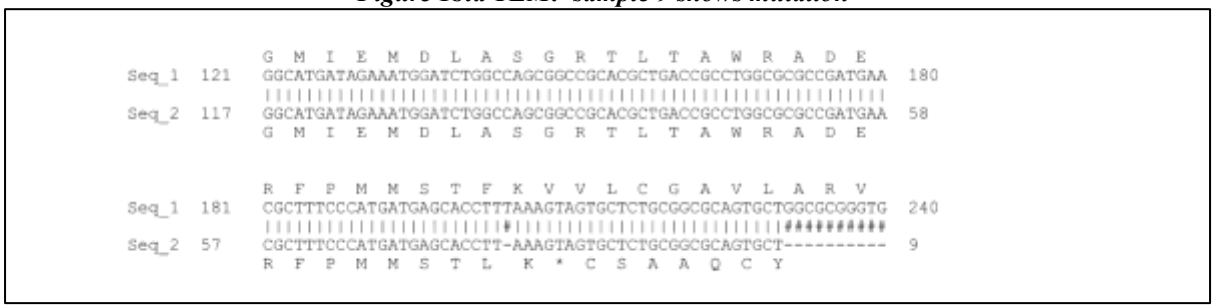

Figure 2 *bla TEM*: sample 47 shows mutation.

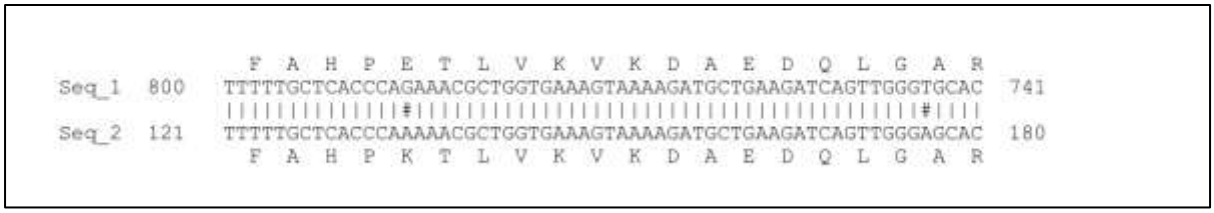

Figure 3 bla TEM: Shows mutation in sample 34.

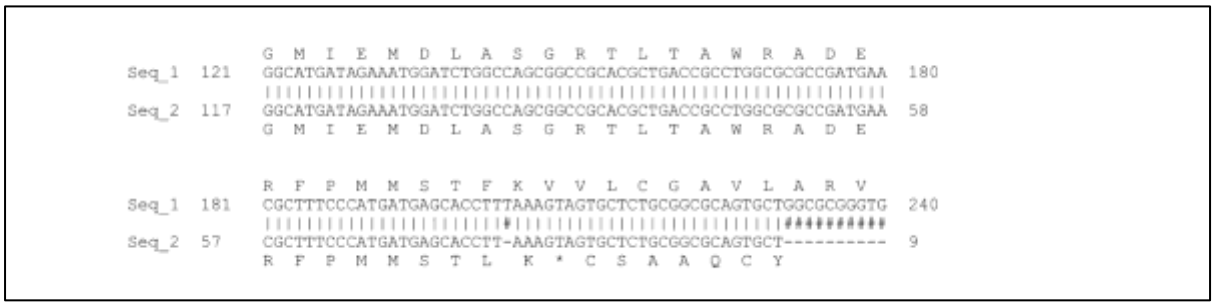

Figure 4 bla TEM: Shows the mutation in sample 47.

Figure 5 bla SHV-1: Shows Mutation in sample 9.

DISCUSSION

Mastitis remains one of the most economically important diseases affecting dairy animals worldwide, leading to significant losses in milk production, treatment costs, and herd management. In the present study, a total of 1,582 milk samples collected from cows and buffaloes were examined for bacterial pathogens associated with mastitis. Among these samples, 154 (9.74%) isolates were identified as *K. pneumoniae*. This finding is consistent with previous studies reporting the prevalence of Klebsiella spp. in mastitic milk ranging from 7% to 15% in dairy cattle. For example, studies conducted in Pakistan and neighboring countries have reported similar prevalence rates of Klebsiella isolates from mastitis cases, with values ranging between 8% and 12%, indicating that *K. pneumoniae* is an important environmental mastitis pathogen in dairy herds. Similarly, studies conducted in India and China reported prevalence values of 9–18%, supporting the increasing role of *K. pneumoniae* in bovine mastitis infections. In the current study, 46 out of 154 *K. pneumoniae* isolates (29.9%) were identified as ESBL-producing bacteria based on phenotypic detection methods (11-16).

This prevalence is comparable with earlier reports that indicated ESBL production in 20–40% of *K. pneumoniae* isolates from dairy animals. Previous studies in veterinary microbiology have also reported similar findings; for instance, ESBL-producing *K. pneumoniae* prevalence ranging from 25% to 35% has been documented in mastitis-associated isolates. The presence of ESBL-producing pathogens in dairy animals is particularly concerning because these bacteria are capable of hydrolyzing a wide range of β-lactam antibiotics, which are commonly used in veterinary medicine for the treatment of mastitis (17-22). The occurrence of ESBL-producing bacteria in dairy farms also represents a potential public health risk due to the possibility of transmission through milk, dairy products, and the farm environment. Molecular analysis of the ESBL-producing isolates in the present study revealed that bla TEM gene have several amino-acid substitutions including E3K (Glutamic acid → Lysine), D10E (Aspartic acid → Glutamic acid), and signal peptide mutations P20R (Proline → Arginine). These substitutions were identified at the N-terminal region of the TEM β-lactamase, which corresponds to the signal peptide (positions 1–23). Mutations in this region are particularly important because they can influence protein secretion, enzyme maturation, and antibiotic resistance phenotypes. The E3K mutation observed in Samples 20 and 34 represents a substitution from a negatively charged glutamic acid to a positively charged lysine. Such charge alterations in the signal peptide region may affect protein translocation efficiency and enzyme stability. Previous studies have demonstrated that mutations within the TEM β-lactamase sequence can significantly alter enzyme activity, folding, and antibiotic resistance properties, particularly when they occur in regions involved in secretion or structural stability. Mutational landscape studies of TEM-1 β-lactamase have shown that even single amino-acid substitutions can influence enzyme activity and bacterial fitness under antibiotic pressure.

Similarly, the D10E substitution (Aspartic acid → Glutamic acid) detected in Samples 9 and 47 represents a conservative acidic residue change. Although conservative substitutions often maintain structural properties, they may still influence enzyme efficiency or secretion when located within the signal peptide. TEM β-lactamase variants frequently accumulate minor substitutions that act as secondary mutations, enhancing enzyme stability or compensating for other resistance-associated mutations.

The P20R mutation identified in Sample 9 is particularly noteworthy because position 20 lies within the signal peptide region of TEM β-lactamase. Signal peptide mutations are known to influence enzyme export and processing. Previous experimental studies reported that substitutions at position 20 in the TEM signal peptide can impair proteolytic processing and reduce enzyme expression, thereby affecting β-lactam resistance levels. Additionally, the N-terminal signal sequence plays a critical role in directing the enzyme to the periplasmic space, where β-lactamase activity occurs; alterations in this region may therefore affect antibiotic resistance phenotypes [].

The presence of multiple substitutions in different isolates in the current study suggests ongoing genetic variability within TEM β-lactamase genes. A single mutation M42L (Methionine to Leucine) was identified in the SHV-1 gene of Sample 9, indicating comparatively limited variability in SHV among the analyzed isolates. Such mutations may contribute to evolutionary adaptation and development of resistance under antibiotic selection pressure. Previous studies have demonstrated that TEM β-lactamases evolve through accumulation of mutations, with many variants differing by only a few amino-acid substitutions yet displaying altered substrate specificity or resistance profiles [].

These findings are consistent with global trends indicating that CTX-M-1 type β-lactamases have become the dominant ESBL enzymes worldwide. Previous molecular studies have reported blaCTX-M-1 detection rates ranging from 45% to 65%, while blaTEM and blaSHV-1genes have been reported at frequencies of approximately

30–50% and 20–40%, respectively. For example, several studies investigating ESBL-producing *K. pneumoniae* isolates from dairy animals have shown that blaCTX-M-1 genes were present in more than half of the ESBL isolates, indicating their widespread distribution (23-27).

The high prevalence of the blaCTX-M-1 gene in the present study supports these findings and highlights its major role in mediating resistance to β-lactam antibiotics in mastitis-associated *K. pneumoniae* isolates. The antimicrobial susceptibility testing performed in this study revealed a high level of resistance to commonly used antibiotics, particularly cefotaxime (100%), amoxicillin (86.9%), and ceftriaxone (82.6%). Similar resistance patterns have been reported in previous studies, where ESBL-producing *K. pneumoniae* isolates demonstrated high resistance to third-generation cephalosporins and penicillin derivatives. For instance, earlier investigations reported resistance rates of 80–100% for cefotaxime and 70–90% for amoxicillin among ESBL-producing isolates. These findings highlight the growing challenge of antimicrobial resistance in mastitis pathogens and emphasize the need for appropriate antibiotic stewardship and surveillance programs in dairy farms (17-20). Logistic regression analysis in the present study indicated that previous antibiotic usage was significantly associated with ESBL-producing isolates (AOR = 2.27, p = 0.037). Animals with a history of antibiotic treatment were more than twice as likely to harbor ESBL-producing *K. pneumoniae*. Similar associations have been reported in earlier studies, which identified antibiotic misuse and frequent antimicrobial treatment as major drivers for the emergence and spread of ESBL-producing bacteria in livestock populations. In contrast, other variables such as animal species, breed type, stage of lactation, parity, and milking system did not show statistically significant associations with ESBL occurrence in the present study (28-32). These findings are in agreement with previous research indicating that management practices and antimicrobial usage patterns are more critical determinants of antimicrobial resistance than animal-related factors alone. Overall, the findings of this study demonstrate the presence and dissemination of ESBL-producing *Klebsiella pneumoniae* harboring blaCTX-M-1, blaTEM, and blaSHV-1 genes among mastitis cases in dairy animals in Mardan, Pakistan. The detection of multiple β-lactamase genes in these isolates highlights the increasing threat of antimicrobial resistance in veterinary pathogens. These results emphasize the need for improved mastitis management practices, rational antibiotic usage, and continuous molecular surveillance of antimicrobial resistance in dairy farms to prevent the further spread of ESBL-producing bacteria and protect both animal and public health (33-35).

The current study found a mastitis prevalence of 21.9%, aligning with 22.4% and 18.15% reported by references from Pakistan. A diminished prevalence of mastitis was also documented in other nations, specifically 3.3% in China, 12% in Japan, 22.7% in Ethiopia, and 23% in Canada, respectively. A prevalence of mastitis at 49% was documented by Ref. from Pakistan, consistent with reports from other countries such as Kenya, Ethiopia, and Uganda (33-36). The parallels and disparities among diverse research may stem from variances in breed traits, as well as epidemiological, environmental, and management variables. Additional factors contributing to the elevated incidence of mastitis may include inadequate management and maintenance of sanitary conditions, as well as poor mastitis control strategies in the studied regions. The incidence of mastitis cases is rising globally, with *K. pneumoniae* and *S. aureus* often identified pathogens. The emergence of antibiotic resistance has constrained treatment options for resistant diseases. These studies noted that the predominant ESBL-producing *K. pneumoniae* isolates exhibited significant sensitivity to amikacin (80%), ceftazidime (71%), and tetracycline (71%), corroborating our findings, which also indicated high sensitivity to ceftazidime (80%), amikacin (72%), and tetracycline (65%). In contrast, (23) identified gentamicin as 90% sensitive, but tetracycline was shown to be 13.4% resistant.

These characteristics of ESBL-producing *K. pneumoniae* in bovine milk were previously documented in Japan. The detection of ESBL-producing *K. pneumoniae* isolated from bovine mastitis in this investigation aligns with previous findings. This microbiota is frequently observed in the human population. Numerous findings indicate that consumable cattle may harbor and disseminate drug-resistant infections, including ESBL isolates, to humans. Several research have explicitly documented the cross-transmission of ESBL-producing Enterobacteriaceae between food-producing animals and the human population. The existence of analogous AmpC and ESBL-producing Enterobacteriaceae clones in food animals and humans provides indirect evidence of cross-transmission.

CONCLUSION

The findings confirm that *K. pneumoniae* is an important mastitis-associated pathogen and that a considerable proportion of the isolates possess ESBL-producing capability. Molecular analysis further revealed the presence of β-lactamase resistance genes including blaCTX-M111, blaTEM, and blaSHV-1-1-1, indicating the circulation of multiple antimicrobial resistance determinants among the isolates. The high level of antimicrobial resistance observed among these bacteria highlights the growing challenge of ineffective antibiotic therapy in dairy herds. Additionally, the study suggests that inappropriate or frequent use of antibiotics in dairy farming may contribute to the emergence and spread of resistant bacterial strains. Therefore, the implementation of rational antibiotic use, improved mastitis management practices, and continuous surveillance of antimicrobial resistance is essential to control the dissemination of ESBL-producing pathogens and to protect both animal health and public health.

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Author Contribution
HK Conceptualization, data collection and designed the study. AJ Reviewed and supervised. HK Help in data collection, providing lab facility, funding in the form of reagents. HK Statistical data analysis and visualization of data. HK Manuscript revision and study co supervision. All authors read and approved the final manuscript.

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