Heliyon 10 (2024) e35876

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Genomic diversity and nutritional analysis of multi-drug resistant extended spectrum β-lactamase Producing-*Klebsiella pneumoniae* genes isolated from mastitic cattle milk in district peshawar, Pakistan

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ARTICLE INFO

Keywords:
MDR
K. pneumoniae
Pathogenesis
Antimicrobial resistance
Genomic diversity
Genotyping

ABSTRACT

The increasing incidence of resistance extended spectrum-beta lactamase (ESBL) producing *Klebsiella pneumonia* become worldwide issue. The current study aimed to determine the genomic diversity of ESBL-producing *K. pneumoniae* in milk samples collected from cows with mastitis as well as their antibiotic sensitivity profiles and genetic identification in Peshawar, Pakistan. The california mastitis test (CMT) was initially used to verify the presence for mastitis in 700 collected milk samples. The molecular identification of the *16SrRNA* gene confirmed 120/700 (17.14 %) propagation of *K. pneumonia.* Out of these isolates MDR ESBL-producing isolates were 60/120 (50 %). The lactose were found (M = 3.96 ± 0.28, SD = 2.19), followed by fats (M = 3.12 ± 0.11, SD = 0.90), protein (M = 5.97 ± 0.24, SD = 1.84), sodium (M = 55.74 ± 2.07, SD = 15.81), potassium (M = 138.5 ± 1.53, SD = 11.71), chloride (M = 0.74 ± 0.03, SD = 0.24), calcium (M = 10.27 ± 0.31, SD = 2.42), and chlorine (M = 2.80 ± 0.22, SD = 1.70), respectively. Amikacin (80 %), ceftazidime (71 %), and tetracycline (71 %) were shown to be the most effective antimicrobials against all of the isolates. The occurrence of the *blaSHV* gene was observed at 56.00 % whereas the *blaTEM* gene and *blaCTX-M* gene were 36.00 %, and 30.00 %. The distribution of *blaCTX-M* subgroup genes was followed by *blaCTX-M*-1 (38.00 %), *blaCTX-M*-9 (22.20 %), and *blaCTX-M*-15 (61.10 %). Co-occurrence of *blaCTX-M*+ *blaSHV* was (15.00 %), *blaCTX-M*+ *blaTEM* were (6.60 %), and *blaSHV* + *blaTEM* were (10.00 %), respectively. The inappropriate, prolonged and common use of antibiotics may apply selective pressure for propagation and the occurrence of resistant isolates.

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https://doi.org/10.1016/j.heliyon.2024.e35876
Received 27 January 2024; Received in revised form 31 July 2024; Accepted 5 August 2024
Available online 6 August 2024

1. Introduction

According to the agricultural economy, Pakistan is largely dependent on livestock, which accounts for 11 % of Pakistan's GDP (Agricultural Gross Domestic Product) [1]. Pakistan is among the top four producers of milk [2], along with China, India, and the United States, demonstrating the value of livestock to agriculture and, consequently, to the national economy. Because of the livestock industry's quick expansion and easy access to sufficient inputs throughout Pakistan, poverty has been reduced [2]. More than 62 % of individuals who live in rural regions depend on agriculture for their living, either directly or indirectly. Pakistan has 0.9 million camels, 26.5 million sheep, 27.3 million buffaloes, 29.6 million cows, and 53.8 million goats as part of its overall livestock population. The major sources of milk production are cattle and buffalo, which have a population of 35–40 million and produce between 18,000 and 31,000 million tons of milk annually [3]. In Pakistan, various factors affect livestock productivity, including the non-availability of vaccines, improper health management system, and inadequate nutritional sources, while limited and prehistoric husbandry practices are fatal for the dairy sector [4]. Besides other factors, different livestock diseases like mastitis, foot-and-mouth disease (FMD), endo and ectoparasitic infections, blackleg, hemoglobinuria, and hemorrhagic septicemia play a pivotal role in livestock-related productivity losses in Pakistan [5,6]. According to an estimate, the annual financial losses due to livestock diseases are approximately 200 million US dollars in Pakistan [7,8].

Bovine mastitis is caused by various pathogens including *Staphylococcus aureus* [9], *Mycoplasma bovis* [10], *Klebsiella pneumonia* [11], and many other pathogens. The symptoms of bovine mastitis include inflammation of mammary quarters, decreased milk production, changes in milk color, and composition, and sudden onset of the cardinal signs in mammary quarters [12]. Mastitis causes significant economic losses due to decreased milk production, expensive treatment options, higher labor expenses, milk withheld for human use after treatment, and premature culling [13]. For the majority of dairy farmers, subclinical early identification is crucial to reducing production losses and improving recovery chances. The presence of flakes, clots, and pus in the milk, as well as local and systemic responses and alterations, are used to diagnose clinical mastitis [13]. It's a multi-pathogenic infection in which *K. pneumoniae* is the most noticeable pathogen [14]. They are difficult to prevent, thus responsible for decreased yield and increased mortality rate resulting in significant losses to the dairy sector [6,15].

K. pneumoniae is a Gram-negative bacterium that belongs to the family *Enterobacteriaceae* and is known for developing pyogenic infections [14,15]. The inappropriate and extensive use of antibiotics in agriculture, animals, and humans leads to a rise in antibiotic resistance through a variety of mechanisms like drug modification, inactivation of their target position, efflux mechanisms, metabolic pathways, and acquisition of antibiotic-resistant genes [16]. Antibiotics should be used responsibly in animals and crops, according to the Office International des Epizooties (OIE) and World Health Organization (WHO) [17]. Globally, extended spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* like *Escherichia coli* and *K. pneumoniae* have been frequently reported in mastitic infected milk, milk tanks, and healthy livestock populations from Germany, Tunisia, France, United Kingdom, India, Japan [18], Italy, China, and Pakistan [19]. Moreover, Kaza et al. [20] reported ESBL genes in all their studied *K. pneumoniae* isolates in India. Similarly, Wareth et al. [21] study in Germany also frequently reported the ESBL resistance genes.

ESBL-producing *K. pneumoniae* is becoming a severe global problem. Their incidence varies from one region to the next; however, scientific evidence is scarce on the relationship between antibiotic composition and ESBL generation frequency in *K. pneumoniae* isolates [22]. Furthermore, there is still controversy over the limited data and varied genetic information of *K. pneumoniae* strains and bovine mastitis-linked nutritional analyses [23]. Currently, the distribution of ESBL-producing *K. pneumoniae* among cow milk samples is undocumented in Peshawar, Pakistan. In light of these considerations, the current investigation is directed to estimate the nutritional analysis, the prevalence of ESBL-producing *K. pneumoniae*, and molecular identification of their resistance genes linked to bovine mastitis in dairy farms in Peshawar, Pakistan.

2. Materials and methods

2.1. Study location

The study was performed in District Peshawar (The capital of Khyber Pakhtunkhwa), Pakistan from June 2021 to February 2022.

2.2. Sample collection and processing

Total seven hundred (700) 10 mL milk samples from 700 cows were randomly collected from dairy farms located in Peshawar in a sterile falcon tube properly sealed and labeled. All samples were transported at 4 °C for bacteriological analysis to the microbiology laboratory, College of Veterinary & Animal Husbandry, Abdul Wali Khan University, Mardan. The milk samples were initially screened for mastitis on day of collection by the california mastitis test (CMT).

2.3. Nutritional analysis

Utilizing the Association of Official Analytical Chemists' protocol, a nutritional analysis of milk was conducted. The experiment was conducted at the Veterinary Research Institute (VRI) in Peshawar's Department of Livestock Research and Development. About 1 mL of each milk sample were dried at 105 °C for 24 h to remove moisture, and then burned at 550 °C for 6 h to assess the amount of ash present. This technique was used to measure many other components, including crude fat, sodium, potassium, chloride, crude protein, calcium, and lactose [24–26].

2.4. Isolation and molecular characterization of K. pneumoniae

The entire CMT-positive milk samples were initially cultured in 100 mL Luria Bertani broth (LB broth) media and incubated at 37 °C for 18–24 h. After incubation, the growth was sub-cultured on *K. pneumoniae*-specific simon citrate inositol agar (SCI agar) at 37 °C for 24–48 h according to Ref. [27]. Colony Morphology, microscopy, and biochemical tests were performed for the identification of *K. pneumoniae* according to Ref. [28]. Total nucleic acid from the entire ESBL isolates was extracted through WizPrepTM gDNA Mini Kit (Seongnam-si, Gyeonggi-do, 13209, Republic of Korea). Briefly, *K. pneumoniae* isolates were cultivated in 10 mL nutrient broth media, about 20 μL of culture was added into 1.5 mL eppendrof tube and centrifuged at 7500 rpm for 10 min. After this, the supernatant was removed and 180 μL GT1 buffer was added. Then, 200 μL of GT2 buffer and 20 μL of proteinase K were added. Followed by vortexing, the tube was incubated at 56 °C for 10 min. Then, 200 μL of 100 % ethanol was added into the tube and centrifuged for 1 min at 13000 rpm. The washing was done by 500 μL W1 buffer and 700 μL W2 buffer with centrifugation at 13000 rpm. The extracted DNA was eluted by adding 100 μL of elution buffer. The extracted DNA was stored at −20 °C for further processing. Molecular detection of *K. pneumoniae* was done by using polymerase chain reaction (PCR), and two pairs of *K. pneumoniae 16SrRNA* gene specific primers Pf (5′-ATT TGA AGA GGT TGC AAA CGA T- 3′)/Pr1 (5′-TTC ACT CTG AAG TTT TCT TGT GTT C-3′) and Pf/Pr2 (5′-CCG AAG ATG TTT CAC TTC TGA TT-3′) with amplification at 58 °C and 260 base pair (bp) fragment size, were used according to Ref. [29]. The primers were synthesized by Sangon Biotech Co. (Shanghai, China).

2.5. ESBL confirmation and their antibiogram analysis

The entire *K. pneumoniae* isolates were evaluated by double-disc synergy and phenotypic confirmation as described earlier [30] with slight modifications. Briefly, third-generation cephalosporin including amoxicillin + clavulanate acid (10 μg + 10 μg each), amoxicillin (10 μg), and ceftazidime (30 μg) disc was inserted 25 mm apart from each other in a lawn culture. The inhibition zone increases ≥5 mm for amoxicillin + clavulanate acid (10 μg + 10 μg) and amoxicillin (10 μg) were confirmed as ESBL producers. The entire ESBL isolates were screened for 11 different antibiotics including different classes, i.e., β-lactams (Ampicillin 10 μg, amoxicillin-clavulanic acid 20 μg/10 μg), phenicol (Chloramphenicol 30 μg), cephalosporin's (Ceftazidime 30 μg, Cefepime 30 μg), carbapenemase (Imipenem/Meropenem 10 μg), aminoglycosides (Gentamycin 10 μg, Amikacin 30 μg), tetracycline (Tetracycline 30 μg), quinolones (Ciprofloxacin 05 μg, Levofloxacin 30 μg), sulfonamides (Trimethoprim-Sulfamethoxazole 1.25 μg/23.75 μg).

2.6. Genomic characterization of ESBL resistance genes

Molecular detection of six (06) ESBL-encoding genes *blaSHV*, *blaTEM*, *blaCTX-M*, *blaCTX-M*-1, *blaCTX-M*-9, and *blaCTX-M*-15 were performed through PCR by using gene-specific primers, as given in Table 1. PCR Primer-specific bands were visualized in 01 % agarose gel electrophoresis using Gel Doc^{TM}EZ (BIO-RAD).

2.7. Statistical analysis

The mean of nutritional parameters was analyzed by a One-way ANOVA test and descriptive statistics. The expected and detected *K. pneumoniae* values links were analyzed through a chi-square test by SPSS version 20 and $p \leq 0.05$ values were considered statistically significant.

3. Results

3.1. Isolation and characterization of ESBL-producing K. pneumoniae

The results of the current study indicated that among the 700 symptomatic mastitis cow milk samples initially n = 350 (50.00 %)

Table 1
Primers (F, Forward primer; R, Reverse primer) with its base pair (bp) band size and amplification temperature (°C) for molecular detection of *K. pneumoniae* ESBL resistance genes.

Gene Name	Primers	Amplification	Band Size	References
blaSHV	F-GGGTTATTCTTATTTGTCG R-TTAGCGTTGCCAAGTGCTC	55°C	567bp	[31]
blaTEM	F-ATAAAATTCTTGAAGACGAAA R-GACAGTTACCAATGCTTAATC	54 °C	1086bp	[31]
blaCTX-M	F-CGCTTTGCGATGTGCAG R-ACCGCGATATCGTTGGT	55 °C	550bp	[32]
blaCTX-M-1	F-TCAATGGGCCGATGTCACTG R-CGCCGACGCTAATACATCG	54 °C	500bp	[33]
blaCTX-M-9	F-GTGACAAAGAGAGTGCAACGG R-ATGATTCTCGCCGGCTGAAGCC	56 °C	463bp	[34]
blaCTX-M-15	F-GTGATACCACTTCACCTC R-AGTAAGTGACCAGAATCAG	54 °C	390bp	[35]

were found positive in CMT, while on SCI agar the *K. pneumoniae* growth was observed in n = 120 (17.14 %) samples, further confirmed by molecular characterization of *16SrRNA* gene (Supplementary Fig. 1). Among the entire n = 120 isolates of *K. pneumoniae* the frequency of ESBL-producing *K. pneumoniae* was found n = 60 (50.00 %), which were processed for further investigation (Fig. 1).

3.2. Nutritional analysis

In mastitis cow's milk samples, the level of observed nutritional parameters were lactose (M = 3.96 ± 0.28, SD = 2.19), fats (M = 3.12 ± 0.11, SD = 0.90), protein (M = 5.97 ± 0.24, SD = 1.84), sodium (M = 55.74 ± 2.07, SD = 15.81), potassium (M = 138.5 ± 1.53, SD = 11.71), chloride (M = 0.74 ± 0.03, SD = 0.24), calcium (M = 10.27 ± 0.31, SD = 2.42), and chlorine (M = 2.80 ± 0.22, SD = 1.70) with p-value< 0.05 (Fig. 2).

3.3. Antibiogram analysis of ESBL-producing K. pneumoniae

Antibiogram results revealed that among the 60 ESBL-producing *K. pneumoniae* isolates majority were found highly sensitive to amikacin (80 %) followed by ceftazidime and tetracycline (71 %) whereas less effective antibiotics were found in ceftriaxone (78 %), eropenem (68 %), followed by trimethoprim-sulfamethoxazole (54 %), as shown in Fig. 3.

3.4. Diversity of ESBL-producing K. pneumoniae resistance genes

The ESBL encoding genes were visualized by primer-specific bands (Supplementary Fig. 2). Among the 60 ESBL-producing *K. pneumoniae* isolates the *blaSHV* gene was observed n = 34/60 (56.00 %) whereas the *blaTEM* gene and *blaCTX-M* gene were n = 22/60 (36.00 %), and n = 18/60 (30.00 %). The distribution of *blaCTX-M* subgroup genes was followed by *blaCTX-M*-1 n = 7/18 (38.00 %), *blaCTX-M*-9 n = 4/18 (22.20 %), and *blaCTX-M*-15 n = 11/18 (61.10 %). Co-occurrence of *blaCTX-M*+ *blaSHV* was n = 09/60 (15.00 %), *blaCTX-M*+ *blaTEM* were n = 04/60 (6.60 %), and *blaSHV* + *blaTEM* were n = 04/60 (10.00 %) respectively, as shown in Fig. 4. The occurrence results of ESBL-producing genes in various locations in Peshawar are presented in Fig. 5 and Table 2.

3.5. Statistical investigation

The number of samples (n) was set to 150 and the degree of freedom was set to n^{-1} for chi-square analysis. The chi-square test revealed a significant amount of relationship between anticipated and observed *K. pneumoniae* values, proving our null hypothesis where $p \leq 0.05$.

4. Discussion

Despite adopting various preventative measures like isolation of infected animals, teat dropping, and dry cow therapy, mastitis is still prevalent among dairy herds worldwide [23,36]. M astitis-causing *Enterobacteriaceae* including *K. pneumoniae* [37] is an important Gram-negative pathogenic bacterium of animals and humans concern [38]. Mastitis caused by *Klebsiella* species is responsible for a huge loss in the dairy industry including; reduction and recycling of milk, diagnostic and treatment expenses, mortality, isolation or removal of diseased animals, and poverty. Annual financial losses of about $185 per animal are recorded due to mastitis [39].

A variety of animals, including goats, cows, buffalo, sheep, and even people, may produce milk, which is a nutrient-rich diet. However, this type of milk's richness of nutrients which includes a wide spectrum of macro and micronutrients as well as vitamins and

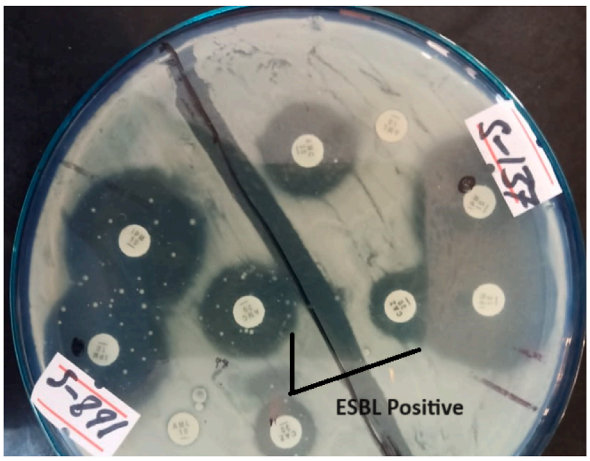

Fig. 1. Randomized ESBL positive isolates showing double-disc synergistic effect.

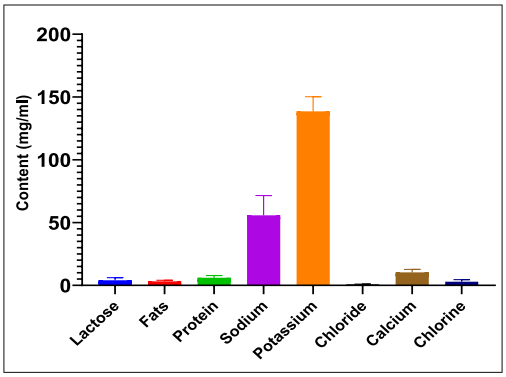

Fig. 2. Nutritional parameters of mastitis-infected cow's milk.

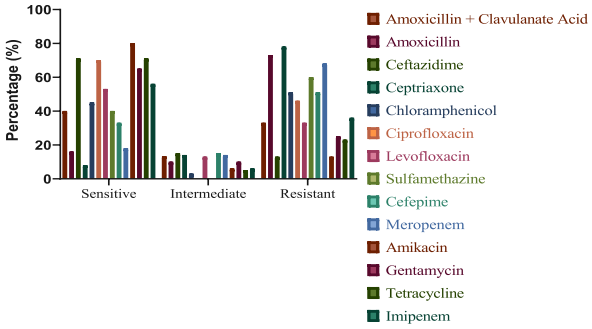

Fig. 3. Antibiogram analysis of 14 tested antibiotics against ESBL producing-*K. pneumoniae* isolated from mastitis of cow milk. The results show that isolates were extremely sensitive to amikacin while extremely resistant to ceftriaxone (78 %) and meropenem (68 %). (See details in Supplementary Table 1).

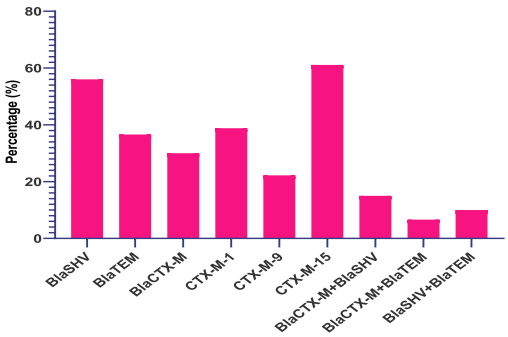

Fig. 4. Frequency of ESBL-producing genes of *K. pneumoniae* isolates, (A) *blaSHV*, (B) *blaTEM,* (C) *blaCTX-M*, (ABC) *blaSHV* + *blaTEM* + *blaCTX-M,* (AB) *blaSHV* + *blaTEM*, (AC) *blaSHV* + *blaCTX-M*, (BC) *blaTEM* + *blaCTX-M, blaCTX-M* sub-types *blaCTX-M*-1, *blaCTX-M*-9, and *blaCTX-M*-15.

minerals creates an ideal environment for the development of various bacteria [40]. Either all bacteria have immediate access to nutrients, or only some populations can break down the major components to release nutrients and metabolites that other germs can use. Dairy product development is directly impacted by the microbial makeup of milk. As seen by the reduced lactose content of milk with mastitis [41]. Similar to this, we discovered the highly increased level of protein, sodium, and chlorine level as reported by Refs. [24–26], While fat, lactose, potassium, chloride, and calcium content were reported to decrease in the current investigation of cow's milk samples contaminated with bovine similarly [24–26]. Another recent study in Pakistan also reported the decrease nutrient content in mastitic cow's milk samples [42].

In the current study, the prevalence of mastitis was recorded as 21.9 %, coinciding with [43] 22.4 %, and 18.15 % by Ref. [44] from Pakistan. A lower prevalence of mastitis was also reported in other countries like 3.3 % in China [45] 12 % in Japan [46] 22.7 %, and 23 % in Ethiopia and Canada, respectively [47,48]. A higher prevalence of mastitis, 49 %, was recorded by Ref. [49] from Pakistan as reported in other countries like Kenya [50] Ethiopia [51], and Uganda [52]. The similarities and differences among various studies

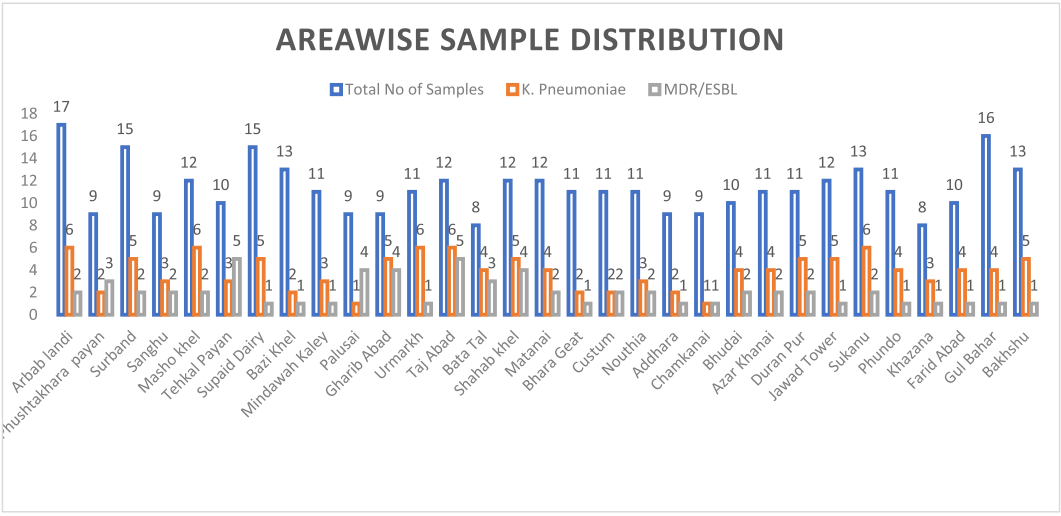

Fig. 5. Shows total number *K. pneumoniae* isolates, ESBL producers, and MDR *K. pneumoniae* in overall samples.

might be due to variations in breed factors, and epidemiological, environmental, and management factors [53]. Other factors responsible for the high prevalence of mastitis may be the improper management and maintenance of hygienic conditions, and ineffective mastitis control programs in the study areas [54]. Globally, mastitis associated cases are instantly increasing, among which *K. pneumoniae* [55] and *S. aureus* [56] are frequently isolated. In the current study, 17.14 % frequency of *K. pneumoniae* was isolated as reported by other studies [55,57]. Other similar studies also reported a diverse group of *K. pneumoniae* strains associated with mastitis [58,59]. *Klebsiella* has been isolated from 11.3 to 35.1 % among coliform mastitis [60,61]. The high prevalence of *K. pneumoniae* in the livestock population is multi-factorial depending upon weather conditions, environmental factors, lack of hygienic conditions, bedding management, livestock crowding, and pre-milking animal preparation, the introduction of rodents, herds, new animals, and lack of cleanliness of installations [60]. The *K. pneumoniae* is also mainly a neglected pathogen in health and veterinary professionals [61]. The continuous presence of a diverse population of *K. pneumoniae* in dairy farms might favor the increased genetic diversity [60].

The development of antimicrobial resistance limited the treatment option against resistant pathogens [62]. The said study observed that the majority of the ESBL-producing *K. pneumoniae* isolates were highly sensitive to amikacin (80 %), ceftazidime (71 %), and tetracycline (71 %), in agreement with our findings [42] also reported high sensitivity of ceftazidime (80 %), amikacin (72 %), and tetracycline (65 %). In contrast [63] found gentamicin (90 %) sensitive, while in agreement [64], found tetracycline (13.4 %) resistant. These features among ESBL-producing *K. pneumoniae* in bovine milk were earlier reported in Japan [65]. The presence of ESBL-producing *K. pneumoniae* isolated from bovine mastitis in this study is consistent with the finding of [65,66]. This microbiota is commonly reported in the human population [67]. However, various reports claim that edible livestock might store and transmit drug-resistant pathogens as ESBL isolates to the human population [68] several studies clearly described the cross-transmission of ESBL-producing *Enterobacteriaceae* between food-producing animals/food and the human population [69]. The presence of similar types of AmpC and ESBL-producing clones of *Enterobacteriaceae* in food animals and the human population supports indirect proof of such cross-transmission [70].

The presence of various β-lactamases resistant genes including *blaTEM*, *blaCTX-M*, *blaSHV*, *blaCTX-M*-1, *blaCTX-M*-9, and *blaCTX-M*-15 among mastitis milk samples in the current study coincides with a study from India, reported that ESBL producing *K. pneumoniae* from bovine milk containing *blaCTX-M*, *blaTEM*, and *blaSHV* resistant genes [65]. Another study [71] reported the existence of *blaCTX-M*-1 gene and [68] reported *blaCTX-M*-15 from ESBL producing *K. pneumoniae* isolated from bovine mastitis. *K. pneumoniae* harbored *blaSHV*-12, and *blaTEM*-1 β-lactamases from cases of bovine mastitis in Egypt [70]. Among mastitis, isolated resistance phenotypes observed in ESBL-producing *Klebsiella* isolates studied were similar to those found in *CTX-M*-2-producing *Enterobacteriaceae* [57] in Japan. The high prevalence of β-lactamases resistant genes among mastitis milk-derived *K. pneumoniae* might be because *Klebsiella* generally has a class A chromosomal β-lactamases, and the isolates that lack the enzymes are rare [72]. On the other hand, β-lactam antibiotics block the development of cell walls in the final stage and have a high prevalence due to increased misuse and considerable variation day today [73]. The emergence of such resistance to β-lactamase inhibitors further narrows the available therapeutic options to tackle ESBL infection [74].

The maintenance of hygienic conditions in diary forms is difficult due to the continuous production of organic matter like urine, feces, milk, and different weather conditions. The worst and most challenging maintenance of good sanitation in dairy farms creates a favorable environment for a genetically complex mastitis-causing microbial population, making it difficult to treat and control. The mastitis causing *K. pneumoniae* in diary-farms become exposed to different environmental conditions like moisture, nutrients, pH, temperature, and diverse microbiota, responsible for mutation and horizontal gene transfer of resistant genes due to which genetically diverse group of *K. pneumoniae* is observed [75]. The high prevalence of multidrug resistance, ESBL-producing *K. pneumoniae* from bovine mastitis milk, needs great attention towards the improper and empirical use of various antibiotics among the livestock

Table 2
Area-wise diversity of ESBL producing *K. pneumoniae* resistance genes in district Peshawar, Pakistan. The positive (+) sign represents the existence of genes while the negative (−) sign represents the non-existence of genes.

S. No.	ESBL producing genes						Location	Resistance phenotypes to antibiotics
	blaCTX-M	CTX-M-1	CTX-M-9	CTX-M-15	blaTEM	blaSHV		
S-07	-	-	-	-	+	+	Arbab landi	AMC,AML,CAZ,FD,C,VA,AK,CN
S-10	+	+	-	-	-	-	Arbab landi	FEP,VA,AML,FD,SXT,C,LEV,CAZ,AK
S-13	+	-	-	+	-	+	Phushtakhara	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-16	-	-	-	-	-	+	Surband	AML,IMP,LEV,TE,FD,SXT,FEP,VA,C
S-18	-	-	-	-	+	-	Surband	AML,FD,SXT,FEP,IMP,LEV,TE,VA,C
S-19	+	-	-	+	-	+	Sanghu	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-22	-	-	-	-	+	+	Sanghu	AML,FD,SXT,FEP,VA,C,TE,IMP,CN,LEV
S-51	-	-	-	-	-	+	Masho khel	AML,FD,SXT,IMP,LEV,TE,FEP,VA,C
S-53	+	+	-	+	-	-	Masho khel	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-54	-	-	-	-	+	-	Supaid Dairy	AML,FD,SXT,TE,AK,CAZ,FEP,VA,C
S-55	-	-	-	-	-	+	Bazi Khel	AML,FD,SXT,FEP,VA,C,TE,IMP,CN,LEV
S-60	-	-	-	-	+	-	Bazi Khel	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-61	+	-	-	+	-	+	Gharib Abad	AML,FD,SXT,FEP,TE,AK,CAZ,VA,C
S-63	-	-	-	-	+	+	Gharib Abad	AML,FD,IMP,LEV,TE,SXT,FEP,VA,C
S-65	-	-	-	-	-	+	Gharib Abad	AML,TE,AK,CAZ,FD,SXT,FEP,VA,C
S-67	-	-	-	-	+	-	Gharib Abad	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-71	+	+	-	+	-	+	Uarmarkh	AML,FD,SXT,FEP,VA,TE,AK,CAZ,C,
S-73	-	-	-	-	+	-	Taj Abad	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-79	-	-	-	-	-	+	Taj Abad	AML,FD,SXT,IMP,LEV,TE,FEP,VA,C
S-81	-	-	-	-	-	+	Taj Abad	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-84	-	-	-	-	-	+	Taj Abad	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-87	-	-	-	-	+	-	Taj Abad	AML,FD,SXT,IMP,LEV,TE,FEP,VA,C
S-88	+	-	+	+	-	+	Taj Abad	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-90	-	-	-	-	-	-	Bata Tal	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-112	-	-	-	-	-	+	Bata Tal	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-116	-	-	-	-	-	-	Bata Tal	AML,FD,IMP,LEV,TESXT,FEP,VA,C
S-119	+	-	-	+	+	+	Matanai	AML,FD,SXT,FEP,VA,C,IMP,LEV,TE
S-124	-	-	-	-	-	-	Matanai	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-129	-	-	-	-	-	+	Bhara Geat	AML,FD,SXT,FEP,TE,AK,CAZ,VA,C
S-137	-	-	-	-	-	+	Gul Bahar	AML,FD,SXT,FEP,VA,C,IMP,LEV,TE
S-139	+	+	-	-	+	-	Bakhshu	AML,FD,SXT,FEP,VA,C,TE,CAZ
S-146	-	-	-	-	-	+	Custum	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-147	+	-	+	-	-	+	Custum	AML,FD,SXT,FEP,VA,C,IMP,LEV,TE
S-153	-	-	-	-	-	-	Nouthia	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-161	-	-	-	-	-	+	Nouthia	AML,FD,SXT,FEP,VA,C,IMP,LEV,TE
S-167	-	-	-	-	-	-	Addhara	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-175	+	-	+	+	+	-	Azar Khanai	AML,FD,SXT,FEP,VA,C,IMP,LEV,TE
S-179	+	-	-	-	-	+	Azar Khanai	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-182	-	-	-	-	-	+	Duran Pur	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-186	-	-	-	-	+	-	Duran Pur	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-189	+	-	-	+	-	-	Sukanu	AML,FD,SXT,FEP,VA,C,TE,CIP
S-193	-	-	-	-	+	-	Sukanu	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-196	+	-	-	+	-	+	Phundo	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-199	+	+	-	+	+	-	Phundo	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-207	-	-	-	-	-	+	Khazana	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-212	-	-	-	-	+	-	Farid Abad	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-219	–	–	–	–	–	+	Custum	AML,FD,SXT,FEP,VA,C,IMP,LEV,TE
S-233	+	+		–	–	–	Nouthia	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-237	–	–	–	–	+	+	Nouthia	AML,FD,SXT,FEP,VA,C,IMP,LEV,TE
S-239	+		+	–	–	–	Addhara	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-245	–	–	–	–	–	+	Azar Khanai	AML,FD,SXT,FEP,VA,C,IMP,LEV,TE
S-252	–	–	–	–	+	–	Azar Khanai	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-257	–	–	–	–	+	+	Duran Pur	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-263	–	–	–	–	+	–	Duran Pur	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-296	–	–	–	–	–	+	Sukanu	AML,FD,SXT,FEP,VA,C,TE,CIP
S-303	–	–	–	–	–	+	Sukanu	AML,FD,SXT,FEP,VA,C,TE,AK,CAZ
S-307	–	–	–	–	–	+	Phundo	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK
S-309	–	–	–	–	+	–	Phundo	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-340	+	+	–	–	–	–	Khazana	AML,FD,SXT,FEP,VA,C, TE,IMP,CN,LEV
S-346	–	–	–	–	+	+	Farid Abad	AML,FD,SXT,FEP,VA,C,LEV,CAZ,AK

population. The presence of ESBL resistance genes among bovine mastitis-causing *K. pneumoniae* constrains us from giving special attention as this might be responsible for clinical infection among humans through the intake of food like milk.

The most significant conclusion from our research is that raw milk could represent a risk to food safety and public health due to MDR ESBL-producing *K. pneumoniae*. Finally, even though exclusive uses of lactamase inhibitors are to treat animals in Pakistan (Asia),

it is still advisable to check the presence of ESBL-producing bacteria in milk and other foods derived from animals. As information is the first step in defending an animal's health, additional research into the association between ESBL-producing *K. pneumoniae* and milk is required. Currently, it is a need of the hour to develop veterinary diagnostic laboratories for routine screening of animals and perform antibiogram analysis to overcome the empirical uses of antibiotics and resistance towards extended-spectrum cephalosporin and other groups of antibiotics.

Funding

The authors would like to extend their gratitude to King Saud University, Riyadh, Saudi Arabia for funding this research through researchers supporting the project (**RSP-2024-R406**) and we are also thankful to the Higher Education Commission of Pakistan for funding the research experimental work under NRPU project (Sr. Number: 8633).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Saddam: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Muhsin Jamal:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sadeeq Ur Rahman:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Muddasir Khan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Abdul Qadeer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Mohamed H. Mahmoud:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to extend their gratitude to King Saud University (Riyadh, Saudi Arabia) for funding this research through Researchers supporting Project number (RSP-2024-R406).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35876.

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