ChemBioNexus
ISSN: 2024-2025
ISSN: 2024-2026

Drug Susceptibility Trends in Mastitis-Associated *Klebsiella* Species from Dairy Herds

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DOI:
Received: 6th May, 2025
Revised: 12th August, 2025
Accepted: 25th August, 2025

Keywords:
Bovine mastitis
Klebsiella pneumoniae
Antimicrobial resistance
Colistin-tigecycline
Dairy cows

Abstract
Mastitis remains a major challenge for the dairy industry, causing significant economic losses due to reduced milk yield, diminished milk quality, and increased veterinary costs. *Klebsiella pneumoniae* is recognized as a critical mastitis-causing pathogen due to its rapid disease progression, poor therapeutic response, and association with multidrug resistance (MDR). This study aimed to isolate *Klebsiella* species from mastitis-affected dairy cows and assess their antimicrobial susceptibility profiles. A total of 160 milk samples were collected from clinically confirmed mastitis cases across four dairy farms in the Lahore region. Among these, 34 isolates (21.25%) exhibited characteristic growth on *Klebsiella* selective media and lactose fermentation on MacConkey agar. Biochemical identification (API® 20E) confirmed all isolates as *Klebsiella pneumoniae*. Antibiotic susceptibility testing was conducted using the Kirby-Bauer disc diffusion method following CLSI guidelines. Results demonstrated that nearly all isolates were MDR, showing complete resistance to amoxicillin and piperacillin and resistance to most tested antibiotics. Notably, all *K. pneumoniae* isolates were fully sensitive only to the combination of colistin and tigecycline, followed by variable sensitivity to cotrimoxazole, minocycline, ceftipime, imipenem, moxifloxacin, ceftazidime, and ceftriaxone. The findings emphasize the high prevalence of MDR *K. pneumoniae* in bovine mastitis and highlight the potential effectiveness of colistin-tigecycline combination therapy for future mastitis outbreaks. Strengthened antimicrobial stewardship and improved farm management practices are crucial to mitigate resistance development in dairy systems

Introduction

Mastitis is important ailment concerning economics of dairy herds. Mastitis causes swelling of the udder which frequently outcomes in the form of changes in the chemical, physical and bacteriological characteristics of milk amid other signs[1]. Different surveys of important dairy disease in Pakistan have declared mastitis as most prevalent problem in lactating herds[2–4].

It has been projected that the only mastitis causes about 70% of all preventable damages during milk production. The mastitis is not a localized problem as it harmfully disturbs animal health, milk production and the economics of milk production on a world level. Problem of mastitis is faced by both the developed and the developing countries of the world and results in huge economic losses[5].

Mastitis has two major forms; clinical and subclinical mastitis. Clinical mastitis is frequently apparent and can be easily diagnosed by alteration in the color and composition of milk, decreased quantity of milk produced along with signs and symptoms of disease such as agony, inflammation and soreness, with or without heat in infected mammary quarters. On the other hand, subclinical form of mastitis is not easily diagnosed as signs and symptoms are not apparent[6]. Subclinical form of mastitis often results in 70% decrease in milk production[7,8].

Bovine mastitis is classified into two categories: contagious mastitis which are caused by contagious pathogens *i.e., Streptococcus agalactiae* or *Staphylococcus aureus*. These pathogens are present on skin of animals and inside udder of animals and communicated from infected animals to others during or after milking. Other form of mastitis is environmental caused by environmental pathogens *i.e., Klebsiella* sp., *Streptococcus uberis*, and *Escherichia coli*. These bacteria are prevalent in surroundings of animals as sheds, feces, feed and organic bedding[7]. Different control measures *i.e.,* dipping of teats after milking, antibiotic therapy of dry cows, and segregating diseases animals are practices worldwide. These control measures have decreased contagious mastitis while environmental pathogens does not decrease with these practices are now major concern for commercial dairy herds[9]. Coliform pathogens (*Klebsiella* sp. and *Escherichia Coli*) among environmental bacteria are major cause of mastitis and considered accountable for about 50% of acute mastitis infections[10]. *Klebsiella pneumoniae* has affected a number of dairy animals. Due to poor response of *Klebsiella pneumoniae* to antibiotic treatment, fast development to toxic shock, and death, *Klebsiella pneumoniae* has a much greater impact on agricultural economics than *Escherichia coli*[10]. Transmission of *Klebsiella* sp. occurs between milking due to presence of *Klebsiella* sp. in sawdust and feces[11]. Apart from preventive vaccination, the most effective method for controlling *Klebsiella* sp. mastitis is prevention via proper management of the animals and clean environment for their housing[12]. Continuous and indiscriminate use of antimicrobials in dairy herds as treatment protocol or growth promoter is important reason for increased resistance of pathogenic organisms. This is greatest hazard to both humanity and animals worldwide[13].

Miserably, there is absence of adequate surveillance data on antibiotic resistance in the country. Appearance of multi drug resistant (MDR) pathogens and their dissemination from food animals to the community carries huge public health concerns in Pakistan[14,15]. Multi-drug resistant pathogens are a tenacious hazard in current health care system, food safety and animal health. Development of new antibiotics is required to replace existing conventional and ineffective antimicrobials[16].

This research was assumed to isolate *Klebsiella pneumoniae* associated with mastitis so suitable antibiotic may be evaluated for mastitis treatment. Therefore, current research project was undertaken to evaluate antibacterial susceptibility profile of *Klebsiella* sp. isolated from mastitis milk samples in different dairy farms of Lahore Pakistan.

Methodology

Sample collection

A total of 160 milk samples of cows suffering from mastitis were collected from four different dairy farms of Lahore region (table 1). Milk samples were collected following the guidelines of National Mastitis Council Inc. (1990). Milk samples were collected from two types of animals, *i.e.,* cows with clinical mastitis and cows having subclinical mastitis. The cows with subclinical mastitis were screened through Surf Field Mastitis Test (SFMT) and only those cows were selected who were positive to SFMT. All the milk samples (20 ml/cow) were collected aseptically for microbiological examination in sterilized bottles. Milk samples were stored at 4°C until use for bacterial isolation. A total of 90 milk samples were collected from udder of cows having clinical mastitis and 70 milk samples were collected from udder of cows having subclinical mastitis. Surf Field Mastitis Test was performed for the identification of subclinical mastitis following the procedure elaborated by Muhammad *et al.,* (2010)[17]. Briefly, equal volume of mastitis milk samples and 3% solution of a common detergent (*Surf Excel by Unilever, Pakistan*) were added in the receptacle of the Surf Test paddle. The mixture was rotated for minimum 15 seconds and observed for gel formation (*i.e.,* thickening of the mixture).

Isolation of *Klebsiella* from milk samples

Collected milk samples were gently shaken 8 times (following National Mastitis Council Inc. 1990 guidelines) to get even spreading of the microbes. A volume of 10 µl milk sample was streaked on *Klebsiella* Selective Agar media. The plates were incubated for 24 hours at 37°C.

Identification and Purification of Bacterial Colonies

Initially isolates were identified by cultural and morphological characteristics of *Klebsiella* sp. on *Klebsiella* Selective Agar media. These *Klebsiella* isolates were then grown on MacConkey agar to confirm their lactose fermenting ability. Single colonies of *Klebsiella* sp. were obtained, through several rounds of streaking on *Klebsiella* selective agar for gram staining, biochemical identification and molecular identification.

Table 1: Distribution of milk samples collected from various farms of Lahore

Sr. No.	Dairy Farms	Total Lactating Animals at Farms	Cows suffering from Mastitis	
			Cows having clinical mastitis	SFMT Positive cows having subclinical mastitis
1	Farm 1	110	21	16
2	Farm 2	120	22	18
3	Farm 3	115	19	16
4	Farm 4	134	28	20
Total		**479**	**90**	**70**

Morphological characterization of Bacterial Isolates[18]

Bacterial single colonies were studied for colony characteristics like size, color, elevation, shape and margins of the colonies. To observe the cell morphology such as the shape and arrangement of bacterial cells gram staining was performed.

Biochemical identification of bacterial isolates using API® 20E Strip method

API® 20E strip (*BioMérieux, France*) was used to study of biochemical characteristics of the *Klebsiella* sp. Isolated colonies were picked with the help of inoculation loop and transferred in 10 ml of PBS, while bacterial suspensions turbidity was adjusted according to 0.5 McFarland turbidity standard. API® 20E strip was inoculated with freshly made bacterial suspension as per manufacturer's recommendations. Covered strips were incubated for 24 hours at 37°C. As incubation period is over, colors was noted for each test and matched with color key provided with API® 20E kit. Results were interpreted by following API® 20 E Quick Index Booklet and API WEB™ online service[19].

Antibiotic susceptibility testing

To carry out the antibiotic susceptibility testing, disc-diffusion assay[20] was used to evaluate the antibiotic resistance pattern of isolates of *Klebsiella* sp. Various groups of antibiotics used in the antibiogram analysis included: penicillins (amoxicillin and piperacillin), cephalosporins (ceftriaxone, ceftazidime and ceftipime), carbapenems (imipenem), quinolones (moxifloxacin), tetracycline (minocycline), Sulfa drugs (cotrimoxazole), polmyxins (colistin) and tigecycline combination.

Disc diffusion assay

A panel of antibiotics mentioned above were used to evaluate the resistance pattern of different isolates against antibiotic discs.

1. Bacterial cultures were standardized in by adjusting the optical density at 0.5 (OD_{600}=0.5) at 600 nm.
2. Sterile cotton swabs were used for uniform spreading of bacterial suspensions on the surface of Muller Hinton agar plates.
3. Once agar surfaces dried, antibiotics discs were placed on plate surfaces with sterile forceps (Figure 1).
4. Later on plates were incubated at 37°C for 16 to 24 hours aerobically.
5. Vernier caliper was used to measure zone of inhibition in millimeters (mm).
6. Results of disc diffusion were understood by comparing with standards provided by Clinical & Laboratory Standards Institute (CLSI).

Study of the Mastitis prevalence among the cows

The overall prevalence of the mastitis among the cows was studied according to the breed, age and the number of lactating cows under study.

Results

Physical characteristics of Cow udder and Milk Samples Collected

In this study, a total of 160 mastitis milk samples were collected from both clinical and subclinical mastitis positive cows. Milk samples from cows showing signs and symptoms of mastitis (clinical mastitis) were watery, bloody, and blood tinged, having clots, flakes or pus. Taste of milk was rancid. Whereas, milk samples from cows showing no signs and symptoms of mastitis (subclinical mastitis) were normal in color, consistency or taste. For such samples, SFMT was performed and the gel formation of milk with surf (Figure 2) indicated that cows were suffering from subclinical mastitis. Physical characteristics of milk samples such as color, consistency and taste were also noted after sample collection and are given in table 2.

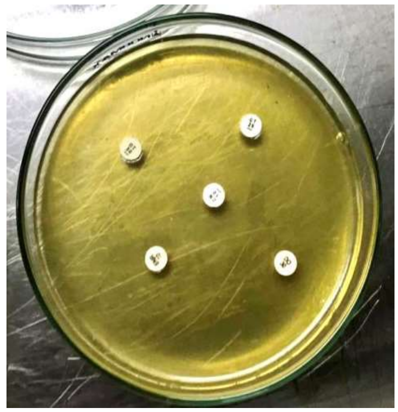


Figure 1: A representative of antibiotic discs on swabbed Mueller Hinton Agar

Isolation and Identification of *Klebsiella* sp.

A total of 160 collected milk samples were cultured on *Klebsiella* selective agar. Among these 160 milk samples, only 34 samples (21.25%) produced purple magenta colored colonies on *Klebsiella* selective agar, having characteristics of *Klebsiella* sp.

Among these 34 samples, 22 mastitis milk samples were collected from clinical mastitis cows and 12 mastitis milk samples were collected from subclinical mastitis cows (table 3). These 34 isolates of *Klebsiella* were then grown on MacConkey agar which produced characteristic pink colored colonies on MacConkey agar. The growth of pink colored colonies of *Klebsiella* sp. isolates confirmed their lactose fermenting ability on MacConkey agar (Figure 3).

Morphological characterization of *Klebsiella* sp.

Colony and cell morphology of these isolates of *Klebsiella* sp. were studied and noted for each *Klebsiella* isolate (table 4.3). Colonies of *Klebsiella* isolates were large (2-3mm), circular and convex. Color of colonies was purple magenta on *Klebsiella* selective agar and pink on MacConkey agar. Colonies were mucoid and translucent opaque. All the 34 isolates were found gram negative rods under microscopic examination (100X lens).

Biochemical identification of bacterial isolates using API® 20E Strip method

According to API® 20E strip method, all the 34 x bacterial isolates were identified as *Klebsiella pneumoniae*. As per results, it was proved that all isolates utilized citrate as sole carbon source, fermented various sugars including glucose, lactose, arabinose, inositol, mannitol, mannose, sorbitol and sucrose. Isolates were also found positive to Voges–Proskauer (VP) test and negative to indole, H_2S gas production and Methyl red (MR) tests (Table 4).

Antibiotic resistance/sensitivity pattern of *Klebsiella* isolates

Disc diffusion assay (Kirby-Bauer method) was used to study the antibiotic sensitivity/resistance pattern of all the bacterial isolates. Resistance or sensitivity to a particular antibiotic is shown by the zones of inhibition. Results of antibiogram analysis are given in Table 4. All the *Klebsiella* isolates (100%) were found resistant to amoxicillin and piperacillin while 24 isolates (70.59%) were found resistant to moxifloxacin and 10 isolates (29.41%) were found sensitive to moxifloxacin (Figure 4).

Figure 2: Consistency of milk sample indicating subclinical mastitis

Table 2: Physical characteristics of cow udder & milk samples collected

Sr. No.	Physical characteristics	Cows suffered with Clinical Mastitis	Cows suffered with Subclinical Mastitis
1	Cow udder	Inflammatory changes in infected udder quarters such as pain, inflammation, red, either hot or not	Normal
2	Color of Milk	Watery, bloody or blood tinged	Normal
3	Consistency of milk	Flakes, clots or pus	Normal
4	Taste	Unstable or rancid taste	Normal

Table 3: *Klebsiella* Isolates from clinical and subclinical samples

Sr. No.	Type of mastitis	No. of Isolates	Codes of Samples
1	Clinical Mastitis	22	KP1, KP2, KP3, KP6, KP7, KP8, KP9, KP10, KP11, KP12, KP13, KP14, KP19, KP20, KP21, KP22, KP25, KP26, KP27, KP28, KP29, KP30,
2	Subclinical Mastitis	12	KP4, KP5, KP 15, KP16, KP17, KP18, KP23, KP24, KP31, KP32, KP33, KP34

Figure 3: Representative pink colored colonies of *Klebsiella* on MacConkey agar

In case of ceftriaxone and ceftazidime, 27 isolates (79.41%) and 25 isolates (73.53%) were found resistant to the drug respectively whereas 6 isolates (17.64 %) and 9 isolates (26.47%) were found sensitive to the same drug as shown in figure 4 and table 4 and 5. A total of 17 isolates (50%) bacterial isolates were found resistant to Ceftipime and 17 isolates (50%) were found sensitive to the same drug (Figure 4). 22 isolates (64.71%) were susceptible and 11 isolates (32.35%) were resistant to minocycline. Cotrimoxazole also inhibited the growth of 23 isolates (67.65%) and 11 isolates (32.35%) bacterial isolates showed resistance to Cotrimoxazole (Figure 4). Only 14 isolates (41.18%) were susceptible to imipenem and 20 isolates (58.82%) were resistant to imipenem. However all of 34 isolates (100%) were susceptible to colistin and tigecycline combination (Figure 4). Very few isolates were found intermediately resistant to antibiotics. As in case of each ceftriaxone and minocycline, 1 isolate showed intermediate resistant.

Study of the prevalence of Mastitis among the cows

Out of 479 lactating cows present at 4 different dairy farms in Lahore regions, 160 (33.40%) animals were positive to mastitis. Out of these 160 mastitis positive cows, 90 cows (56.25 %) were suffering from clinical mastitis and 70 cows (43.75%) were suffering from sub clinical mastitis. However prevalence of clinical mastitis was 18.78% and subclinical mastitis was 14.61 % in herd. (Table 5).

Breed wise prevalence of Mastitis

Out of 479 cows present at farms, 124 cows were Holstein Friesian (HF), 116 cows were Sahiwal breed and 239 cows were X bred. 45 (36.29%) HF cows out of 124 were mastitis positive, 35 (30.17%) Sahiwal cows out of 116 were mastitis positive, and 80 (33.47%) X bred cows out of 239 were mastitis positive (Figure 5).

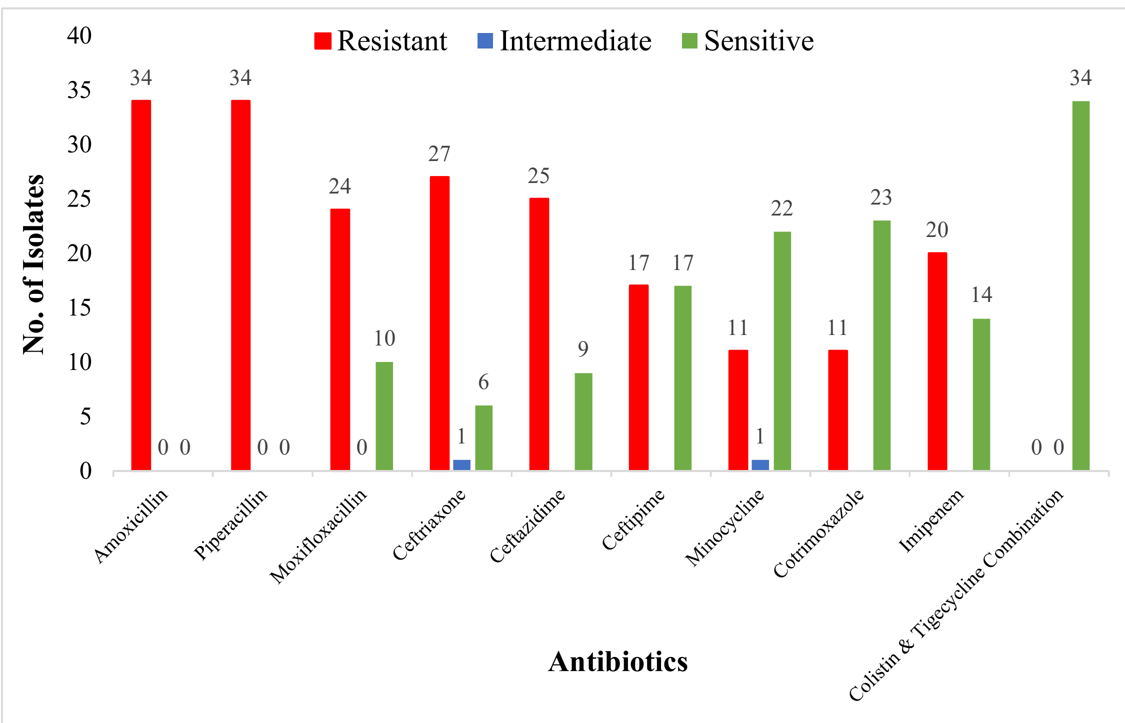


Figure 4: Antibiotic susceptibility profiling of *Klebsiella* sp.

Age wise prevalence of Mastitis

Age wise prevalence of mastitis was noted and details are at figure 6. Age group was ranging from 3 year to 8 years of age. At the age of 8 years, 5 x cows out 10 (50%) were having mastitis. Animals at the age of 7 years, 7 cows out 15 (46.67%) were having mastitis. While animals at the age of 6 years, 53 cows out of 118 (44.92%) were having mastitis. At the age of 5 years, 49 cows out 132 (37.12%) were having mastitis. At the age of 4 years, 35 cows out 145 (24.14%) were having mastitis. At the age of 3 years, 11 cows out 59 (18.64 %) were having mastitis (Figure 6).

Table 4: Antibiotic susceptibility profiling of *Klebsiella* isolates

Sr. No	Antibiotic	Resistant	%	Intermediate Resistant	%	Sensitive	%
1	Amoxicillin	34	100.00			0	0
2	Piperacillin	34	100.00			0	0
3	Moxifloxacillin	24	70.59			10	29.41
4	Ceftriaxone	27	79.41	1	2.9	6	17.64
5	Ceftazidime	25	73.53			9	26.47
6	Ceftipime	17	50.00			17	50.00
7	Minocycline	11	32.35	1	2.9	22	64.71
8	Cotrimoxazole	11	32.35			23	67.65
9	Imipenem	20	58.82			14	41.18
10	Colistin & Tigecycline Combination	0	0			34	100.00

Lactation wise prevalence of Mastitis

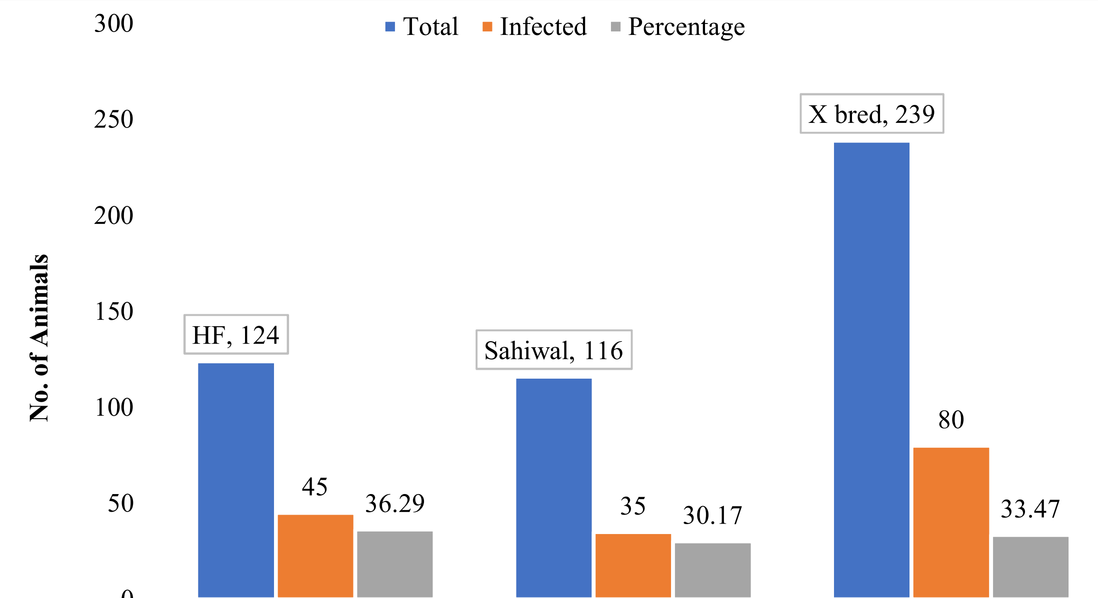
Lactation wise prevalence of mastitis was noted and details are given at figure 7. No of lactation was ranging from 1st to 5th in herds. Total 64 cows of 1st lactation were present on these farms, out of which 9 cows (14.06%) were having mastitis. Animals with 2nd lactation, 52 cows out 145 (35.86%) were having mastitis. While animals in 3rd lactation, 47 cows out of 137 (34.3) were having mastitis and 7 cows out 15 (46.67%) were having mastitis during 5th lactation.

Table 5: Prevalence of Clinical and subclinical Mastitis

Sr. No.	Mastitis	No. of Samples	Percentage	Prevalence out of total herd (479 cows)
1	Clinical	90	56.25%	18.78%
2	Subclinical	70	43.75%	14.61%
Total		160		

Discussion

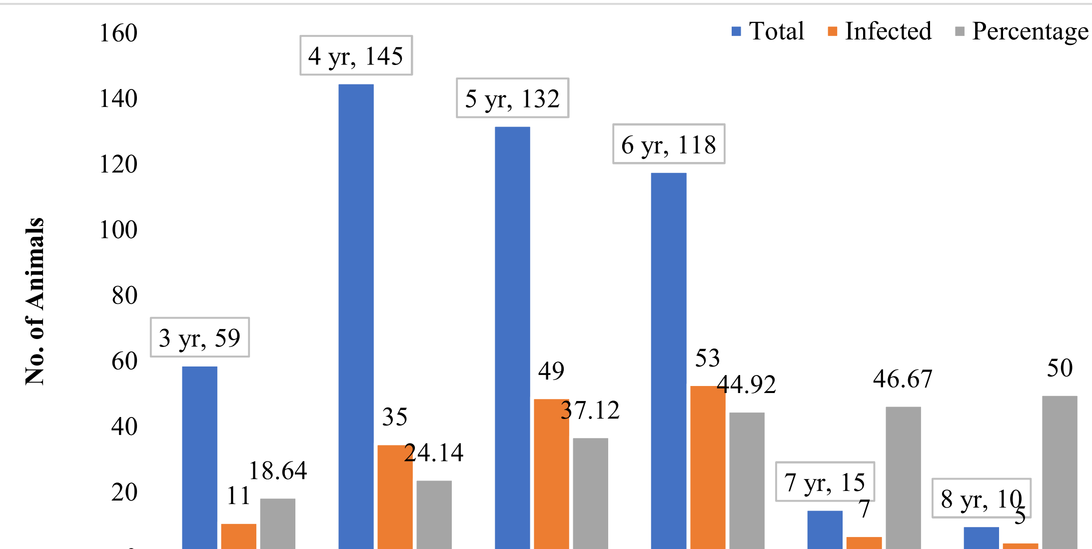
Mastitis is the swelling of udder parenchyma of lactating cows, which has severely affected both milk yield and milk quality. *Klebsiella pneumoniae* associated mastitis has caused economic losses in numerous ways including rejection of infected milk, therapeutic cost, labor cost, mortality, discarding of infected animals and lower fertility rates[21]. Antimicrobial drugs along with hygienic measures are important tools for control of mastitis. However, pathogens has developed resistance to different antibiotics and same have been revealed by investigators over the time, which is serious concern as therapy is becoming no more effective against mastitis pathogens[10].



	HF	Sahiwal	X bred
Total	124	116	239
Infected	45	35	80
Percentage	36.29	30.17	33.47

Breeds of Cows

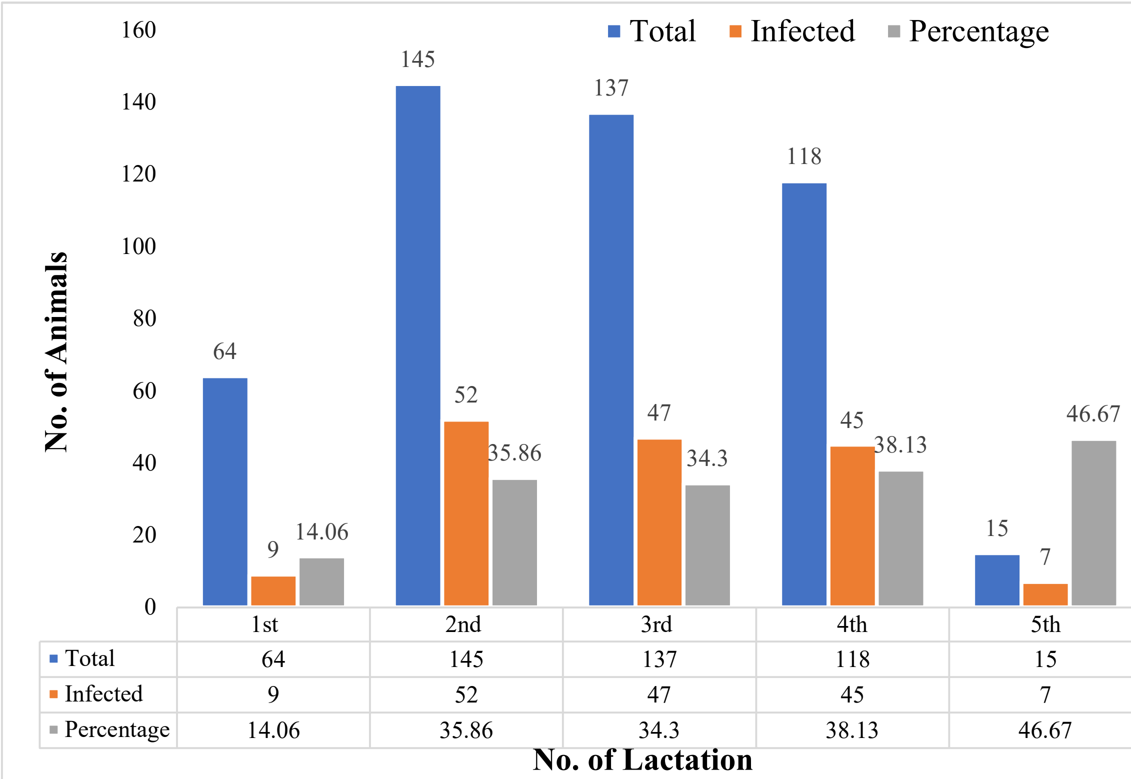
Figure 5: Breed wise prevalence of Mastitis in cattle



	3 yr	4 yr	5 yr	6 yr	7 yr	8 yr
Total	59	145	132	118	15	10
Infected	11	35	49	53	7	5
Percentage	18.64	24.14	37.12	44.92	46.67	50

Age (Yrs)

Figure 6: Age wise prevalence of Mastitis in cattle



	1st	2nd	3rd	4th	5th
Total	64	145	137	118	15
Infected	9	52	47	45	7
Percentage	14.06	35.86	34.3	38.13	46.67

Figure 7: Lactation wise prevalence of Mastitis in cattle

The present study was designed to isolate and identify *Klebsiella* sp. using conventional isolation, biochemical and molecular identification techniques. The second part of the study was to find out most effective antimicrobial for the treatment of *Klebsiella* associated mastitis through antibiotic susceptibility profiling of the isolated bacteria. For this purpose, milk samples were collected from the lactating cows suffering from both clinical and subclinical mastitis. All the lactating cows (n=479) belonging to 4 x different dairy farms in Lahore were examined for clinical and subclinical mastitis. Out of these lactating cows, 160 x animals (33.4%) were mastitis positive. Similar results have been reported in the region by Girma *et al*., (2012)[22] in which the mastitis prevalence in cows was reported 23.2 to 81.1%. Among 160 x mastitis cows, 34 x (21.23 %) cows were suffering from *Klebsiella* associated mastitis. This high prevalence of *Klebsiella* associated mastitis (21.23%) is similar to other survey conducted worldwide for prevalence of *Klebsiella* associated mastitis in America, Asia and Europe with prevalence from 33.5 to 45.0%[23–25]. Botrel *et al.,* 2010[26] reported higher prevalence in France (92%) and Egypt (51.67%). A lower prevalence in Africa (15–16%) and a very lower prevalence in Canada were also reported. Similar prevalence of *Klebsiella* associated mastitis (12.5%) was reported in Bangladesh[27].

Klebsiella pneumoniae causes both clinical and subclinical mastitis. Clinical mastitis can cause irregular milk production, enlarged quarters, malnutrition, and fever in cows. There are no apparent indicators of disease in subclinical mastitis. In this study, 160 milk samples were collected from mastitis positive cows. Out of these 160 mastitis positive animals, 18.78% (90 x cows) were found with clinical mastitis and 14.61% (70 x cows) were found with sub clinical mastitis. Kibebew (2017)[28] reported similar data on prevalence of subclinical mastitis in Pakistan and India as 17 to 93% in cows and 4 to 48% in buffaloes. This prevalence was also in line with data reported in different districts of Pakistan as Bachaya *et al.,* 2011[29] reported prevalence of mastitis in Tehsil Jatoi about 60%, with decreasing prevalence in Muzaffargarh, Kot Adu and Ali Pur was 48%, 41.6% and 30.4 % respectively.

Subclinical mastitis is also more prevalent in animals as it has no obvious sign and symptoms and it is difficult to diagnose. Different standardized protocols are used for its diagnosis as bacterial culture, PCR, CMT, white side test and SFMT[11]. In this present study, SFMT was used for detection of subclinical mastitis. Total 389 x lactating cows were screened for subclinical mastitis out of which 70 x cows (17.99%) were found positive for subclinical mastitis. Similar prevalence of subclinical mastitis with SFMT have been reported in different districts of Pakistan[30] and Bangladesh[31]. Previous studies conducted by had isolated *Klebsiella* sp. on *Klebsiella* selective agar. Purple magenta color colonies of *Klebsiella* isolates have been reported on *Klebsiella* selective agar[32,33]. Similarly in present study, *Klebsiella* isolates were isolated from mastitis positive milk samples by growing on *Klebsiella* selective agar, where these *Klebsiella* isolates produced characteristics purple magenta color colonies on *Klebsiella* selective agar. *Klebsiella* isolates produced pink color colonies on MacConkey agar and fermented lactose.

Mastitis in dairy animals is costliest disease which directly affects lactating animals so it is a concerning diseases for dairy farmers and milk processing industry. However, it has become concerning for consumer and public health stakeholders because of antibiotic residues and their resistance, milk quality and welfare of animals. This disease is difficult to control as numerous pathogens infects udder of lactating cows. Furthermore, due to absence of immunization antimicrobials are major tool for its treatment. Therefore, antibiotic sensitivity test is important for success of treatment[34]. The current study tested the resistance/sensitivity profile of 34 *Klebsiella isolates* against ten commonly used antibiotics such as amoxicillin, piperacillin, ceftriaxone, ceftazidime, ceftipime, imipenem, moxifloxacin, minocycline, cotrimoxazole, colistin and tigecycline combination, in dairy cows using the antibiotic disc diffusion test. The disc diffusion technique is the most commonly used method for assessing animal pathogen susceptibility, particularly in clinical practice where the right therapy must be determined[10,24]. The 34 *Klebsiella* isolates obtained from mastitis milk samples were tried against 10 antimicrobials for their antibiotic profiling. The results of antibacterial susceptibility testing revealed that all the *Klebsiella* isolates tried, were susceptible to Colistin and Tigecycline combination followed by decreasing sensitivity to Cotrimoxazole, Minocycline, Ceftipime and Imipenem. Sensitivity of Imipenem to most of *Klebsiella* isolates tested has proved that carbapenems are still potent against *Klebsiella* sp. similar recommendation of CLSI guidelines were published in 2016, which has kept this antibiotic among class of antibiotics effective against *Klebsiella pneumoniae.* All the *Klebsiella* sp. isolates were found to be completely resistant to Amoxicillin and Piperacillin. Current sensitivity pattern is similar to higher resistance to these antibiotics reported by previous studies[13,35]. The resistance noticed may be developed due to prolonged and indiscriminate use of antibiotics which led to development of resistance in microbes. Moxifloxacillin, Ceftriaxone and Ceftazidime were ineffective against 70.59%, 79.41% and 73.53% isolates of *Klebsiella* sp.

Though, current study found that, following antimicrobials were found effective against the isolates *Klebsiella* sp. form mastitis milk samples. Ceftipime was found effective against 50% of the bacterial isolates, Minocycline was found effective against 64.71% of *Klebsiella* isolates, Cotrimoxazole was found effective against 67.65% of *Klebsiella* isolates, Imipenem was found effective against 41.18% of *Klebsiella* isolates and Colistin & Tigecycline Combination was found 100% effective against all the tested *Klebsiella* isolates. Depending on antibiotic susceptibility pattern in this study, Colistin & Tigecycline combination should be drug of choice in case of *Klebsiella* sp. associated infection in Lahore Pakistan.

Abera *et al.,* (2010)[37] investigated the prevalence of mastitis caused by *Klebsiella pneumoniae*, associated risk factors and patterns of antimicrobial resistance in Ethiopia. Results showed significant variations among cattle of different age groups, lactation stages and housing systems. Similarly 3 factors *i.e.,* age, lactation and breed were selected as the parameters to study mastitis prevalence of in dairy farms under study. In present study, a total of 479 cows were studied to check the effect of different parameters on mastitis prevalence. Out of these cows, 124 cows were from Holstein Frisian (HF) breed, 116 x cows were from Sahiwal breed cows and 239 x cows were from Cross bred breed. 45 HF cows out of 124 were mastitis positive (36.29%), 35 x Sahiwal cows out of 116 were mastitis positive (30.17%), and 80 x cross bred cows out of 239 were mastitis positive (33.47%). This research discovered a link among breed and the mastitis occurrence. Mastitis was more likely in imported and cross breed cattle than in local breed[38,39]. Animals having mastitis were belonging to 6 x age groups which were 3, 4, 5, 6, 7 and 8 years of age. At the age of 8 years, about 50% cows were having mastitis. Prevalence was less at younger age as prevalence was 46.67% at 7 years of age, prevalence was 44.92% at 6 years of age, prevalence was 37.12% at 5 years of age, prevalence was 24.14% at 4 years of age and prevalence was 18.64% at 3 years of age. This study correlated that mastitis prevalence was high in older animals as they have weak immunity and other factors which was in line with other studies[40]. Animals having mastitis were belonging to different number of lactation as 1st, 2nd, 3rd, 4th or 5th lactation. Mastitis prevalence is found variable in animals of different lactation. As total 64 x cows of 1st lactation were present on these farms, out of them 9 x cows (14.06%) were having mastitis. 52 x cows of 2nd lactation out of 145 (35.86%) were having mastitis. While animals in 3rd lactation, 47 x cows out of 137 (34.3%) were having mastitis and 7 x cows out 15 (46.67%) were having mastitis during 5th lactation. This study correlated that in early lactation, lower mastitis prevalence was noticed than cows of mid lactation, however no apparent pattern was found for different lactation stages as early lactation or late lactation. Previous investigations[38,41,42] have also found evidence of a tendency of increasing prevalence at an early stage.

Conclusion

This study proves that *Klebsiella pneumoniae* is causative agent of 34% mastitis positive cows, which causes both clinical and subclinical mastitis. From the results it is also evident that age, lactation and breed of animal are some of the factors that are responsible for the mastitis prevalence. The study also suggests that in case of mastitis outbreak, morphological and biochemical identification of pathogen associated with mastitis is necessary and only effective antibiotic should be used against pathogen after antibiotic susceptibility testing (AST) for the isolated bacteria. This study finds that use of effective antibiotics will reduce therapeutic cost of mastitis at dairy farms as well as curb development of MDR strains, which are emerging threat for food security in Pakistan and worldwide. The study suggests that most effective antibiotic for *Klebsiella* associated mastitis is colistin and tigecycline combination. Findings of study are landmark and warrant further investigation to evaluate effective antibiotics for treatment of *Klebsiella* associated mastitis in different regions of Pakistan, so these studies can be used as reference studies in case of future outbreaks of *Klebsiella* associated mastitis in different regions of Pakistan.

Acknowledgement

Authors acknowledge the facilities provided by the Department of Microbiology, Government College University, Faisalabad, Pakistan for providing the lab facilities for the conduct of this research work.

Author Contribution

Tahir Zakria and Amber Khan performed the experimental work of this project and Muhammad Amjad supervised the overall research work and helped in the preparation of this manuscript.

Conflict of Interest

Authors declare no conflict of interest.

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