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RESEARCH ARTICLE

Epidemiological Features, Biochemical Indices, Antibiogram Susceptibility Profile and Biofilm Factor Genes of *Klebsiella pneumoniae* Isolated from Bovine Clinical Mastitis Cases

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A B S T R A C T

Bovine mastitis, a growing issue in dairy farms, with Klebsiella species being associated with severe mastitis. This study was designed to evaluate the prevalence, biochemical changes, antibiogram susceptibility profile, and biofilm-producing capacity of *Klebsiella pneumoniae* isolated from Holstein cows with clinical mastitis at a private farm in Alexandria, Egypt. The overall isolation rate of *Klebsiella* species was 28%, which was further characterized by polymerase chain reaction (PCR) targeting the 16S-23S internal transcribed spacer gene as *K. pneumoniae.* Clinical mastitis significantly impacted milk composition and blood biochemistry, causing a decline in milk composition, reduction of antioxidant capacity, and increased enzymatic levels compared to normal controls. *K. pneumoniae* isolates showed 100% resistance to beta-lactam antibiotics, followed by resistance rates of 57, 43 and 43% to chloramphenicol, streptomycin, and sulfamethoxazole–trimethoprim, respectively, while ciprofloxacin and ceftriaxone showed complete susceptibility. The study found that all *K. pneumoniae* strains expressed the fimA, mrkA, and mrkD fimbrial genes, and the ecpA gene. The study highlights the high prevalence of multidrug-resistant and biofilm-producing *K. pneumoniae* strains in bovine clinical mastitis, emphasizing the need for improved antimicrobial usage and antibiofilm approaches to overcome the poor treatment response, as well as measuring antioxidant level and enzymatic activity in milk improved milk health screening.

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INTRODUCTION

Bovine mastitis significantly impacts dairy farm's economic losses, putting pressure on human needs, particularly in African countries where livestock is a significant national resource *(*Zerfu *et al.,* 2021). Mastitis in cattle is caused by bacterial pathogens, including *Streptococcus agalactiae* and *Staphylococcus aureus* and coliforms. Klebsiella is a gram-negative opportunistic bacteria in the Enterobacteriaceae family, causing severe mastitis (Munoz *et al.,* 2007). Klebsiella species-induced udder infections significantly impact milk yield, clinical signs, and treatment response, leading to substantial economic losses (Fuenzalida and Ruegg, 2019). *K. pneumoniae* is a significant pathogen in humans associated with many nosocomial infections, responsible for up to 20% of pneumonia, sepsis, and urinary tract infections (Rice, 2010).

Klebsiella-associated udder pathogenicity is linked to biofilm formation and antibiotic resistance, with *K.*

pneumoniae being part of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp*) bacterial group frequently associated with antimicrobial resistance (AMR) (Rice, 2010). The Enterobacterales classifies it as opportunistic, hypervirulent and generally antibiotic-resistant (Sydow *et al.,* 2022). This is a Gram-negative, facultative anaerobic bacteria found in both human and animal natural flora (Gorodnichev *et al.,* 2023). It causes widespread infections in animals and humans, causing significant morbidity and mortality in farm animals and pneumonia, urinary tract inflammation, meningitis, wound infections, liver abscesses, and sepsis in humans (Chen *et al.,* 2022). AMR in *K. pneumoniae* leads to severe adverse complications due to the presence of resistance genes encoding a single antibiotic resistance phenotype. Furthermore, *K. pneumoniae* with multiple drug resistance (MDR) capacity has 10–1,000 times higher tendency to form biofilms than planktonic bacteria (Gorodnichev *et al.,* 2023).

K. pneumoniae's ability to produce biofilms, embedded in a self-produced extracellular polysaccharide matrix, enhances protection against host immune cells and reduces antibiotic therapy efficacy (Daini *et al.,* 2005). *K. pneumoniae* clinical strains contain numerous genes, including type I fimbriae, type III fimbriae, and capsular polysaccharide genes, which are crucial virulence determinants for initial colonization and biofilm formation (Sanchez *et al.,* 2013). *Escherichia coli* common pilus (ECP), an adhesive protein, has been detected in *K. pneumoniae* genome, playing a crucial role in cell adherence, biofilm creation, and bacteria-bacteria interactions (Lebeaux *et al.,* 2014). To the best of our knowledge, very few studies have described biofilm formation for *Klebsiella* isolates recovered from bovine mastitis cases.

In diagnostic laboratories, Biochemical tests in diagnostic laboratories often misdiagnose *Klebsiella*, leading to misdiagnosis with other bacterial species and some *E. coli* isolates being detected using polymerase chain reaction (PCR) (Alcántar-Curiel *et al.,* 2013). Sequencing the rpoB gene, a subunit of RNA polymerase could aid species detection but is time-consuming and unsuitable for economical diagnostic routines (Massé *et al.,* 2020). *K. pneumoniae* is the most prevalent Klebsiella species found in bovine clinical mastitis cases. Some other *Klebsiella* species were also reported but with much less importance, such as *K. oxytoca* and *K. variicola* (Ahmed *et al.,* 2016).

Over the years, there has been a significant focus on the development of indirect tests, particularly those used for screening intra-mammary infections (Ahmed *et al.,* 2016). Because the infection of the intra-mammary gland is multifactorial, the inflammation of the epithelial cells causes the release of several substances and enzymes that reduce the quality of the milk. These substances and enzymes are excellent and significant biomarkers of udder health. Additionally, various milk chemistry changes can be used to track the udder's health. This study investigates *Klebsiella* prevalence in Holstein Friesian cows with clinical mastitis in Alexandria, Egypt, and its impact on milk quality through milk biochemical characteristics estimation.

MATERIALS AND METHODS

Animals: A private dairy farm located in Alexandria governorate, Egypt, was followed for the emergence of clinical mastitis cases. A machine milking system was implemented twice daily, with milk production data records. Clinical examination of lactating cows detects clinical mastitis through visual inspection of udders and quarters, detecting disproportional symmetry, fibrosis, inflammatory swellings, injuries, and enlargement of supra-mammary lymph nodes. Milk was screened for abnormal physical changes, and cows with clinical mastitis were isolated in a separate pen for treatment.

Sampling: A study collected 50 milk samples from 16 dairy cows with clinical mastitis from October 2022 to September 2023, ensuring they had not received antibiotic treatment for at least a month. Samples were collected from infected quarters, discarded the first few milk strips, and transferred to the laboratory on ice after aseptic collection. Milk samples were aseptically taken from mastitis cows (n=50) and negative control cows (n=50). Milk samples underwent centrifugation at 7000 rpm for 5 minutes. Clear supernatant was pipetted into glass tubes for biochemical analysis.

Bacterial isolation: Each milk sample was streaked onto plates of nutrient agar, MacConkey agar (Oxoid, UK), and eosin methylene blue medium (Oxoid, UK), then the plates were incubated aerobically at 37°C for 24 h. Media were prepared according to standard procedures recommended by Constable *et al.* (2016). The presumptive isolates were confirmed at the species level based on PCR, targeting the *K. pneumoniae* species-specific 16S rDNA-23S rDNA ITS region.

***K. pneumoniae* species-specific PCR amplification**

Genomic DNA extraction: DNA extraction from the samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). PCR amplification was performed by targeting the 16S rDNA-23S rDNA ITS region. The 25-µL reaction containing PCR Master Mix, primers, water, and DNA template was performed in an Applied Biosystem 2720 thermal cycler. The PCR products were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH). A ladder (Fermentas, Germany) was used to determine the fragment sizes. The gel was photographed using a gel documentation system (Alpha Innotech, Biometra).

Antimicrobial susceptibility testing: The antimicrobial sensitivity of pure *K. pneumoniae* cultures was evaluated for various antibiotics using a Kirby-Bauer disk diffusion assay (Zadoks *et al.,* 2011). The Clinical and Laboratory Standards Institute categorizes antibiotic values into resistant, intermediate and sensitive categories, with antibiotic selection based on field use and potential determinant phenotypes, including ampicillin, ceftriaxone, ciprofloxacin, chloramphenicol, gentamicin, kanamycin, cephalexin, tetracycline, penicillin G, streptomycin, and sulfamethoxazole–trimethoprim. The disks were obtained from Nissui Pharmaceutical (Tokyo, Japan). The MAR index was calculated according to Bhutto *et al.* (2012).

Biofilm formation

PCR assay for the detection of biofilm-associated genes: PCR was used to identify *K. pneumoniae* strains and detect biofilm-associated genes like mrkA, mrkD, ecpA, and fimA. DNA was extracted from bacterial colonies, with primer sequences and conditions listed in Table 1.

Biochemical analysis: Milk samples were used to determine aspartate transaminase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), Urea, total protein, albumin, triglycerides (TG), malondialdehyde (MDA), and total antioxidant capacity (TAC), phosphorus, sodium, chloride, potassium, and calcium levels using the colorimetric method according to kits supplied by Chema Diagnostica (Monsano, Italy).

Statistical analysis: Chi-square test was used for the analysis using SPSS version 24.0 (IBM. Corp., Armonk, NY). The statistical significance of the comparison between the mastitis and control groups was assessed using Student's t-test. Results were presented as the mean standard error of the mean (SEM), and P-values less than 0.05 were considered statistical significance.

RESULTS

Clinical and epidemiological features of clinical mastitis in cows: The prevalence of clinical mastitis at the cow's level was 6.4% meanwhile the quarter-level prevalence was 5% based on clinical examination of udder and physical changes observed in milk.

The farm's clinical mastitis incidence was significantly influenced by cow parity (P= 0.005), with the 2nd parity group having the highest prevalence (48%), followed by multiparous cows (≥3) while primiparous cows showed the lowest percentage (12%) of clinical mastitis. The lactation stage significantly impacts clinical mastitis case distribution (P= 0.04), with early and late stages being the most susceptible (48 and 36%, respectively). The emergence of clinical mastitis showed a strong significant relationship with cow's age (P= 0.04) where adult cows were more infected compared with old cows. The frequency of clinical mastitis cases revealed a significant association with the season of the year (P= 0.03). The higher frequency of clinical mastitis was during summer (40%) followed by winter (28%) and spring (20%) Fig. (1).

Regarding *K. pneumoniae* infection with the various risk attributes (Fig. 1), the 2nd parity group had the highest percentage (50%) of *K. pnuemoniae* infection followed by primiparous cows. Early lactation stage was found to possess (50%) of *K. pneumoniae* infection. Unlike the prevalence of clinical mastitis, the emergence of *K. pneumoniae* clinical mastitis showed a higher infection rate (57%) in old cows compared with (43%) adult cows. *K. pneumoniae* infection was most frequent during summer (50%) followed by winter (28.5%) and spring (21.5%) while, no *K. pneumoniae* infected mastitis cases were found during autumn.

Bacterial isolation of *Klebsiella* species: *Klebsiella* colonies were identified on nutrient agar as large, circular, smooth and convex colonies, whereas, on MacConkey's agar, they appeared as large, shiny, pink, mucoid, dome-shaped colonies, and on Eosin Methylene Blue agar, they appeared as mucoid pink colonies. Specifically, *Klebsiella* colonies were suspected in 14 milk samples, representing 28% of the total quarters with clinical mastitis.

Molecular identification of *K. pneumonia:* All 14 isolates (100%) yielded the target PCR products at 130 bp.

Antibiotic sensitivity profile: The obtained results revealed that all *K. pneumoniae* strains were resistant to antimicrobials of the beta-lactam family, including ampicillin, amoxicillin with clavulanic acid, and penicillin G (100% each). Moreover, the strains were highly resistant to chloramphenicol, streptomycin, and sulfamethoxazole–trimethoprim (57, 43 and 43%, respectively). In contrast, all *K. pneumoniae* strains were susceptible to ciprofloxacin and ceftriaxone (100% each); meanwhile, most strains were susceptible to kanamycin, cephalexin, and tetracycline (71% each).

Antimicrobial resistance patterns of the *K. pneumoniae* isolates against 12 antimicrobial agents are presented in Table 2. A greater proportion of *K. pneumoniae* isolates (92.8%) had MDR, whereas 12 of the 14 isolates (85.7%) displayed MAR for at least three different antimicrobial classes. The MAR index was higher than 0.2 in all *K. pneumoniae* isolates. *K. pneumoniae* showed significant resistance levels to antimicrobial agents belonging to the beta-lactam family, ceftriaxone, ciprofloxacin, kanamycin, cephalexin, and tetracycline ($P<0.05$); meanwhile, sulfamethoxazole–trimethoprim and chloramphenicol had higher levels of significant difference ($P<0.01$).

Biofilm formation activities

Detection of biofilm genes in *K. pneumoniae* isolates: The *ecpA* gene was highly prevalent among *K. pneumoniae* strains where all *K. pneumoniae* strains were PCR-positive. 100% of the *K. pneumoniae* strains possessed the following biofilm-associated genes-mrkA, mrkD, ecpA, and fimA- and provided their characteristic bands.

Changes in biochemical parameters in milk: The biochemical results of the mastitis and control cow groups are shown in Fig. 3. The data revealed a significant increase in the values of AST (3-fold), LDH (1.8-fold), ALP (2.7-fold), urea (2.6-fold), total protein (5.3-fold), albumin (3.1-fold), phosphorus (0.2-fold), sodium (0.5-fold), chloride (0.6-fold) and MDA(3.5-fold) in the diseased group compared to the control group, and marked decrease in the TG (0.5-fold), TAC (0.7-fold), calcium (0.3-fold) and potassium (0.5-fold) level in the diseased group compared to the control group.

DISCUSSION

This study investigated *Klebsiella* isolate prevalence, antimicrobial profile, biofilm-producing capacity, and molecular characterization from clinical mastitis cows in Alexandria, Egypt. The study found a 6.4% prevalence rate of clinical mastitis in 16 cows, compared to a quarter-level prevalence of 5%. Similarly, a study in Beni Suef, Egypt, found a clinical quarter level prevalence of 5.81%, and an

Table 1: Primers sequences, target genes and amplicon sizes.

	Target gene	Primers sequences	Amplified segment (bp)
Biofilm genes of *K. pneumoniae*	*FimA*	CGGACGGTACGCTGTATTTT GCTTCGGCGTTGTCTTTATC	436
	mrkA	CGGTAAAGTTACCGACGTATCTTGTACTG GCTGTTAACCACACCGGTGGTAAC	475
	ecpA	GCAACAGCCAAAAAAGACACC CCAGGTCGCGTCGAACTG	477
	mrkD	CCACCAACTATTCCCTCGAA ATGGAACCCACATCGACATT	226
K. pneumoniae	*16S-23S ITS*	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130

Table 2: Antimicrobial resistance patterns of the 14 *K. pneumoniae* isolates against 12 antimicrobial agents.

No. of patterns	No. of antimicrobial agents	No. of antimicrobial classes	Antimicrobial resistance pattern	No. of *K. Pneumoniae* isolates	MAR index of isolates
1	7	4	AMC, AMP, P, CL, STR, KAN, TE	2	0.583
2	7	5	AMC, AMP, P, CL, STR, CHL, SXT	1	0.583
3	6	4	AMC, AMP, P, CL, STR, CHL	1	0.5
4	6	3	AMC, AMP, P, GEN, STR, SXT	2	0.5
5	6	3	AMC, AMP, P, GEN, KAN, CHL	2	0.5
6	5	3	AMC, AMP, P, CHL, SXT	2	0.416
7	5	3	AMC, AMP, P, CHL, TE	2	0.416
8	4	2	AMC, AMP, P, SXT	1	0.333
9	3	1	AMC, AMP, P	1	0.25

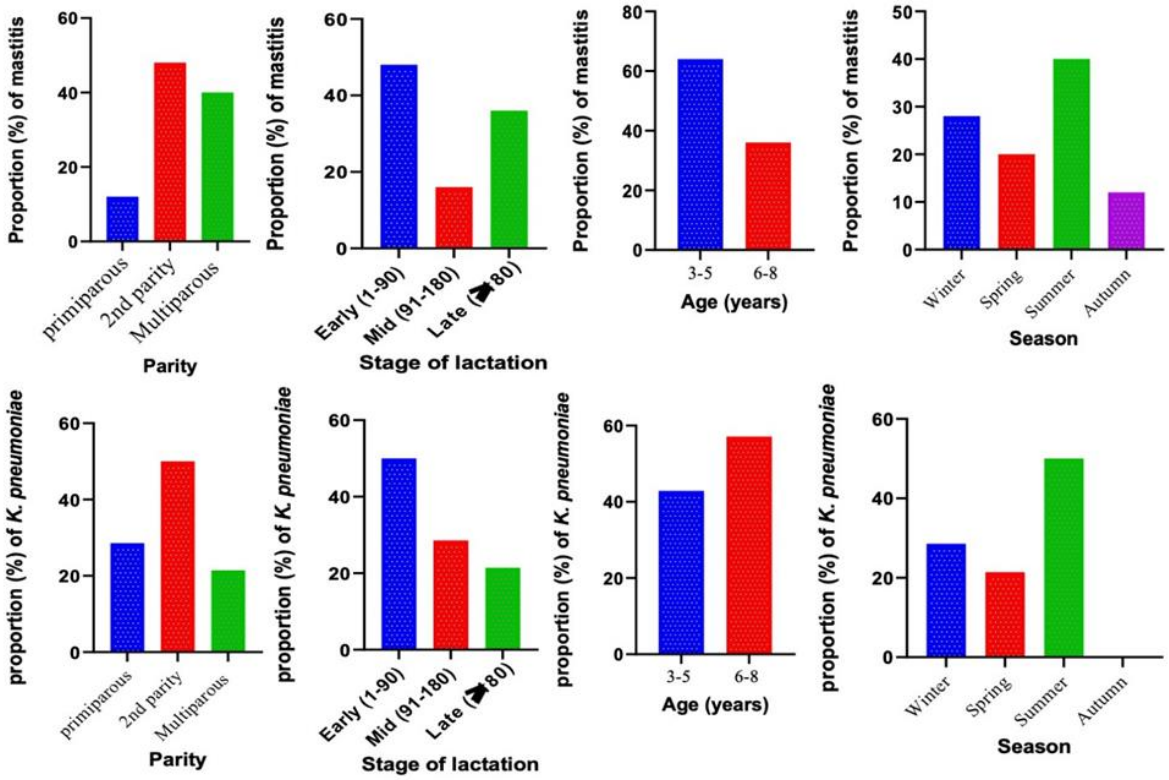

Fig. 1: The association between the occurrence of clinical mastitis, *K. pneumoniae* infection and various risk factors.

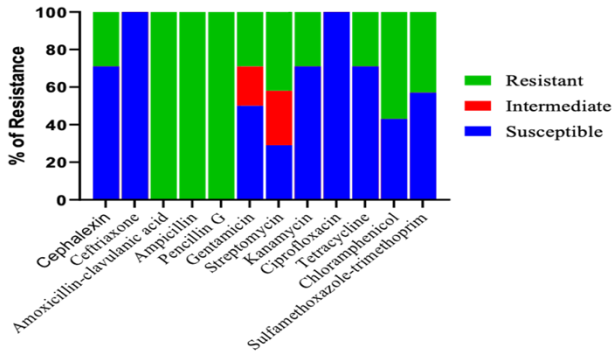

Fig. 2: Antibiogram of *Klebsiella pneumoniae* isolates.

individual level prevalence of 9.87% of clinical mastitis (Kaczorek-Łukowska *et al.*, 2021). We found a 28% prevalence rate of *Klebsiella* isolates in milk samples. A 14.6% prevalence rate of *Klebsiella* species was observed in dairy farms surrounding Cairo, Egypt (Abebe *et al.*, 2016). This variation in the prevalence rates might be related to geographic locality, the number of samples, biosecurity, and herd immunity.

Results revealed a strong significant relationship with cow's age where adult cows had higher infection rate than old ones which came in agreement with Kaczorek-Łukowska *et al.* (2021), aligning with Salauddin *et al.* (2019). Old cows have a higher infection rate of *K. pneumoniae* may be due to their large pendulous udder,

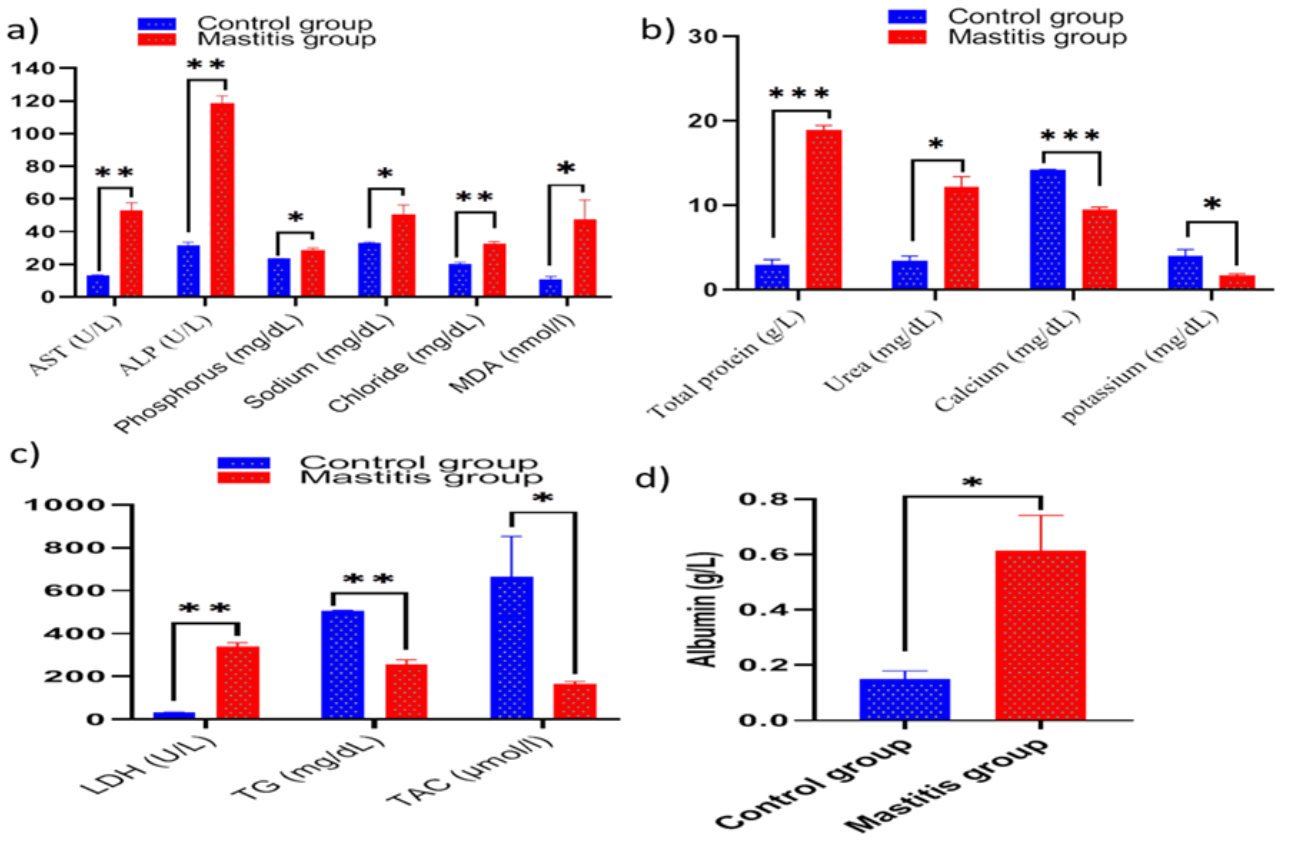

Fig. 3: The effect of clinical mastitis on the biochemical parameters and oxidative stress markers in cow's Milk. Data are presented as means ± SD. *P<0.05 vs control group. **P<0.01 vs control group. ***P<0.001 vs control group *AST: aspartate transaminase; ALP: alkaline phosphatase; MDA: malondialdehyde; LDH: lactate dehydrogenase; TG; triglycerides: TAC; total antioxidant capacity.

which is susceptible to injuries. Cow parity significantly influenced clinical mastitis cases, with the highest percentage (48%) occurring in the 2nd parity group, aligning with Kaczorek-Łukowska *et al.* (2021). Early stage was found to be the most susceptible stage (48%) to clinical cases of mastitis involving 50% of *K. pneumoniae* infected samples. Consistent with our findings, Osman *et al.*, 2014 attributed this higher susceptibility during the early lactation stage to the marked reduction of antioxidant defense mechanisms and higher physiological demands. Results showed increased cases of *K. pneumoniae* infection during summer (50%) followed by winter (28.5%) while, no *K. pneumoniae* infected mastitis cases were found during autumn. Gomaa (2021) observations suggest that favorable hot environmental conditions may enhance the proliferation of pathogens during hot weather.

The study identified 100% of Klebsiella isolates at the species level using PCR targeting the *K. pneumoniae* 16S-23S ITS gene. The findings align with Massé *et al*. (2020). Our study revealed that all *K. pneumoniae* isolates were 100% resistant to ampicillin, penicillin G, and amoxicillin/clavulanate, as per previous research (Mirzaie and Ranjbar, 2021); however, The isolates did not exhibit resistance to third-generation cephalosporins, ceftriaxone, fluoroquinolones, and ciprofloxacin, a finding consistent with previous research (Tremblay *et al.*, 2014).

Udder pathogens' antimicrobial resistance varies across studies, with a high rate of 43% observed in this study despite the European Committee of Antimicrobial Susceptibility Testing reporting no resistance to streptomycin (McAloon *et al.*, 2022). We found a resistance rate of 43% in *K. pneumoniae* isolates against trimethoprim/sulfamethoxazole. Dallal *et al*., 2018 found that most *K. pneumoniae* isolates were trimethoprim and sulfamethoxazole-resistant (88.2 and 91.7%, respectively) in Egypt. In our study, 57% of the *K. pneumoniae* isolates under study developed resistance to chloramphenicol, which is lower than the rate (82.6%) reported by Abebe *et al*. (2016). We found that most *K. pneumoniae* strains were susceptible to kanamycin, cephalexin, and tetracycline (71%). We found that 92.8% of *K pneumoniae* isolates were MDR, with 84.7% displaying MAR for at least three antimicrobial classes, consistent with a previous study conducted in Egypt, reporting that 91.7 and 95.77% of *K. pneumoniae* isolates were MDR, respectively (Amira *et al.*, 2016).

K. pneumoniae's pathogenicity is attributed to its ability to form biofilms, which enhances its resistance to antimicrobials, in addition to antibiotic resistance (Tremblay *et al.*, 2014). The molecular level of the biofilm formation ability of Kklebsiella. species recovered from bovine clinical mastitis cases is under-documented. Interestingly, the PCR-based detection of the *fimA*, *mrkA*, and *mrkD* fimbrial genes showed that 100% of the *K. pneumoniae* strains carried the three genes under study. These results agree with those reported by Alcántar-Curiel *et al*. (2013), who found the *mrkA* fimbrial gene in 100% of *K. pneumoniae* strains, whereas 78% carried the *fimA* gene.

The study of milk's biochemical components in mastitis infections could enhance our understanding of the pathogens involved and potentially aid in the discovery of

their biomarkers. Our findings showed that compared to milk from non-infected cows, mastitis milk had significantly higher levels of AST, LDH, ALP and urea. These findings align with those of Lebeaux *et al.* (2014). Milk's elevated enzyme levels are primarily due to increased microcirculatory vessel permeability in inflamed areas, leukocyte, and degenerating parenchyma cell leakage. Higher levels of milk immunoglobulin and albumin have been observed in cows with mastitis (Coulona *et al.*, 2002). Mastitis increases TP in milk due to immune response activation, causing inflammation-associated blood proteins to enter the mammary gland. Also, the decrease in triglyceride levels in mastitis milk in this study is coordinated with another study which revealed a decrease of milk fat in mastitis (Chang *et al.*, 2011) and may be due to the lipolysis of milk globule membranes by leucocyte lipases or plasmin, thereby reducing the mammary gland's secretion capacity and fat concentration.

The milk of cows with mastitis had rising levels of sodium, chloride, and phosphate and decreasing levels of potassium and calcium. These results were in line with those of El Zubeir *et al.* (2005). Mastitis in cows increases milk conductivity, leading to increased sodium and chloride levels. Inflammatory cytokines reduce parathormone secretion, potentially causing calcium levels to drop. Maintaining isotonic milk osmolality is believed to cause a reduction in milk potassium concentration, while phosphorus' role in enhancing milk immune cell phagocytic activity contributes to the increase.

Milk's oxidative state, measured by MDA, is crucial for udder health assessment. Total antioxidant capacity provides a clear understanding of the body's antioxidant status (Farghali *et al.*, 2021). Our findings were in line with those of Zigo *et al.* (2019). Increased lipid peroxidation can be seen during inflammatory processes in the mammary gland, which can cause MDA levels to rise. TAC activity in milk may have decreased due to their depletion from combating free radicals during the oxidative process.

Conclusions: The study revealed a high isolation rate of *K. pneumoniae* strains (28% in bovine clinical mastitis cases), an increasing rate of resistant strains to antibiotics, and their high biofilm formation capacity. It suggests a link between AMR and biofilm production, potentially explaining the poor treatment response in *K. pneumoniae*-infected animals and the severe, aggressive, and long-lasting clinical mastitis. Also, it offers a reference for preventing and treating bacterial mastitis in dairy cows, while also preventing global transmission of zoonotic pathogens.

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Authors' contributions: All authors participated in the conception and design of the study; AAS, HEME, SIK, AH, and EBA collected the samples, performed laboratory work, and drafted the manuscript. MMAE, AIAZE, and MBS shared in the laboratory works, analyzed the data, and revised the paper. All authors have read and approved the final version of the manuscript for submission.

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Availability of data: The data can be obtained from the author upon request.

Ethical approval: Samples from clinical mastitis cows were collected using scientific procedures and routine bacteriological and molecular diagnostic methods under the ethics committee of Zagazig University, Egypt (approval number: ZU-IACUC/2/F/275/2022).

Consent for publication: Not applicable.

Competing interests: The authors declare that they have no competing interests.

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