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Prevalence, Associated Risk Factors and Isolation of the Major Causative Agent of Subclincal Mastitis in Dairy Cattle

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ABSTRACT

Mastitis is caused by a variety of microorganisms, including bacteria, protozoa, yeasts/fungi, and algae, which can penetrate and multiply in the udder, producing pathogenic substances that cause inflammation. Subclinical mastitis caused by Staphylococcus aureus has important public health implications. The objective of this study to observe prevalence of subclinical mastitis and associated risk factors in dairy cattle, isolation andidentification of S. aureus, risk factors associated with subclinical mastitis as well to determine antimicrobial resistance patterns. A total of 400 milk samples were collected from different farms in the Faisalabad district. The California mastitis test was used to determine whether subclinical mastitis was present. Standard microbiological methods were then applied to 42 positive milk samples in order to isolate and identify *S. aureus*. The disc diffusion method was used for antibiogram analysis in order to evaluate antibiotic resistance. The prevalence of subclinical mastitis (SCM) at the cow and quarter levels was 22% and 10.5%, respectively. Vancomycin, erythromycin, gentamicin, ciprofloxacin, and chloramphenicol showed the highest susceptibility to antimicrobial susceptibility testing, whilst tetracycline showed the highest rate of resistance. The prevalence of subclinical mastitis in cows was substantially associated with factors such as age, parity, and milk production. These results highlight the necessity of more stringent aseptic management in dairy farms to stop animal-to-animal disease transmission and ensure the production of clean, safe food for human consumption.

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INTRODUCTION

Mastitis is a multi-etiological disease linked to the cattle production system and rearing environment. It is characterized by physical, chemical, and bacterial alterations in the mammary gland tissue (Kibebew 2017). The main cause of Mastis are inflammation of the udder gland tissue and the organism's defense response to certain elements, and the presence of the microbes within the mammary gland (Abrahmsén et. 2014). When the organism are immunocompromised, the infection spreads. Mastitis primarily impacts milk production, which results in financial losses for dairy farmers worldwide. This production disease is regarded as a major disease of dairy cows, resulting in reduced milk output, loss of milk quality and quantity, losses from rejected milk, premature culling, treatment costs, and additional labor costs (Tommasoni et al. 2023).

The three kinds of mastitis, sub-clinical, clinical, and chronic depend on the animal's breed, age, immunity, stage of lactation, and causative organisms (Samad 2022). Because there are no obvious changes in the milk and it is difficult to identify, sub-clinical mastitis (SCM) significantly reduces milk production. Gross observations

of clinical mastitis (CM) include udder enlargement, flake-containing milk, clots, and watery milk. In dairy animals, chronic mastitis is an uncommon type that causes ongoing inflammation of the mammary gland (Zigo et al. 2021).

A significant reduction in milk production, thinning and discoloration of milk, the presence of flocculent material, and elevated body temperature are all signs of clinical mastitis (CM), which presents as an abrupt onset of varying degrees of erythema, oedema, hyperthermia, and discomfort in the affected mammary quarter (Neculai-Valeanu and Ariton 2022). Subclinical mastitis is the most prevalent kind, characterized by an increased number of somatic cells in the milk without any visible abnormalities in the udder or milk (Sumon et al. 2020).

A few common bacterial infections are responsible for the majority of mastitis cases. Both environmental pathogens (*Escherichia coli, Klebsiella spp., coagulase-negative staphylococci CNS*, and *S. uberis*) and infectious pathogens (*S. aureus, S. agalactiae, Mycoplasma spp*., and *C. bovis*) can cause mastitis. *Staphylococcus aureus*, a well-known bacterium that causes numerous serious infections in human and animals worldwide, is the cause of mastitis. The primary cause of both preclinical and symptomatic cow mastitis is *S. aureus*. Because it may produce many enterotoxins in milk and dairy products, it is the second most common cause of food poisoning in humans (Ewida and Al-Hosary 2019).

Antimicrobial therapy is a crucial technique in the management of mastitis since bacteria are the most frequent cause of the condition. However, using antimicrobials misssue increases the risk of bacteria developing antibiotic resistance. These days, the development and spread of antimicrobial resistance (AMR) bacteria and the risk they pose to humans are worldwide issues of great concern in both human and veterinary medicine (Ferri et al. 2017). Dairy farms may include human pathogenic bacteria that are resistant to antibiotics, and the use of antibiotics in food animal production and their inappropriate treatment of mastitis contribute to the development and spread of antimicrobial resistance (Oliver et al. 2011). Timely detection and comprehension of the range of infections linked to mastitis are essential for its prevention and management (Ashraf et al. 2020).

The gold standard for diagnosing mastitis is the identification of intramammary pathogens present in milk. Nevertheless, this is expensive, time-consuming, and only partially applicable in field settings. The International Dairy Federation (IDF) recommends that the most popular tests to identify changes in milk due to an inflammatory process are the somatic cell count (SCC) and the microbiological state of the quarter (Adkins and Middleton 2018). Both direct and indirect procedures, such as the California Mastitis Test (CMT) and Somatic Cell Count (SCC), are used to diagnose subclinical mastitis. While SCC serves as a vital indicator of inflammation and milk quality with high accuracy in identifying infected quarters, the CMT offers a quick, affordable, cow-side screening method by gelling with DNA (Zeweld and Tarekegn 2025). The primary aim of this study was to estimate the prevalence of subclinical mastitis in dairy catel, investigate the main associated risk factor, and isolate the bacteria responisble for infection.

MATERIALS AND METHODS

Ethical Consideration

The Ethics Review Committee (ERC) of Government College University Faisalabad provided ethical permission for the study (GCUF/ERC/19/20) prior to its beginning. The methods used to acquire the samples were in accordance with international standards for biological samples from cows (De Baere et al. 2023).

Sample Collection

This study included 100 healthy milking dairy cows with 400 functional quarters chosen at random from different farms. Milk samples were obtained during nighttime milking in the milking parlor, with cows confined and teats completely cleansed and wiped. Milk samples (3–4 ml) were collected aseptically from each quadrant (Singh et al. 2024).

Screening of the milk sample

The first milk samples were taken during nighttime milking and examined for signs of subclinical mastitis. Each udder was individually wiped and dried after being cleansed with water and a biocide solution prior to testing. Two milliliters of milk were extracted from each quarter of the udder for testing and combined with an equivalent volume of commercial CMT reagent (RAIDEX GmbH, Dettingen on the Erms River, Germany) after the first stream of milk was discarded (Tawfik et al. 2022). The paddle was gently moved in a horizontal plane for a few seconds to achieve mixing. On a scale of 0 to 4, the viscosity of the final combination was assessed. Scores of two, three, and four were categorized as positive, while scores of zero and one were categorized as negative and trace, respectively. A cow was deemed to have subclinical mastitis if it tested positive for at least one quarter with a score of two or above. A sterile tube containing ten milliliters of milk was taken from a positive quarter and stored at 4 °C until it reached the lab. Within a day, milk samples were delivered to the microbiology lab, where they were processed right away.

Isolation and Identification of *S. aureus*

The milk samples that were positive for the screening were centrifuged for five minutes at 3000 rpm after being incubated for twenty-four hours at 37 °C. After removing the cream layer, the sediments were spread out onto mannitol salt agar, blood agar, and nutrition agar plates. The streaked plates were then incubated at 37 °C for 24 to 48 hours. The suspected growing colonies were identified using the morphological and biochemical techniques. Gram staining was performed for confirmation of bacteria. Round convex golden-yellow colonies of *S. aureus* were collected and kept in a medium containing 10% glycerol (v/v) at -80 °C for further analysis (Pérez et al., 2020).

Antimicrobial Susceptibility Test

Antibiotic susceptibility was evaluated using the disc diffusion technique in accordance with the guidelines

provided by the Clinical and Laboratory Standards Institute (CLSI). As shown in the table, various antibiotics were used to determine the isolates' susceptibility patterns (Gaur et al. 2023).

Table 1: Interpretive Criteria for Zone of Inhibition Diameter of different antibiotics

Antibiotic	Disc conc	Resistance (mm)	Sensitive (mm)
Vancomycin	30 g	≤14	≥15
Gentamicin	10 µg	≤12	≥15
Tetracycline	10 g	≤14	≥19
Erythromycin	15 µg	≤13	≥18
Ciprofloxacin	5 g	≤15	≥21
Oxacillin	1 µg	≤10	≥13
Chloramphenicol	30 µg	≤12	≥18
Amoxicillin/clavulanic acid	10–20 µg	≤19	≥20

Associated risk factors for subclinical mastitis

In this study, the role of factors including age and parity in subclinical mastitis was examined. Each milking dairy cow that was employed at random was chosen to be included in the study; these parameters were appropriately and successfully gathered, documented, and used in the study. Additionally, adherence to dry cow therapy and milking cleanliness were personally inspected and documented (Salum et al. 2022).

RESULTS

Prevalence of subclinical mastitis

The prevalence of subclinical mastitis was found in 22% of cows and 10.5% of quarters in this study. The detail is given in table 2.

Table 2: Prevalence of subclinical mastitis (SCM) at cow and quarter levels

Type of mastitis	Number of examined cows	SCM No.	%	Number of examined quarters	SCM No.	%
Subclinical mastitis (SCM)	100	22	22.0	400	42	10.5

Prevalence of *S. aureus* Subclinical Bovine Mastitis

Using the California mastitis test (CMT), 42/100 (10.5%) milk samples had subclinical mastitis (Table 1). Additionally, *S. aureus* was detected in 42 out of 100 bovine milk samples; tests for coagulase, catalase, and Gram staining all produced positive results for the *S. aureus* isolates.

Table 3: Results of CMT Screening in the Collected Samples

CMT Grades	Examined Quarters (no)	Positive *S. aureus* isolates (no)	*S. aureus* isolates (%)
+++	18	9	50
++	12	6	50
+	12	4	33
Total	42	19	100

Antimicrobial Susceptibility Profiles of *S. aureus* Isolates

The *S. aureus* isolates' antimicrobial susceptibility profile showed that they were susceptible to gentamicin, ciprofloxacin, vancomycin, Amoxicillin/clavulanic acid, and chloramphenicol. Nevertheless, a significant resistance rate of 37% (7/19) to erythromycin and 58% (11/19) to tetracycline and 32% (6/19) to oxacillin was seen in this study. The detail is given in table 4.

Table 3: Antimicrobial Susceptibility Pattern of *S. aureus* Isolates

Antibiotic	Sensitive	Resistance
Vancomycin	19 (100%)	0 (0%)
Gentamicin	10 (100%)	0 (0%)
Tetracycline	8 (42%)	11 (58%)
Erythromycin	12 (63%)	7 (37%)
Ciprofloxacin	19 (100%)	0 (0%)
Oxacillin	13 (68%)	6 (32%)
Chloramphenicol	19 (100%)	0 (0%)
Amoxicillin/clavulanic acid	19 (100%)	0 (0%)

Risk Factors Linked to subclinical mastitis prevalence in dairy cows

The prevalence and risk factors for sub-clinical mastitis in dairy cows that are milking are shown in Table 5. In terms of the risk variables noted, parity number had a significant role in the development of subclinical mastitis since it was found that the likelihood of developing subclinical mastitis increases with parity number. The management factors were the reason for the increased incidence of subclinical mastitis. One of the predictors was age, which raised the risk of subclinical mastitis. However, in this study, the influence of age led to a lower percentage of subclinical mastitis than parity.

Table 5: Prevalence and associated risk factors with subclinical mastitis in milking dairy cows

Risk Factors	Number of cows examined	Cows with mastitis	Prevalence
Age			
1–5	48	11	22.91%
6–10	46	9	19.56%
>10	6	2	33.33%
Parity			
1–3	67	6	8.9%
4–6	29	13	44.82%
>6	4	3	75%
Milking hygiene			
Poor hygiene	100	22	22%
Good hygiene	0	0	0%
Dry cow therapy			
No dry cow therapy	100	22	22%
Adherence to dry cow therapy	0	0	0%

DISCUSSION

Subclinical mastitis is a common disease that costs cattle companies income all over the world. The effectiveness of current antibiotic therapy has been adversely affected by the possibility of antibiotic resistance spreading to humans and the rise in antibiotic resistance among many bacterial illnesses. This seems to be an emerging challenge and a major public health concern (Shafeeq et al. 2021).

The prevalence of subclinical mastitis at the cow and quarter levels in the current study was 22% and 10.5%, respectively, which is somewhat lower than earlier findings in Kosovo, which were 25.6% and 12.2%, respectively, as reported by Sylejmani et al. (2016). The

reason for the slight decrease in the prevalence of SCM in this study is most likely due to the country's ongoing subsidisation of cow's milk based on quality, which encourages farmers to engage and take measures to improve the quality of milk, both in terms of cow maintenance and farm environment. In contrast Hiitio et al. (2017) reported a 19% prevalence of subclinical mastitis in Finland in 2010, but Themistokleous et al. (2019) found a 34.5% prevalence in Greece. The variation in mastitis prevalence between different studies may be caused by a variety of diagnostic methods, sampling strategies, and mastitis measurements, as well as factors including the lactation stage, parity number, and breed of animals utilised in the studies. Subclinical mastitis is more common in dairy livestock due to a number of predisposing factors, including (1) contaminated milking equipment, (2) inappropriate housing, (3) poor hygiene, and (4) poor animal handling. In addition, treatment failure was invariably brought on by the development of drug-resistant bacteria, a persistent infection followed by fibrosis, and an inadequate antibiotic dosage (Michira et al. 2023).

Out of 42 milk samples that tested positive for CMT, 19/ 42 (45%) of cows with subclinical mastitis had *S. aureus* isolated from them. In contrast to our findings, *S. aureus* was identified from animals with subclinical mastitis at a rate of 36% (Algammal et al. 2020). And another study conducted by Shahid et. (2023) reported that 40% (40 out of 100 CMT-positive milk samples). The use of contaminated milk equipment and contaminated milkers' hands are the main ways that *S. aureus* spreads between animals.

Furthermore, the antimicrobial susceptibility tests revealed that 19 isolates were susceptible to ciprofloxacin, gentamicin, amoxicillin/clavulanic acid, vancomycin, and chloramphenicol. Nevertheless, 32% (6/19) of the isolates showed oxacillin resistance. According to the previous study condcuted by Zihad et al. (2021) explained that the isolated *S. aureus* was shown to be completely resistant to penicillin, oxacillin, and vancomycin (21%). On the other hand, tetracycline, gentamycin, norfloxacin, and levofloxacin were all effective against all isolates.An important step in the development of new antibiotic combinations was the identification of clavulanic acid, a naturally occurring β-lactamase inhibitor from Streptomyces clavuligerus. The β-lactam antibiotics sulbactam and clavulanic acid are strong β-lactamase inhibitors but have comparatively little bactericidal activity.

However, erythromycin sensitivity was found in 63% (12/19) of all isolates. In a similar vein, 63.1% susceptibility to erythromycin was noted in another investigation (Algammal et al. 2020). Erythromycin is a macrolide antibiotic that exhibits strong antibacterial activity against both Gram-positive and Gram-negative bacteria, including *Escherichia coli, streptococci*, and *staphylococci*.

In the current study (11/19), 58% of bacteria were resistant to broad-spectrum antibiotics such tetracycline, resulting in the establishment of resistant strains due to their widespread use. 59.5% of strains were resistant to tetracycline, according to a prior conducted by Foster et al. (2017). The study conducted by shahid et al. (2023) showed that (24/40), 60% of bacteria were resistant to tetracycline. Tetracycline resistance was brought on by *S. aureus's* ribosome protection protein, which binds to tetracycline in a competitive manner.

Age group, parity, milking cleanliness, and dry cow therapy were the risk factors taken into account for this study. Age, parity, milking cleanliness, and dry cow therapy were among the chosen potential risk variables in the current study that had an impact on the incidence of mastitis, which is consistent with the findings of Zeryehun et al. (2013) in Addis Ababa, Ethiopia. The age factor in this study was divided into three groups: 1–5 years old, 6–10 years old, and >10 years old. The prevalence of these groups was 22.91%, 19.56%, and 33%, respectively. Therefore, age in connection to subclinical mastitis in this study appears to indicate that the likelihood of contracting mastitis increases with age. According to Tewari (2014) in India, age increases the incidence of subclinical mastitis (Sanotharan et al. 2016), and Batticaloa in Sri Lanka revealed that the prevalence of various age groups was documented in ranges of below 5 years (45%), 5-8 years (62.5%), and above 8 years (75%). Older animals may be more susceptible to SCM infection than younger ones, and their teat canals may be more dilated and partially or permanently open. Consequently, there is a significant risk of skin and environmental infections entering the teat canal (Shittu et. 2012).

Parity was another risk factor. It was separated into three groups: 1-3 parity numbers, 4-6 parity numbers, and >6 parity numbers. The prevalence of these groups was 8.9%, 44.82%, and 75%, respectively. The prevalence of CMT positivity demonstrated an increasing tendency as the number of parties increased. The current study's findings are comparable to those of (Sanotharan et al. 2016), Batticaloa District in Sri Lanka, which discovered that a greater prevalence of CMT positive was identified in >5 parity numbers (75%), while the prevalence was 28.4% and 53.3% in 1-2 and 3-5 parity numbers, respectively.

Increased pathogen penetration of the tear duct and accumulated prior infection may be the cause of the greater incidence in cows three years of age and older (Mgonja et al. 2023). The genetic resistance of bacteria, milking cleanliness, milking techniques, management system, and the investigator's technical expertise may all contribute to the variation in the prevalence of dairy farms included in this study (Moshi et al. 1998). Poor environmental hygiene and sanitation, particularly in the house where the cows reside due to the bare floor, faecal pollution, and damp conditions, were these linked risk factors. The milking process, which included scrubbing teats with dirty water, sharing towels for wiping and drying after cleaning the udder, and milking the cows by hand, resulted in poor milking hygiene.

Since the rate of infections rises with age and parity numbers, the likelihood of mastitis infections increased since the majority of the cows were older than five years and had more than three parity numbers. Additionally, neither a teat dip antimicrobial application following milking nor dry cow therapy was administered to the cow during drying and drying off the cows. Despite this, milking was done with hands that were not well washed, which served as a source of mastitis infections (Akter et al. 2020).

Conclusions: In conclusion, *S. aureus*-induced subclinical mastitis is regarded as a serious public health issue. According to the current study, bovine sub-clinical mastitis significantly impairs the health of the cows and the dairy cows' ability to produce milk. If proper hygiene practices are maintained during the milking process and in the cow's surroundings, subclinical mastitis may be less likely. To detect subclinical mastitis early, CMT can be performed often as a prophylactic measure. By identifying the pathogenic bacterial agents and the antimicrobial sensitivity data, the best antibiotic for a certain bacterium can be found.

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