PREVALENCE, ANTIBIOTICS SUSCEPTIBILITY PROFILING, AND ASSOCIATED RISK FACTORS OF COLIFORM MASTITIS AT COMMERCIAL DAIRY FARMS

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ABSTRACT: This study aimed to determine the prevalence of coliform sub-clinical mastitis in bovines, their associated risk factors, and antimicrobial susceptibility patterns of *E. coli*. A total of 102 pure and local breed lactating cows and buffaloes were included in this study. Milk samples (n=102) were screened by the Surf Field Mastitis Test (SFMT). The *E. coli* isolated from mastitic milk were cultured on MacConkey's agar and confirmed through PCR. Antibiotic susceptibility profiling was done through the disc diffusion method. The associated risk factors and antibiotic susceptibility profile were analyzed by chi-square, logistic regression, and ANOVA. Out of 102 screened animals, 26% (n = 13/51) pure breed and 32% (n=16/51) local breed were positive for SCM. The higher prevalence of SCM with significant association was noted in local breeds (32%), early lactation stages (57.14%), and brick block type floor (29.35%). The different risk factors showed statistically significant associations ($p<0.001$) such as cylindrical teat shape (32.97%; $p<0.001$), daily manure removal (23.32%; $p<0.001$), pipeline water source (25.46%; $p<0.001$), use of oxytocin for milk let-down (37.85%; $p<0.001$) and post milk teat dipping (40.82%; $p<0.001$). Amoxicillin, cloxacillin, gentamicin, colistin sulphate, procaine-penicillin, enrofloxacin, ampicillin, oxytetracycline, florfenicol, and streptomycin were used for anti-bio-gram profiling against *E. coli* isolates. Enrofloxacin (81%), gentamicin (76%), and ampicillin (67.7%) were found to be most effective *in vitro*. The study emphasizes breed variations, age, and management practices as influential factors for the prevalence of SCM. The effective control strategies, early detection, and prudent antibiotic use are recommended to alleviate SCM.

Keywords: Molecular epidemiology; Risk factors; ampicillin; Antibiotic profiling, antibiogram profiling.

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INTRODUCTION

Mastitis is the inflammation of the parenchyma of the mammary gland. It is caused by multiple etiological agents, such as bacteria, viruses, fungi, and yeast, leading to substantial economic losses in the dairy industry (Goulart and Mellata, 2022). It not only reduces milk production but also leads to detrimental changes in the milk composition (Grispoldi *et al*., 2019). The most prevalent managemental illness of dairy animals is SCM (Bobbo *et al*., 2017; Viguier *et al*., 2009). During SCM, milk production decreases by 10-26% (Dhakal *et al*., 2007). *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Escherichia Coli*, and *Corynebacterium pyogenes* are repeatedly isolated from the infected milk of SCM lactating dairy animals. 80% subclinical mastitis in bovines is due to coliform microbes (Fahim *et al*., 2019). Coliform mastitis is more prevalent in early-stage lactation animals than the animals that are in mid-lactation stage animals (Abegewi *et al*., 2022). *E. coli* type mastitis is usually a commensal type (Momtaz *et al*., 2012; Suojala *et al*., 2011).

S. aureus and *E. coli* are the leading causes of bovine SCM (Hinthong *et al*., 2017). SCM in dairy animals is commonly detected after laboratory examination of the milk, as there is no gross swelling of the udder or apparent changes in the milk (Baloch H *et al*., 2016). In Pakistan, SCM is detected by CMT and SFMT under field conditions (Ahmad *et al*., 2012; Rizwan *et al*., 2021). To treat SCM, most antibiotics are used (Aqib *et al*., 2017). These antibiotic residues are transferred to humans through milk consumption (Rizwan *et al*., 2016). Tylosin, amoxicillin, enrofloxacin, and penicillin are most commonly used to treat SCM in the field. Amoxicillin shows lower efficacy against coliform bacteria than norfloxacin (Abegewi *et al*., 2022). Procaine penicillin is the most commonly used against SCM (Khan *et al*., 2018). Few studies have reported the risk factors associated with SCM on Pakistani dairy farms. These studies have generally focused on larger farms and mainly cattle. This study aimed to provide data

on prevalence, associated risk factors, and antibiotic resistance for coliform SCM on dairy farms in Multan, Pakistan.

MATERIALS AND METHODS

Study design and collection of milk samples: This study was conducted in cows and buffaloes of the district Multan of Pakistan. A total of 102 lactating cows and buffaloes, irrespective of breed, age, number of lactations, and feeding management, were included. A standardized collection method was employed to obtain milk samples from each lactating animal. After careful physical examination of the teats by proper palpation, the teat ends were thoroughly washed using 70% ethanol and then dried off. Then a total of 10ml milk sample was collected from each teat. The SFMT was employed as a diagnostic tool for the detection of SCM. The test is based on the principle of detecting an increased number of leukocytes and SCC through the observation of viscous mass formation at different levels. The positive milk samples were inoculated onto MacConkey's agar and incubated at 37°C for a period of 24 hours. After noticing microbial growth on the culture plate single isolated colony was picked and sub-cultured again on MacConkey's agar for further purification of the isolate by observing the colony morphology. Different tests, including *in-vitro* antibiogram profiling of ten *E. coli* isolates against ten commonly used antibiotics, were performed. The following antibiotics were used: amoxicillin (2ml), cloxacillin (2ml), gentamicin (3ml), colistin sulphate, procaine-penicillin (1ml), enrofloxacin (2ml), ampicillin (3ml), oxytetracycline (2ml), florfenicol (2ml), and streptomycin (3ml).

Risk factors associated with SCM: Data for the associated risk factors with SCM were collected through a predesigned questionnaire through observation and owners' interviews.

Statistical analysis: The data collected were analyzed by chi-square using SPSS 22 version. Values were calculated for the association of different risk factors by logistic regression, while the zone of inhibition in the antibiogram study was analyzed by repeated one-way ANOVA. The significance will be checked at 5% probability ($p<0.05$).

RESULTS

Prevalence of SCM: The prevalence of SCM was analyzed and compared between purebred and local animals. We tested 51 purebred and 51 locally bred animals, of which 13 (26%) and 16 (32%) were positive for SCM, respectively. The age-wise prevalence of SCM was observed and compared between purebred and locally bred animals. In pure and local breed cows for ages 3-5 years, 5-8 years, and 8-12 years, the prevalence of SCM was 46.1%, 54% and 50%, respectively. Whereas, SCM prevalence was high for a group of animals that were in 30 to 60 days of lactation in case of pure breed animals, and the same prevalence ratio for local breed animals.

Floor, manure, and teat shape associated risk factors: Risk factors associated with SCM in lactating cows and buffaloes were studied. Samples in case of separate brick-block floor and soil floor were tested. Of the total samples from separate brick-block floors, 28.35% tested positive. Of the samples with soil floor were also tested, which 19.27% were found positive. We observed samples with manure removal once a day, of which there were 27 positive samples (24.32%). In the case of manure removal 2 times a day, 93 samples were observed, of which 21 tested positive (22.58%). Lastly, we observed 84 samples with manure removal multiple times a day, with 18 positive samples (21.42%). Of the 77 samples observed whose teats were round-shaped, 21 tested positive (27.27%). Of the 201 samples with bowl shaped teats were also observed, out of which 59 were positive (29.35%). Lastly, 212 samples with cylinder-shaped teats were observed, out of which 55 were positive for SCM (31.97%).

Milk letdown methods and water source associated risk factors: Out of the samples in which the source of water was a pipeline, 26.46% were positive for SCM. 20.75% were positive from samples in which the source of water was a well. The positivity ratio was 26.62% in the case of calves and 38.85% in the case of oxytocin used for the removal of milk.

Farm cleanliness, post-milking teat dip, and dry period associated risk factors: We observed samples with farm cleaning once a day, out of which 20.82% tested positive for SCM. 61 samples were tested with cleaning twice a day, out of which 26.22% were positive for SCM. In case of post-milking teat dip, we observed 85 samples in which post-milking teat dip was done, out of which, there were 8.23% tested positive for SCM. We observed samples in which post-milking teat dip was not done, out of which 40.82% tested positive for SCM. This study also involved testing a total of 145 cows, with varying lengths of dry periods. Among cows in the dry

period of 0 to 1.5 months, 41 out of 145 (28.27%) tested positive for SCM. For those in the dry period of 1 to 3 months, 46 out of 196 (23.46%) cows tested positive for SCM. Similarly, for those in the dry period of more than 3 months, 23 out of 109 (21.1%) cows tested positive for SCM.

Microbiological investigation: The mastitis-positive milk samples were transferred to the laboratory for further analysis. The samples were primarily cultured on MacConkey agar to identify the causative agents. In SCM, 12 pure isolates of *E. coli* were cultivated during culturing. The extraction of genomic DNA was done using by DNA extraction kit.

Antibiogram profiling: In antibiogram profiling, we used 10 isolates of *E. coli*, of which 60% were resistant and 40% were sensitive against amoxicillin. In the same way, out of 5 isolates of *E. coli*, 83.4% were resistant and 16.7% were sensitive against cloxacillin. Out of 4 isolates, 0% were resistant,25% were intermediate, and 75% were sensitive against gentamicin. Out of 6 isolates, 16.67% isolates were resistant, 66.67% isolates were intermediate, and 16.67% were sensitive against oxytetracycline. Out of 9 isolates,33.33% isolates were resistant and 66.67% isolates were sensitive against florfenicol. Out of 7 isolates, 28.6% isolates were resistant and 57.1% were sensitive against colistin sulphate. Of the 3 isolates, 0% were resistant, 33.4% isolates were intermediate, and 66.7% were sensitive against ampicillin. Out of 8 isolates,12.5% were 244 resistant and 50% were sensitive against enrofloxacin. From 9 isolates, 88.9%were 245 resistant and 11.2% were sensitive against streptomycin. From 7 isolates that were tested, 100 of % isolates were resistant, no isolate was intermediate, and 0% isolates were sensitive against procaine penicillin.

DISCUSSION

Mastitis is a highly prevalent disease in lactating cattle, buffalo, sheep, and goats, which leads to substantial economic losses associated with expenses related to veterinary services, treatment, and labor, as well as reduced milk production, increased occurrence of subsequent mastitis, culling of animals, and poor milk quality (Maalik *et al*., 2019). Moreover, mastitic milk contains bacteria and their toxins above a critical level, which can be detrimental to human health (Ahmad *et al*., 2021). The present study employed SFMT as a screening test, which is a cost-effective, field-applicable diagnostic test, for the detection of intra-mammary infections (IMIs). In the studies by Hameed *et al*. (2012) and Hussain *et al*., (2013), the prevalence of SCM was found to be lower in pure-breed animals compared to non-descript animals, which is consistent with this study. Our findings indicate that the prevalence of SCM was greater in local breeds as compared to purebred animals. My Study reported a higher prevalence of SCM in local breeds than pure breeds using SFMT as a screening tool. Our findings suggest that age and high milk yield are linked to an increased risk of SCM. There are more chances of SCM during early lactation as compared to late lactation. Prevalence of SCM was high during the first stage of lactation, which is in accordance with recent studies. Animals with the first stage of lactation were more prone to SCM, which is not in accordance with our studies (Rahman *et al*., 2009).

Research conducted by the WHO has suggested high milk yield and advanced age as important risk factors for the development of SCM. The prevalence of SCM in cows and buffaloes was found to increase with the age of the animals, with the highest prevalence observed in the 12 years or above age group. In earlier studies, the age-wise prevalence of SCM in purebred and local-bred animals at different stages of ages from 3-5, 5-8, and 12 years was found to be 3.3%, 18.7%, 21.8%, and 29.2%, respectively (Rahman *et al*., 2009). However, our studies have reported different percentages, with the prevalence found to be 46.1%, 54% and 50%, respectively. In conclusion, the prevalence of SCM varies among breeds and is influenced by genetic predisposition factors, management practices, milk yield, and age. Therefore, it is crucial to implement effective measures for mastitis control and prevention, including regular monitoring, proper management practices, early detection, and treatment. Further research is needed to explore the underlying mechanisms contributing to the observed associations between mastitis and breed, milk yield, and age. The likelihood of mastitis in dairy animals is higher when they are housed on brick block flooring, findings of my studies in accordance with previous research work (Rahman *et al*., 2009).

In this study, the results of risk factors associated with teat dipping are presented in accordance with previous research work (Mbindyo *et al*., 2020). According to this, the positive ratio of SCM was 8.23% when teat dip was done, and when not used was 40.82% so when no teat dip was performed, there were more chances of mastitis to occur. This study related to dry period length shows that there is a high chance of SCM during the 1.5 to 3 months stage of the dry period. When the calving interval is more than a year, there are more chances of SCM to occur, which is in accordance with my studies. The findings of this research work related to dry period length and calving interval were in accordance with (Demil *et al*., 2022) dry period and calving interval findings. Different studies showed that manure removal played an important role in the chances of occurrence of SCM in lactating animals. Results showed 18.65% and 25.96% positive ratios on manure removal once and twice a day, respectively, which is in accordance with Bari *et al*. (2022 findings. This study shows that there are

more cases of SCM on cleaning twice a day as compared to cleaning once a day, which is, however, not by Bari *et al*. (2022) research finding. Studies also showed source of water played a crucial role in the occurrence of SCM. Positive ratio of SCM is 26.46% and 20.75% in the case of pipeline and well, respectively, in this study. This finding is by Rizwan *et al*., (2016) research finding. This study also shows that the chances of SCM are higher in animals in which oxytocin (38.85%) is used for milk let down as compared to calves (36.62%), which is by previous work by Napolitano Fabio *et al.* (2022).

Teat shape also affects the prevalence of SCM to some extent, with positive ratios of 27.27%, 29.35%, and 31.97% in the case of round, flat, and cylinder-shaped teats, respectively, which is slightly different from Hussain *et al*., (2013) research finding. Treatment of SCM in lactating animals with antibiotics still characterizes the base of any SCM control strategy (Rizwan *et al*., 2022). However, the misuse or irrational use of antibiotics in preventing and controlling SCM in lactating animals has led to the emergence of antibiotic-resistant strains. Consequently, investigation of etiological agents and their antibiotic susceptibility test profile will help the veterinarian to treat the affected lactating animals with SCM (Grispoldi *et al*., 2019).

Antimicrobial resistance is becoming more prevalent all over the world, which is causing increasing concern. Antimicrobial drug use in significant amounts for control of disease in animals that produce food is considered to be a factor in the persistence and emergence of zoonotic pathogens with antimicrobial resistance. The most successful medications in the *in-vitro* susceptibility testing were amoxicillin, cloxacillin, gentamicin, oxytetracycline, florfenicol, colistin sulphate, ampicillin, enrofloxacin, streptomycin, and procaine-penicillin were used against all isolates. Enrofloxacin and gentamicin were mostly used against *E. coli*, followed by cloxacillin, ampicillin, amoxicillin, and florfenicol. Oxytetracycline efficacy was moderate. Procaine penicillin was the least sensitive antibiotic, with no antibiotics showing susceptibility. Streptomycin was the most resistant antibiotic against *E. coli*. High sensitivity to enrofloxacin and gentamicin has also been observed in previous studies (Dhakal *et al*., 2007). Similar findings were reported by Kaliwal *et al*. (2011) and Awadkar and Kulkarni (2012). Oxytetracycline showed moderate resistance in the present study, that comparable with the findings by Awadkar and Kulkarni (2012), Alekish *et al*. (2013), Mahami *et al*. (2011), and Dhakal *et al*. (2007). Penicillin resistance (100%) observed in this study is much more than other researchers work reported by Pitkala *et al*. (2004), Rajala-Schultz *et al*. (2009), and Kenar *et al*. (2012).

In Pakistan, the prevalence of SCM in dairy animals was considered to be high, according to the current investigation, which may interfere with the effectiveness of milk production. In this study, *E. coli* were isolated from cows that were affected with SCM. *E. coli* is were most important cause of SCM in bovines. This indicates that SCM was most prevalent, linked with poor management on the farm and unsanitary practices of milk. To increase milk production, farms should improve their milking practices and management. Advised to use dry cow therapy while treating the cases of SCM that are at the early stage of mastitis. Use an antibiotic sensitivity test to look for the drug of choice for SCM in the dairy animals of the study areas. Further investigations on other causes of SCM are required to control this economically important disease in the study area.

Conflict of interest: There is no conflict of interest among the authors.

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