Molecular Epidemiology of *Staphylococcus aureus* Isolated from Subclinical Mastitis in Lactating Camels of the Cholistan Desert, Pakistan

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ABSTRACT: Subclinical mastitis (SCM) in camels is a significant constraint to milk production and animal health, particularly in arid areas such as the Cholistan Desert, Pakistan. *S. aureus* is the primary pathogen of SCM due to the diversity of its virulence factors, which complicates the treatment and control of SCM. This study aimed to determine the prevalence of SCM and the molecular epidemiology of *S. aureus* isolated from SCM in lactating camels in Cholistan. A total of 221 lactating camels were screened for SCM by SFMT. Positive milk samples were microbiologically processed for the isolation and identification of *S. aureus*. Further, molecular characterization of the isolates was performed to detect the presence of virulence genes. The overall prevalence of SCM was 10.4% (23/221; *P<0.05*). Among the management systems, stall-fed camels exhibited a higher prevalence (12%; *P<0.05*) in comparison with the others, in which grazing camels (9%; *P<0.05*). Age-wise, the prevalence was observed at 13% in camels aged 5–6 years, 11% in those aged 7–8 years, and 14.5% in the 9–10-year age group (P < *0.05)*. *S. aureus* was isolated from 24% of SCM-positive milk samples. Molecular analysis showed the presence of major virulence genes: *coa* (coagulase) at 87.2%, *hla* (alpha-hemolysin) at 80%, and *hlb* (beta-hemolysin) at 82.9%, indicating their pathogenicity (*P<0.05*). This study highlights the prevalence of SCM in lactating Camels of the Cholistan desert, with S. aureus being a major pathogen. The high prevalence of virulence genes underscores the need for selective control measures with a focus on improved milking hygiene and routine screening to control the effects of SCM on camel milk productivity.

Keywords: Cholistan, Epidemiology, Lactating camels, Subclinical mastitis.

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INTRODUCTION

The camel population in Cholistan, Pakistan, is estimated to be around 110,000 heads, and camel milk is an important food source in dry areas. It is commonly consumed by the nomads in the Cholistan desert (Pakistan). Its nutrient density, high in protein, fat, minerals, and particularly vitamin C, presents health benefits that can be particularly advantageous in those regions where milk is not easily accessible. Its unique composition, as well as its curative and preventive effects on diseases such as jaundice, tuberculosis, asthma, anemia, and food allergies, emphasizes its specific importance in human nutrition (Faye and Bonnet, 2012). Despite the camel milk being nourishing, the productivity and quality of the camel milk are drastically reduced due to SCM, which is an inflammatory disease of the mammary gland that compromises both health and production in all its forms. About $21 was reported to be lost per clinical case in Pakistan, and SCM has been reported as one of the diseases contributing to the highest economic losses (Fareed et al., 2016; Makinde et al., 2015). SCM is responsible for over 70% of all cases

of mastitis (Abebe et al., 2016). This mastitis form is particularly difficult to diagnose and control; nevertheless, it dramatically reduces milk yield and milk quality.

The main etiological agents of camel mastitis are *S. aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae,* and *E. coli* (Aqib et al., 2017b). Of these, *S. aureus* is the primary pathogen responsible for CM and SCM, resulting in massive economic losses (He et al., 2016). Worldwide, the rate is 20.35% and in Pakistan, the incidence is even higher at 52.3% (Ahmad et al., 2012; Sarwar, 2013). Indeed, *S. aureus* in micro and macro-environments causes severe and detrimental intramammary infections (IMI) that compromise alveolar physiology through the injury of alveolar and ductal epithelial cells. These altered cells, along with leukocytes, can plug up milk ducts, which in turn causes the production of scar tissue and hence a reduction in milk yield (Radostits et al., 2007; Mbuk et al., 2016). In addition, *S. aureus*-induced IMI can be chronic, as the pathogen can circumvent host immune responses by hiding in neutrophils and other host cells (Petersson-Wolfe et al., 2010). In Pakistan, the prevalence of SCM in camels was reported as 10.4% to 47.14% and in the Cholistan Desert, Ali et al. (2019) reported have reported a prevalence of 48.18%. *S. aureus* is the most commonly found causative pathogen isolated from SCM, with a prevalence 24% to 74.6% (Petersson-Wolfe, 2010). Its virulence is attributed to some virulence genes, such as coa (coagulase), hla (alpha-hemolysin), and hlb (beta-hemolysin) that allow the pathogen to survive and persist in the mammary tissue (Cheung et al., 2021; Ali et al., 2019).

Epidemiological studies on SCM in lactating camels, however, are limited, and the least attempt has so far been made to address this issue as far as Pakistan is concerned. Correct determination of the prevalence and molecular epidemiology of *S. aureus* is necessary for planning the most effective control of transmission. Considering the increasing economic and public health implications of camel mastitis, in particular that caused by *S. aureus*, the current study was conducted to determine the frequency of SCM and to investigate the epidemiology of *S. aureus* in lactating camels in the Cholistan Desert of Pakistan.

MATERIALS AND METHODS

Study area and duration: This study was conducted in the Cholistan desert, which lies in the Bahawalpur division of Punjab, Pakistan. This arid land covers about 26,000 km² and is characterized by high temperature, low rainfall, and little herbage, which is an unfavorable condition for animal grazing (Ali et al., 2009). The study was carried out between March and August 2024 during the spring and summer time, the crucial period for the season of camel lactation and the potential season for mastitis.

Study design and sample size: A cross-sectional study design was employed to assess the prevalence of SCM and to characterize the molecular epidemiology of S. aureus isolates. The sample size was calculated using the formula for estimating a single proportion (Hajian-Tilaki, 2011) as:

$$n = Z^2 \times P \times (1-P)/d^2$$

Where:

n = required sample size

Z = Z-score for 95% confidence level (1.96)

P = expected prevalence (assumed at 10% based on previous studies)

d = desired precision (5%)

To account for potential non-responses and to increase the study's robustness, a total of 221 lactating camels were included in the study.

Animal selection criteria and ethical considerations: Lactating dromedary camels (Camelus dromedarius) of 5-10 years of age were randomly selected from different herds in the Cholistan desert. Camels with clinical signs of mastitis were excluded. All experiments were conducted by the ethical principles and guidelines of the National Research Council.

Screening for SCM and milk sample collection: Screening for SCM was done through the Surf Field Mastitis Test (SFMT). Sterile containers were used to

facilitate the aseptic collection of milk samples from each teat of the udder. An equal volume of milk was added to an equal volume of 3% SFMT reagent and mixed by gentle shaking. Gel formation was considered a positive reaction, an indication of a high SCC and potential SCM. Camels with one or more positive teat(s) were considered as SCM-positive (Karabasanavar et al., 2021). Approximately 10mL of milk was aseptically sampled from each affected teat into sterile screw-capped tubes. Samples were stored in iceboxes and transported to the Laboratory of Microbiology within 6 hours of collection to ensure sample quality.

Isolation and identification of *S. aureus*: Upon arrival at the laboratory, samples were vortexed, and a loopful was streaked onto mannitol salt agar (MSA) plates. Plates were incubated at 37C for 24-48 hours. Colonies exhibiting golden yellow pigmentation and mannitol fermentation were presumptively identified as S. aureus. Gram staining, catalase, and coagulase tests were used for further confirmation (Buchanan & Gibbons, 1974).

Molecular analysis of virulence factors: Positive *S. aureus* isolates were cultured in Brain Heart Infusion (BHI) broth and incubated at 37°C for 24h before use. DNA extraction was performed according to the manufacturer's protocol using the DNeasy Blood & Tissue kit (Qiagen, Germany). Purity and concentration of the extracted DNA were determined on a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). PCR assays for the virulence genes: coa (coagulase), hla (alpha-hemolysin), and hlb (beta-hemolysin) were performed. Gene-specific primers for each gene were designed on known gene sequences (Petersson-Wolfe et al., 2010) and synthesized.

RESULTS

The overall prevalence of SCM in lactating camels was 10.4%, as diagnosed by SFMT. There were no significant differences (p>0.05) between the two management systems. In camels subjected to stall feeding, the prevalence was a little higher (12.0%) than in camels under a grazing system

coa: F: 5'-ATAGAGATGCTGGTACAG G-3'; R: 5'-GCTTCCGATTGTTCGATGC-3'

hla: F: 5'-TTAGCCGAAAAACATCATTTC-3', R: 5'-TTATTCCCGACGAAATTCCAA-3'

hlb: F: 5'-GATCTTTGATATTCGCAAGCA-3', R: 5'-GCTTTATAATGCTGGTGGTG-3'

PCR reactions were set up in a 25µL volume containing 12.5µL of 2×PCR Master Mix (Thermo Fisher Scientific, USA), 1µL each of forward and reverse primers (10µM), 2µL of template DNA, and 8.5µL of nuclease-free water. The thermal cycling conditions were as follows: Initial denaturation at 95°C for 5 minutes, 35cycles of denaturation at 94°C for 30 seconds, annealing at the respective temperatures for each gene for 30seconds, extension at 72°C for 1minute, and final extension at 72°C for 10 minutes.

Gel electrophoresis: The PCR products were subjected to electrophoresis in a 1.5% agarose gel stained with ethidium bromide. A 100bp DNA ladder (Thermo Fisher Scientific, USA) was used as a size marker. Agarose gels were imaged under UV transillumination using a Gel Doc XR+ system (Bio-Rad, USA), and the presence of specific bands matching the target genes was recorded.

Data analysis: Data were analyzed with SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were used to estimate the proportion of SCM and the distribution of virulence genes among the S. aureus isolates. Associations between the prevalence of SCM and the variables age, management system, and presence of virulence genes were assessed using chi-square tests. Differences of p<0.05 were considered to be statistically significant.

(9.1%). Age-specific analysis was conducted, as there were differences in the prevalence of SCM across different age groups. The highest prevalence was 12.9% in camels aged 6-8 years, followed by 11.7% in camels aged 5-6 years, and the lowest was 7.7% in camels aged 9-10 years. However, the differences were statistically non-significant (P>0.05). These observations imply that, although differences may be

observed for SCM prevalence at the levels of the management system and age group, these are not significant for this study. The prevalence of SCM was significantly increased with the increase in parity number. Results reported that significantly higher prevalence of SCM in camels of 2-3 parity. The camels with teat lesions, necrosis at the udder, and skin abrasions and swelling showed a significantly high prevalence of SCM (Table 1).

Table 1: Management, age group, and parity-wise prevalence of SCM in camels

Factor	Prevalence of SCM (%)	Statistical Significance
Overall Prevalence	10.4%	-
Management System		
Stall Feeding	12.0%	P > 0.05
Grazing System	9.1%	P > 0.05
Age Groups		
5-6 years	11.7%	P > 0.05
6-8 years	12.9%	P > 0.05
9-10 years	7.7%	P > 0.05
Parity Number		
2-3 Parity	Significantly higher	P < 0.05
Teat Lesions / Udder Damage	Significantly higher prevalence	P < 0.05

P > 0.05 indicates that the differences observed are not statistically significant for the management system and age group.
P < 0.05 indicates that the differences observed are statistically significant, especially in cases of higher parity and teat lesions or udder damage.

A total of 26.1% of coagulase-positive S. aureus were identified. There is no significant difference in the *S. aureus* prevalence isolated from SCM in different localities of Cholistan (P>0.05).

PCR amplification was performed for three specific virulence genes, i.e., *coa* (coagulase), *hla* (alpha-hemolysin), and *hlb* (beta-hemolysin B) in *S. aureus* isolates. It was interesting to find that each of these genes was detected in 83.3%, 54.1%, and 63.3% of the isolates. These virulence determinants contribute to the increased pathogenic potential of *S. aureus* by enabling tissue invasion and evading host defense mechanisms. These data indicate that S. aureus isolated from SCM in camels may be pathogenic for humans; therefore, it is recommended to introduce preventive measures against this pathogen in camel populations.

DISCUSSION

This study reports, for the first time in Pakistan, the prevalence of SCM and the molecular typing of S. aureus isolated from SCM in lactating camels in the Cholistan Desert of Pakistan. The overall prevalence of SCM (10.4%) is in line with reports in other similar arid semiarid regions elsewhere in Ethiopia, where 10.6% was recorded in Jigjiga City (Abera et al., 2010). These differences could be related to management procedures, environment, and the modality by which they have been diagnosed.

Effect of climatic and ecological conditions: This study also assessed the impact of climate on the prevalence of *S. aureus* isolated from SCM in lactating camels under different ecological stress conditions in Pakistan, which showed a moderate level of prevalence. While the results of previous studies indicated extremely high rates of prevalence in some of the study areas (Aqib et al. 2017b; Ahmad et al. 2012), with the prevalence of SCM (41.67 and

46%), respectively. There are also studies conducted in Kuwait and Kenya that reveal 43% and 60% prevalence rates, respectively (Abdulkadhim, 2012; Wanjohi et al., 2013). In contrast, lower prevalence was reported by Abera et al. (2010), and Abdurahman (2006) also found similar findings to Chara (2011c) and Bekele and Molla (2001). Some of the differences might be due to differences in geographical locations, management, and hygienic conditions of the farms.

Risk factors and management strategies: The investigation revealed that the prevalence of SCM in stall-fed camels (12%) was more than in grazing-fed camels (9%), which may be associated with the susceptible management systems. This is in line with previous studies, which reported that risk factors such as poor udder hygiene, tick invasion, and improper milking practices had high associations with SCM prevalence (Ahmad et al., 2012). Age-specific breakdown showed a higher prevalence of SCM in camels between 6–10 years, which is in line with findings indicating that advanced age is associated with increased susceptibility to SCM (Ahmad et al., 2012). When it comes to the determinants, we found a significant association between teat dipping, age group, parity number, and body condition with SCM, which was expected, as compared to the studies of Aqib et al. (2017a), Husein et al. (2013), and Ahmad et al. (2012). Nonetheless, the factors such as the rearing system, water channel source, tick infestation, and feeding system of the present study did not support the findings of the studies mentioned above. This difference might be associated with intensive rearing practice in the monetized. Persistently low udder hygiene, inadequate feeding, and poor body condition favor the development of infection (Mbuk et al., 2016). The traditional method of tying the teat of camels with a thread to prevent calf suckling is a widespread camel husbandry system that leads to udder injuries and predisposes the teat to bacterial infection (Woubit et al., 2001). Dry animals are more susceptible to mammary infection because the keratin plugs in the teat form late, the concentration of leukocytes is lower, the milk lactoferrin is diluted, the immune system is inactivated, and there is no flushing activity.

Isolation and genomic characterization of *S. aureus:* *S. aureus* was identified in 26.1% of SCM-positive milk samples, confirming that it is a leading cause of camel mastitis. Molecular studies showed that the prevalence rate of *COA*, *HLA*, and *HLB* genes in the tested isolates was high, with a positivity of 83.3% in each of these 3 genes. These results were consistent with previous works in the same sequenced virulence genes for *S. aureus* isolated from SCM in camel (Ahmad et al., 2012). The existence of these genes shows the potential pathogenicity of the isolates and their ability to steer clear from the host immune responses. Aqib et al. (2017) reported a total of 88.05% of *S. aureus* isolates. No significant results were obtained in the prevalence of *S. aureus* on the basis of different localities ($P>0.05$). Aqib et al. (2017) reported that the prevalence of SCM in tehsil Bahawalpur revealed a higher prevalence of coagulase-positive *S. aureus*, followed by Liaqatpur and tehsil Ahmadpur East, with rates of 91.18%, 90%, and 85%, respectively. The results of this study highlight the need for focused control measures to control SCM in camels. Enhancing hygiene, screening camels and herders as part of a regular program, and educating camel herders about how to manage their animals are all important. Moreover, monitoring the patterns of antimicrobial resistance and promoting the appropriate use of antibiotics is important to control the dissemination of resistant *S. aureus.*

Conclusions: This study shows a substantial incidence of SCM in lactating camels of the Cholistan desert, with *S. aureus* as the most prevalent pathogen. The high prevalence of virulence genes suggests that the control of SCM, by implementation of adequate control strategies, such as application of better milking hygienic measures and frequent monitoring to reduce the burden of SCM in camel milk, should be considered. Further investigations, with emphasis on resistance profiling and strategies to combat SCM in camel herds, are recommended.

Recommendations: There should be awareness among the camel farmers about subclinical mastitis and its associated risk factors. Through good milking hygienic measures, the prevalence of mastitis can be reduced.

REFERENCES

Abebe, R., Hatiya, H., Abera, M., Megersa, B., & Asmare, K. (2016). Bovine mastitis: prevalence, risk factors and isolation of Staphylococcus aureus in dairy herds at Hawassa milk shed, South Ethiopia. *BMC Veterinary Research, 12*, 270. https://doi.org/10.1186/s12917-016-0905-3BioMed Central

Abdurahman, O. A. S. (2006). Udder health and milk quality among camels in the Errer valley of eastern Ethiopia. *Livestock Research for Rural Development*. 18(8): 234-239. http://www.lrrd.org/lrrd18/8/abdul18110.htm

Ahmad, R., Khan, A., Khan, M. Z., Hussain, I., & Mahmood, F. (2012). Prevalence and antibiogram of Staphylococcus aureus isolated from subclinical mastitis in camels. Pakistan Journal of Zoology, 44(3): 659–663.

Ahmad, S., Yaqoob, M., Bilal, M. Q., Muhammad, G., & Yang, L. G. (2012). Risk factors associated with prevalence and major bacterial causes of mastitis in dromedary camels (Camelus dromedarius) under different production systems. Tropical Animal Health and Production, 44(1): 107–112. https://doi.org/10.1007/s11250-011-9895-0

Ali, M., Muhammad, G., Arshad, M., Saqib, M., & Hassan, I. J. (2019). Prevalence of subclinical mastitis in camels in the Cholistan Desert of Pakistan. Pakistan Veterinary Journal, 39(1): 1–4.

Ali, M., Shafiq Chaudhry, M., & Farooq, U. (2009). Camel rearing in the Cholistan Desert of Pakistan. Pakistan Veterinary Journal, 29(2): 85–92.

Aqib AI, Ijaz M, Hussain R, Durrani AZ, Anjum AA, Rizwan A, Sana S, Farooqi SH, and Hussain K, 221-225. Identification of coagulase gene in Staphylococcus aureus isolates recovered from subclinical mastitis in camels. Pakistan Veterinary Journal. 37(2): 160-164.

Aqib, A. I., Ijaz, M., Ali, M. M., & Mehmood, K. (2017b). Prevalence and antibiogram of Staphylococcus aureus, a camel mastitogen from Pakistan. Pakistan Journal of Zoology, 49(1): 1–7. https://doi.org/10.17582/journal.pjz/2017.49.1.1.7

Aqib, A. I., Iqbal, M. K., Muhammad, G., & Hassan, M. A. (2017b). Prevalence and antibiogram of Staphylococcus aureus isolated from camel mastitis in Pakistan. Pakistan Journal of Zoology, 49(4):1347–1353.

Bekele, T., & Molla, B. (2001). Prevalence and etiology of mastitis in camels (Camelus dromedarius) in eastern Ethiopia. Journal of Camel Practice and Research, 8(1): 67–70.

Buchanan, R. E., & Gibbons, N. E. (1974). Bergey's Manual of Determinative Bacteriology (8th ed.). Williams & Wilkins.

Cheung, G. Y. C., Bae, J. S., & Otto, M. (2021). Pathogenicity and virulence of Staphylococcus aureus. Virulence, 12(1): 547–569. https://doi.org/10.1080/21505594.2021.1878685

El-Agamy, E. I., & Khatab, A. A. (1992). Physicochemical and microbiological characteristics of Egyptian human milk. Alexandria Journal of Agricultural Research, 37(1): 1–10.

FAO. (2001). Production Yearbook 2001 (Vol. 55). Food and Agriculture Organization of the United Nations.

Fareed, A., Ahmad, T., and Khan, M. A. (2016). Economic loss due to clinical mastitis in crossbred cows. Pakistan Journal of Agricultural Sciences, 53(3): 691–694.

Faye, B., & Bonnet, P. (2012). Camel sciences and economy in the world: current situation and perspectives. In Proceedings of the 3rd ISOCARD Conference (pp. 2–15).

Hajian-Tilaki, K. (2011). Sample size estimation in diagnostic test studies of biomedical informatics. Health Information Management Journal, 40(1): 14–23.

He, Y., Cao, X., & Liu, Y. (2016). Prevalence,

antimicrobial resistance, and virulence genes of Staphylococcus aureus isolated from subclinical mastitis in dairy cows in China. Pakistan Veterinary Journal, 36(3): 356–360.

Karabasanavar. (2021). Evaluation of the Single Strip Mastitis Test for the detection of subclinical mastitis in dairy animals. Veterinary World, 14(3): 684–689.

Mbuk, E. U., Kwaga, J. K. P., Bale, J. O. O., and Okolocha, E. C. (2016). Bacteriological quality of raw camel milk from pastoral communities in Nigeria. Veterinary World, 9(6): 546–550.

Mbuk, E., Kwaga, J., Bale, J., and Barde, I. (2016). Prevalence and antibiotic susceptibility of Staphylococcus aureus isolated from bovine mastitis in Plateau State, Nigeria. Journal of Veterinary Medicine and Animal Health, 8(8): 91–96.

Nagy, P., Juhasz, J., and Reiczigel, J. (2013). Milk production and composition in dromedary camels (Camelus dromedarius): A review. Journal of Dairy Research, 80(2): 185–196.

Petersson-Wolfe, C. S. (2010). Detection of Staphylococcus aureus in camel milk using PCR. Journal of Dairy Science, 93(3): 1062–1068.

Radostits, O. M., Gay, C. C., Hinchcliff, K. W., & Constable, P. D. (2007). Veterinary medicine: A textbook of the diseases of cattle, horses, sheep, pigs, and goats (10th ed.). Saunders Elsevier.

Sarwar, M. (2013). Prevalence and antibiogram of Staphylococcus aureus isolated from subclinical mastitis in camels. Pakistan Journal of Zoology, 45(4): 1049-1053.

Sinha, M. K., Thombare, N. N., & Mondal, B. (2014). Subclinical mastitis in dairy animals: incidence, economics, and predisposing factors. Scientifica. 21(3): 110-115. https://doi.org/10.1155/2014/523984

Smith, K. L., Todhunter, D. A., & Schoenberger, P. S. (2019). Environmental mastitis: cause, prevalence, prevention. Journal of Dairy Science, 68(6):1531–1553.

Swelum, A. A., Alowaimer, A. N., Abouheif, M. A., & Al-Harbi, M. S. (2021). Prevalence and antimicrobial resistance of Staphylococcus aureus isolated from camel milk in Saudi Arabia. Veterinary World, 14(2): 362–368. https://doi.org/10.14202/vetworld.2021.362-368.

Wanjohi, G. M., Gitao, C. G., & Bebora, L. C. (2013). Subclinical mastitis affecting the hygienic quality of marketed camel milk from North-Eastern Province, Kenya. Microbiology Research International, 1(1): 6–15. https://www.netjournals.org/z_MRI_13_012.html

Woubit, S., Bayleyegn, M., Bonnet, P., & Jean-Baptiste, S. (2001). Camel (Camelus dromedarius) mastitis in the Borena lowland pastoral area, southwestern Ethiopia. Revue d'Élevage et de Médecine Vétérinaire des Pays Tropicaux, 54(3-4): 207–212. https://doi.org/10.19182/remvt.9786