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RESEARCH ARTICLE

Comparative Antibiogram Analysis of Vancomycin-resistant S*taphylococcus aureus* (VRSA) Isolated from Small Ruminants in Pakistan

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ABSTRACT

Subclinical mastitis (SCM) is a major challenge in dairy animals due to its association with pathogenic *Staphylococcus aureus* and the emergence of vancomycin-resistant *S. aureus* (VRSA). This study investigated the prevalence of SCM, *S. aureus*, and VRSA in small ruminants, along with the antimicrobial susceptibility profile of VRSA isolates. 384 milk samples were collected from goat breeds, including Beetal and Teddy, and sheep breeds, including Lohi, Kajli, and Thali. Primarily, culturing was done on 5% blood agar followed by mannitol salt agar (MSA). After phenotypic confirmation of *S. aureus*, PCR was performed for genetic confirmation. Then, VRSA isolates were identified using the disc diffusion method, and an antibiotic susceptibility test was performed. The overall prevalence of SCM, *S. aureus*, and VRSA was found to be 38.02%, 34.93%, and 49.01% respectively. A higher SCM prevalence among goats was observed in Beetal goats than in teddy goats. In sheep, SCM was highest in the Lohi, followed by Kajli and Thali. Phenotypic screening revealed higher VRSA prevalence in Beetal goats compared to Teddy. Among sheep, VRSA prevalence was highest in Lohi, followed by Kajli and Thali. Antimicrobial susceptibility testing showed VRSA isolates exhibited the highest resistance to oxytetracycline, cefoxitin, and trimethoprim-sulfamethoxazole. Conversely, linezolid and moxifloxacin remained effective, while gentamicin, amikacin, fusidic acid, ciprofloxacin, and tylosin showed moderate efficacy. Notably, sheep isolates exhibited resistance to fewer antibiotics compared to goat isolates. These findings highlight the alarming emergence of VRSA in small ruminants, emphasizing the need for rational antibiotic use and continuous surveillance.

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INTRODUCTION

Small ruminants, including sheep and goats, represent the most widely distributed livestock species, with goats exhibiting a greater adaptability to arid and semi-arid environments than sheep (Degen, 2007). According to the livestock census 2024-25, the goat and sheep populations were found to be 89.4 and 33.1 million, respectively. Most of Pakistan's rural population depends on the livestock industry for their livelihood, making it a significant component of its agricultural economy. Moreover, small ruminant milk is regarded as a nutritional

powerhouse and one of the functionally active dairy products. Within the human gastrointestinal tract, sheep and goat milk proteins are hydrolyzed into bioactive peptides that exhibit antioxidative, antimicrobial, antiaging, immunomodulatory, and antithrombotic properties (Mohapatra *et al.*, 2019). Also, goat milk's high buffering capacity makes it useful for treating stomach ulcers and is suggested as a good alternative for individuals allergic to cow milk (Bhattarai, 2012).

Mastitis is one of the most common and costly diseases affecting lactating animals. Mastitis is a major economic concern in small ruminants due to the high death rate of lactating animals, treatment expense, decreased milk supply and quality, and the potential for consumer food poisoning. Multiple pathogens are involved in small ruminant mastitis. Still, *Staphylococcus aureus* is the most commonly isolated bacterium from goat and sheep milk (Andrade *et al.*, 2021), and the emergence of different strains of gram-positive and gram-negative bacteria that are resistant to various drugs is posing a threat to public health. Antimicrobials are used to treat bacterial mastitis.

Antimicrobial therapy constitutes a fundamental aspect of modern clinical practice; however, its irrational and excessive use has driven the emergence of resistant *Staphylococcus aureus* strains, thereby complicating therapeutic management. The rapid emergence of resistance and inadequate infection control measures facilitate the dissemination of resistant microorganisms to patients and the surrounding environment. *Staphylococcus aureus* frequently develops multidrug resistance, which aids in antibacterial actions and immune system evasion. Methicillin-resistant *Staphylococcus aureus* (MRSA) exhibits resistance to β-lactam antibiotics as a result of the reduced affinity of drugs for penicillin-binding proteins (PBPs), particularly PBP2a (Anwaar *et al.*, 2023).

Initially, vancomycin, a semi-synthetic beta-lactam antibiotic, was considered efficacious against MRSA, but *Staphylococcus aureus* also resisted vancomycin by acquiring the *vanA* gene. This gene causes the alterations in peptidoglycan synthesis by making the action of vancomycin ineffective, resulting in vancomycin-resistant and vancomycin-intermediate *Staphylococcus aureus* strains (Berger-Bächi and McCallum, 2006). Few cases of vancomycin-resistant Staphylococcal strains have been reported in the veterinary field, as vancomycin is not commonly used in veterinary practice for treatment. The development of resistance in pathogens not only leads to an increased cost of treating mastitis due to this pathogen, but it poses an important public health concern, as humans may be exposed through improper handling or consumption of contaminated meat or milk products (Feingold *et al.*, 2012; Caruso *et al.*, 2016). This study evaluates the phenotypic prevalence of VRSA and comparative antibiogram analysis of VRSA isolated from small ruminant mastitic milk.

MATERIALS AND METHODS

Sampling: A total of 384 milk samples (n = 192 Goat; n = 192 sheep) based on 50% prevalence of VRSA by using protocols of (Thrusfield, 2018) as per guidelines of the National Mastitis Council were collected from 2 districts, including Faisalabad and Muzaffargarh (Fig. 1), using a convenient sampling method. First of all, collected milk samples from small ruminants were processed for sub-clinical mastitis (SCM) by using the California Mastitis Test (CMT) according to (Aljumaah *et al.*, 2011). SCM-based positive samples were collected in Falcon tubes aseptically for further analysis.

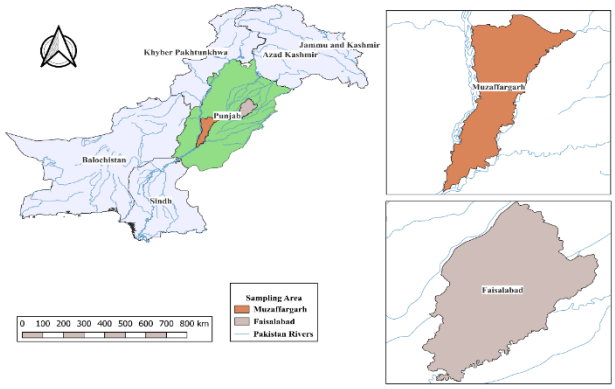

Fig. 1: QGIS map of the study districts

Phenotypic identification and genotypic confirmation of *S. aureus:* For the isolation and identification of *S. aureus*, the samples were subjected to standard microbial techniques (Javed *et al.*, 2023). SCM-based positive milk samples were cultured on 5% blood agar and incubated for 24-48 hours at 37°C. The bacterial colonies were further streaked to Mannitol salt agar (MSA) for phenotypic identification of *S. aureus*. Mannitol fermentation and the appearance of yellow golden colonies confirmed the isolates as *S. aureus*. After this, these isolates were confirmed as *S. aureus* by microscopic and biochemical testing, such as Gram staining and catalase test (Rasheed *et al.*, 2025).

For genotypic confirmation of *S. aureus*, Polymerase chain reaction (PCR) was performed on phenotypically confirmed *S. aureus* by targeting the *nuc* gene with a product size of 270bp using primers (F: GCGATTGATGGTGATACGGTT and R: AGCCAAGCCTTGACGAACTAAAGC) and PCR conditions as used by (Anwaar *et al.*, 2023).

Isolation and identification of VRSA by the Disc Diffusion method: Vancomycin discs were placed on the activated growth of *S. aureus* (0.5 McFarland)

on Muller-Hinton agar plates to identify VRSA. The plates were incubated at 37°C for 24 hours. The growth inhibition zones around the antibiotic disc were measured to compare with standard zones described in Clinical and Laboratory Standards Institute (CLSI, 2019). The isolates exhibiting inhibition zones < 17mm to vancomycin were considered as vancomycin-resistant *Staphylococcus aureus* (VRSA), while isolates exhibiting inhibition zones ≥ 21mm to vancomycin were considered as vancomycin-sensitive *Staphylococcus aureus* (VSSA) strains.

Susceptibility of VRSA to various Antibiotics: The antibiotics used in this study included oxytetracycline (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), Amikacin (30 µg), Tylosin (30 µg), Fusidic acid (10 µg), moxifloxacin (5 µg), cefoxitin (30 µg), linezolid (30 µg), trimethoprim + sulfamethoxazole (1.25 µg, 23.75 µg) were tested against 22 isolates of VRSA from small ruminants (n=11 Goat; n=11 Sheep). The antibiotic discs were placed on Muller-Hinton agar after swabbing with activated VRSA 1x108 CFU/ml growth and then incubated at 37°C for 24 hours. The growth inhibition zones formed around antibiotic discs were measured with a Vernier caliper and compared with standard zones to differentiate VRSA strains as sensitive, resistant, or intermediate (CLSI, 2019).

Statistical Analysis: The prevalence of VRSA was calculated as per the formula narrated by (Thrushfield, 2013). Descriptive statistics were used to categorize the isolates into sensitive, intermediate, and resistant.

RESULTS

Prevalence of SCM and *S. aureus* isolated from small ruminants: Out of the 384 milk samples examined from goats and sheep, the overall prevalence of subclinical mastitis (SCM) was 38.02%. In contrast, among the SCM-positive samples, *S. aureus* was isolated in 34.93% isolates. Between goat breeds, a higher rate of SCM was found in the Beetal breed, 50.00% than that of the Teddy breed, 37.80% and the SCM-associated pathogen was more prevalent in the Teddy breed, 38.70% than in the Beetal breed, 36.36% as given in Table 1.

Among sheep breeds, the highest SCM was found in the Lohi breed, followed by Kajli and Thali, which recorded 40.00%, 26.86%, and 24.00%, respectively. Furthermore, *S. aureus* was more Lohi and Thali (33.33%) than that of the Kajli breed (27.77%), as shown in Table 2.

Prevalence of VRSA on a phenotypic basis in small ruminants: Overall, 49.01% isolates were found resistant to vancomycin on the disc diffusion method, as shown in Table 3. A higher prevalence of VRSA was found in the Beetal breed, 45.00%, than in Teddy, 33.33%. Among sheep breeds, a high proportion of VRSA was observed in Lohi sheep at 70.00%, followed by Kajli at 60.00% and Thali at 50.00% (Table 3).

Table 1. Prevalence of SCM, *S. aureus*, and phenotypic VRSA in goat

Breed	No. of samples	SCM positive (%)	*S. aureus* (%)	VRSA positive (%)
Beetal	110	55 (50.00)	20 (36.36)	9 (45.00)
Teddy	82	31 (37.80)	12 (38.70)	4 (33.33)
	192	86 (44.79)	32 (37.20)	13 (40.62)

Table 2. Prevalence of SCM, *S. aureus*, and phenotypic VRSA in sheep

Breed	No. of samples	SCM positive (%)	*S. aureus* (%)	VRSA positive (%)
Lohi	75	30 (40.00)	10 (33.33)	7 (70.00)
Kajli	67	18 (26.86)	5 (27.77)	3 (60.00)
Thali	50	12 (24.00)	4 (33.33)	2 (50.00)
Total	192	60 (31.25)	19 (31.66)	12 (63.15)

Table 3. Prevalence of SCM, *S. aureus*, and phenotypic VRSA in small ruminants

Breed	No. of samples	SCM positive (%)	*S. aureus* (%)	VRSA positive (%)
(Beetal, Teddy) + (Lohi,Kajli, Thali)	384	146(38.02)	51(34.93)	25(49.01)

Antimicrobial susceptibility and resistance profiling of VRSA isolates from small ruminants: The disc diffusion test against VRSA isolates demonstrated resistance to different antibiotics. In ruminants, the highest resistance was observed against oxytetracycline, followed by cefoxitin and trimethoprim-sulfamethoxazole. On the contrary, antibiotics moxifloxacin and linzolid were susceptible against VRSA isolates in goat and sheep species. Furthermore, gentamicin, amikacin, fusidic acid, ciprofloxacin, and tylosin were moderately effective drugs in small ruminants. Also, sheep VRSA isolates demonstrated resistance to fewer antibiotics than goat VRSA isolates, as shown in Table 4.

Table 4. Antimicrobial susceptibility and resistance profiling of VRSA isolates from small ruminants

Antibiotics discs	VRSA isolates from goats (n =11)			VRSA isolates from sheep (n =11)		
	Sensitive (%)	Intermediate (%)	Resistant (%)	Sensitive (%)	Intermediate (%)	Resistant (%)
Oxytetracycline (30 µg)	2 (18.18)	-	9 (81.81)	2 (18.18)	2 (18.18)	7 (63.63)
Ciprofloxacin (5 µg)	3 (27.27)	2 (18.18)	6 (54.54)	4 (36.36)	5 (45.45)	2 (18.18)
Gentamicin (10 µg)	3 (27.27)	3 (27.27)	5 (45.45)	7 (63.63)	3 (27.27)	1 (9.09)
Amikacin (30 µg)	4 (36.36)	1 (9.09)	6 (54.54)	6 (54.54)	2 (18.18)	3 (27.27)
Tylosin (30 µg)	3 (27.27)	4 (36.36)	4 (36.36)	4 (36.36)	5 (45.45)	2 (18.18)
Fusidic acid (10 µg)	2 (18.18)	4 (36.36)	5 (45.45)	5 (45.45)	1 (9.09)	5 (45.45)

Moxifloxacin (5 µg)	7 (63.63)	2 (18.18)	2 (18.18)	8 (72.72)	2 (18.18)	1 (9.09)
Cefoxitin(30 µg)	2 (18.18)	1 (9.09)	8 (72.72)	2 (18.18)	3 (27.27)	6 (54.54)
Linezolid (30 µg)	8 (72.72)	2 (18.18)	1 (9.09)	9 (81.81)	2 (18.18)	-
Trimethoprim + Sulfamethoxazole (1.25 µg, 23.75 µg)	4 (36.36)	1 (9.09)	6 (54.54)	2 (18.18)	1 (9.09)	8 (72.72)

DISCUSSION

The present study aimed to evaluate the prevalence of SCM, *S. aureus*, and VRSA isolated from small ruminant milk. This study also finds effective antibiotics against VRSA infection. Small ruminants are integral to smallholder farming systems, providing high-quality animal protein and serving as a vital source of financial security, with their primary role being the generation and preservation of cash income. Although small ruminant production contributes significantly to income generation, poverty reduction, and national development, its productivity remains suboptimal due to malnutrition, low genetic potential, poor management, and, most critically, infectious diseases, which represent the major constraint to sustainable production (Gebrewahid *et al.*, 2012). Among these diseases, subclinical mastitis represents a concerning issue affecting the productivity of small ruminants.

According to this study, overall SCM was recorded in small ruminants at 38.02%. In comparison, in goats, SCM prevalence was 44.79% higher than the results of (Gebrewahid *et al.*, 2012; Pirzada, 2016), who reported SCM prevalence of 18.03% and 38% respectively. Current SCM positive results are lower than the results 52.1% reported by (El-Zamkan and Mohamed, 2021), 52.56% demonstrated by (Hussein *et al.*, 2020), and 55.47% by (Javed *et al.*, 2024). Furthermore, at the breed level, SCM prevalence was 50.00% in Beetal and 37.80% in Teddy, which was much higher and in contrast with the findings of (Javed *et al.*, 2025). This variation in prevalence is associated with different management practices, nutritional status, awareness, and diagnostic approaches in different countries.

SCM prevalence in sheep was found to be 31.25% in sheep milk samples isolated from the district Muzaffargarh, which was in agreement with the study of (Sabir *et al.*, 2024), who reported 34.37% SCM in different nomadic sheep flocks. A higher SCM prevalence was found in the Lohi, Kajli, and Thali sheep breeds. The current study reported a higher prevalence of SCM than that of (Hammadi and Yousif, 2013; Rahman *et al.*, 2016), who reported SCM in sheep 28.57% in Baghdad and 26.1 % in Iran, respectively. This variation can be attributed to nomadic rearing practices, which differ substantially from modern farming systems in terms of nutrition, housing, and hygiene standards, thereby increasing the susceptibility of sheep flocks to infections.

S. aureus was found to be a more prevalent pathogen causing mastitis in small ruminants. On a genotypic basis, *S. aureus* prevalence was found to be 34.93% in small ruminants. *S. aureus* is also called "Golden Cluster Seed," hence its nickname "golden staph" due to its formation of golden yellow colonies on MSA (Saad *et al.*, 2024). An Egyptian author reported *S. aureus* prevalence in goats' mastitic milk at 19.3% which was lower than the recent research. In comparison, a higher prevalence of 43.1% of *S. aureus* in goats was reported by (Cortimiglia *et al.*, 2015). In sheep, the association of *S. aureus* with SCM occurrence was 31.66% in this study. Some other studies demonstrated *S. aureus* association with SCM ranged from 27.7% to 33.33% (Alt *et al.*, 2011; Achek *et al.*, 2020).

When treating *S. aureus* infections, vancomycin is one of the best antibiotics. However, VRSA has developed diverse resistance to various antibiotics, and its prevalence in this study was 49.01% in small ruminants. On a comparative basis, VRSA was more prevalent in sheep, 63.15%, than in goats, 40.62%. Another study conducted in Egypt demonstrated VRSA prevalence from sheep carcasses at 20% (Zaher *et al.*, 2023). The comparatively lower prevalence of VRSA in sheep reported in earlier studies than in more recent investigations may be attributed to limited and less frequent use of vancomycin and other critically important antimicrobials in veterinary practice, differences in study design, and sampling size. Other studies reported VRSA 5.73% and 20.75% from bovine milk samples reported by (Muzammil *et al.*, 2023; Ijaz *et al.*, 2024). Beetal goats exhibited higher vancomycin resistance 45.00% compared to Teddy goats 33.33%. Among sheep, the Lohi breed showed the highest resistance to vancomycin 70.00%, followed by Kajli 60.00% and Thali 50.00%, respectively.

Antibiogram results revealed that VRSA isolates of goats were found 81.81% resistant against oxytetracycline, then cefoxitin, and trimethoprim-sulfamethoxazole were found resistant to 72.72% and 54.54% respectively. Meanwhile, moxifloxacin and linezolid were susceptible to VRSA isolates. A similar trend was also observed by (Javed *et al.*, 2021) from bovine and (El-Deeb *et al.*, 2022) from goat VRSA isolates. The opposite trend to the current study was observed in Indonesia and Turkey by (Praja et al., 2023; Cebeci, 2024), regarding oxytetracycline resistance, which ranged from 18.78% to 21.1% from goat milk isolates.

Oxytetracycline (75%) and ciprofloxacin (100%) sensitivity was also seen in bovine VRSA isolates in Pakistan, contrasting our findings by (Javed *et al.*, 2021). Similarly, various antibiotics such as oxytetracycline, ciprofloxacin, and amikacin were all found sensitive to the VRSA isolates among bovine and caprine milk in India by (Bhattacharyya *et al.*, 2016), which is in contrast with our study.

The antibiogram of sheep VRSA isolates revealed that the sheep isolates were found to be 72.72% resistant to trimethoprim-sulfamethoxazole, followed by oxytetracycline, 63.63% resistant, and then cefoxitin, 54.54% resistant. Contrastingly, (Rehman *et al.*, 2020) reported no resistance in VRSA isolates against trimethoprim-sulfamethoxazole and very low resistance of VRSA isolates against oxytetracycline Similarly, the sheep VRSA isolates were susceptible to linezolid (81%), followed by moxifloxacin (72%) and then gentamicin (63%) which is in line with the study of (Muzammil *et al.*, 2023) who found linezolid and gentamicin to be almost 80% sensitive.. These shifting trends reinforce that, despite vancomycin resistance, various alternative antibiotics may remain effective, but their use must be judicious to forestall resistance development.

Conclusion

The study revealed a significant prevalence of subclinical mastitis and *S. aureus* in small ruminants with a notable emergence of VRSA. Higher rates of SCM and VRSA were recorded in Beetal goats and Lohi sheep compared to other breeds. VRSA isolates exhibited multidrug resistance, particularly against oxytetracycline, cefoxitin, and trimethoprim-sulfamethoxazole. These findings underscore the urgent need for prudent antimicrobial use, better herd management, and sustained epidemiological surveillance.

Conflict of Interest: The authors have no competing interests.

Authors' contribution: AA and YRK provided the research idea, performed the experiments, and wrote the manuscript.

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