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RESEARCH ARTICLE

Molecular Dynamics and Antimicrobial Resistance Pattern of β-lactam Resistant Coagulase Positive *Staphylococcus aureus* Isolated from Goat Mastitis

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ABSTRACT

Raw goat milk can be a potential source to transfer antibiotic-resistant pathogens between animals and humans. Due to increased virulence and antimicrobial resistance genes acquired by *Staphylococcus aureus* (*S. aureus*), it is considered a major public health hazard and has a significant impact on the treatment and management of mastitis in dairy goats. For this purpose, Coagulase-positive *S. aureus* (n=103) isolates from mastitis samples in goats were evaluated to check the resistance mechanism against β-lactam antibiotics by disc diffusion assay and by PCR. The isolates were tested for the presence of *coa*, *mec*A, and *bla*Z genes. The results of PCR analysis revealed that out of 384 milk samples collected from goats, 103 were identified as CoPS, 37 isolates carried *bla*Z gene while 19 isolates were identified as methicillin-resistant *Staphylococcus aureus* (MRSA). The absolute frequency depicted that 13 of the isolates harbored both *blaZ* + *mec*A genes in their genetic material. The findings of the antibiogram profile of β-lactam resistant coagulase positive *S. aureus* was found highly resistant against the oxytetracycline, followed by gentamicin and tylosin while the maximum isolates showed sensitivity towards ciprofloxacin, levofloxacin, and moxifloxacin in both groups. The results of antibiotic susceptibility patterns would be critical for evaluating the trends and patterns of antimicrobial resistance (AMR), estimating the contribution of particular genes in drug-specific resistance, and developing control measures to decrease AMR in goats of Pakistan. The genetic analysis of the current study concluded that genetic divergence, the transfer of zoonotic MRSA, and the involvement of *coa*, *mec*A, and *bla*Z genes serve as important virulence factors of *S. aureus* leading to the causation of goat mastitis.

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INTRODUCTION

In small ruminants, mastitis has economic impact in goat farming in terms of decreased yield of milk and quality, mortality, treatment costs, (Gelasakis *et al.*, 2015) and health concerns in public due to the risk of food poisoning in humans (Abdeen *et al.*, 2021). Numerous pathogens can cause mastitis in small ruminants like *Streptococcus* spp, *Enterobacteriaceae*. *Pseudomonas aeruginosa, Mannheimia haemolytica*, *Corynebacteria* and fungi (Contreras *et al.*, 2007) in which, *Staphylococcus aureus (S. aureus)* is the most common microorganism isolated from sheep and goat milk (Queiroga, 2017; Abdalhamed *et al.*, 2018).

S. aureus has ability to directly anchor to mammary tissue epithelium by using adhesive proteins which helps in recognizing macromolecules of host cells. Additionally, presence of teichoic acid polymers also plays an important role in adhesion process and ultimately host cell invasion. Moreover, *S. aureus* also produces variety of virulence factors such as toxins, enzymes and biofilm production which are invoiced in pathogenesis of mastitis (Zaatout *et al.*, 2020). *S. aureus* possesses the *nuc* gene which codes for the thermostable nuclease, to cause DNA and RNA lysis in host cells leading to tissue damage and its spread in the body (Javed *et al.*, 2023; Hu *et al.*, 2013), as well as stimulate the outflow of *S. aureus* when captured by neutrophils extracellular setups and ultimately enable pathogen to escape the host defense mechanism (Kenny *et al.*, 2017). The *nuc* gene is regarded as gold standard for the identification of *S. aureus* (Torres *et al.*, 2019). *S. aureus* contains a variety of virulence factors, including the coagulase enzyme, which has affinity towards plasma fibrinogen to produce fibrin clots and prevents microbes from phagocytosis and various other host defense mechanisms (Andrade *et al.,* 2021). Coagulase enzyme produced by *coa* gene is normally helpful in differentiating coagulase-positive *Staphylococci* (CPS), viz. *S. aureus*, and *S. intermedius* from the CNS species (Pizauro *et al.,* 2019).

Intramammary antibiotics particularly β-lactam agents are recommended for the treatment of mastitis in goats (da Costa Krewer *et al.,* 2015). But studies have reported that their use may become responsible for the selection, pressure, and spread of resistance to many drugs also known as multidrug-resistant (MDR) (Ijaz *et al.,* 2023; Bhargava and Zhang, 2012; Nobrega *et al.,* 2018). *Staphylococci* exhibit resistance to β-lactams including penicillin due to *bla*Z and SCC*mec* genes. Four different types of beta-lactamases are coded by *blaZ* gene that breakdown the ring of β-lactam (Ferreira *et al.,* 2017) and the *Staphylococcal* cassette chromosome *mec* codes a penicillin-binding protein and results in a wide-spectrum resistance to β-lactams (Klimiene *et al.,* 2016). Raw goat milk is a prospective origin of antibiotic resistance in humans. Contamination caused by the microorganisms comes from the farm level and contains the cytological agents necessary for causing subclinical mastitis. Keeping in view the importance of subclinical mastitis, the current study was designed to investigate the prevalence of *S. aureus* strains harboring *coa* and *blaZ* genes that are mainly responsible for high pathogenicity as well as transfer of this antimicrobial resistant strain to human population through consumption of contaminated milk of dairy goats in district Muzaffargarh, Pakistan.

MATERIALS AND METHODS

Sampling area and strategy: Milk samples were taken from various goat flocks, veterinary hospitals located in and around the different tehsils of district Muzaffargarh, Punjab. Therefore, an overall 384 milk samples comprising of both clinical and subclinical mastitis from dairy goats were collected using the convenient sampling technique (Thrusfield, 2007). Screening of samples was done by California Mastitis Test to detect the prevalence of sub-clinical mastitis (Javed *et al.,* 2023). The positive samples for both clinical and sub-clinical mastitis were carried to Medicine Research Laboratory, maintaining a cold chain at 4◦C.

Isolation and confirmation of *Staphylococcus aureus:* Positive milk samples were swabbed on blood agar following the incubation at 37ºC for 24 hours. Further, culturing was done on Mannitol salt agar for differentiation and isolation of *S. aureus.* Coagulase positive *S. aureus* was confirmed by using biochemical tests including catalase and coagulase tests following the guidelines of Bergey's Manual of Determinative Bacteriology.

Molecular characterization of coagulase positive *S. aureus*: Coagulase positive *S. aureus* was genotypically confirmed by targeting *coa* gene using, primers at a 970bp product size (Javed *et al.,* 2023). The PCR product was finally run on 1.5% agarose gel electrophoresis and observed results under UV illuminator.

Methodology for phenotypic identification: For the phenotypic identification of β-lactam resistance in coagulase-positive *S. aureus* isolates, penicillin G disc (10 µg) was used. First of all, fresh culture of *S. aureus* colonies were prepared at the concentration of 1.5×10^8cfu/mL and poured onto Mueller Hinton agar (MHA) following by placement of penicillin G discs by provided the incubation of 37°C for 24 hrs. Isolates showing a zone of inhibition <28mm were considered resistant to β-lactam antibiotics while isolates exhibiting ZOI >29mm were considered sensitive for β-lactam antibiotics according to CLSI guidelines

Molecular confirmation of β-lactam resistant coagulase positive *S. aureus*: Phenotypically β-lactam resistant isolates were further confirmed by targeting the *blaZ* gene using forward primer bla_Z-fwd (5′-CAAAGATGATATAGTTGCTTATTCTCC-3′) and reverse primer bla_Z-rev (5′-TGCTTGACCACTTTTATCAGC-3′) having 421bp product size (El Feghaly *et al.,* 2012). PCR was performed by using the following cyclic conditions i.e. 95°C for initial denaturation for 5 min followed by 30 cycles of amplification (denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min), with a final extension at 72°C for 10 min. The positive samples were visualized at 421bp under UV illuminator and were shipped to well-reputed laboratory for sequencing (Fig. 1).

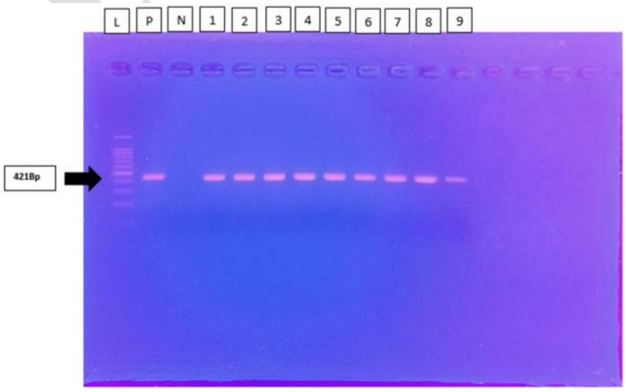

Fig. 1: PCR showing the *blaZ* gene at 421bp: L: Ladder, P: Positive control; N: Negative control, 1-9 positive samples for *blaZ* gene

Evaluation of *blaZ* associated CPS by phylogenetic analysis: By using BLAST, sequences of nucleotide were identified by comparing with already reported sequences. The sequence of our isolates was compared to other extremely comparable published sequences from different countries by using *Clustal W* multiple alignment using BioEdit software. Then a phylogenetic tree on the basis of sequence distance using MEGA-X software's Maximum Likelihood techniques was constructed to check the homology among the sequences.

***In-vitro* antimicrobial susceptibility testing for *S. aureus*:** Fresh culture of coagulase-positive *S. aureus* was adjusted to the concentration at 1.5×10^8cfu/ml and poured onto MHA agar followed by the placement of various antibiotics such as (vancomycin, amikacin, linezolid, gentamicin, tylosin, trimethoprim+sulphamethoxazole, oxytetracycline, ciprofloxacin, levofloxacin, moxifloxacin, and chloramphenicol) aseptically by provided incubation for 24 hours at 37°C and the zones of inhibition (mm) around the antibiotics discs were calculated and compared with the standards provided by CLSI manual (CLSI, 2019).

Data analysis: The prevalence was measured by the given formula;

$$\text{Prevalence (\%)} = \frac{\text{No. of infected animals (n)}}{\text{Total number sampled (N)}} 100$$

The descriptive statistics was applied for the assessment of *in-vitro* antibiotic susceptibility using SPSS version 22.

RESULTS

Phenotypic and genotypic prevalence of coagulase positive *S. aureus*: The current study's findings revealed that a total of 384 (n=172 clinical; n= 212 sub-clinical) milk samples were collected from dairy goats of different regions of the country. Out of 384 milk samples, the phenotypic prevalence of *S. aureus* was 213/384 (55.47%) as confirmed by performing a coagulase test while the genotypic prevalence of *S. aureus* by targeting *coa* gene was found to be 103 (26.82%**)** and were declared as coagulase-positive *S. aureus* (CoPS) (Table 1). The genotypic prevalence of coagulase-positive *S. aureus* was found higher in sub-clinical mastitis as compared to clinical mastitis.

Table 1: Phenotypic and genotypic prevalence of Coagulase positive *S. aureus* isolated from goat mastitis

Sample type	No. of milk samples	Coagulase positive *Staphylococcus aureus* (%)	Genotypic prevalence (%) PCR
Clinical	172	99 (57.56)	39 (22.68)
Subclinical	212	114 (53.78)	64 (30.19)
Total	384	213 (55.47)	103 (26.82)

Prevalence of β-lactam resistant CoPS: All the confirmed Coagulase positive *S. aureus* isolates (n=103) were evaluated for phenotypic as well as genotypic detection of β-lactam resistance. From 103 tested isolates, 26.21% (27/103) displayed resistance towards the β-lactam antibiotics group while 73.79% (76/103) were found as sensitive on a phenotypic basis. The genotypic positive samples for *blaZ* gene were found 35.92% (37/103) while out of 103, remaining (64.07%) isolates out of 66 didn't show positive results towards *blaZ* gene (Table 2).

Table 2: Phenotypic and genotypic prevalence of β-lactam resistant and sensitive Coagulase positive *S. aureus* isolates

Sample type	CPS Isolates (%)	Phenotypic prevalence (%)		Genotypic prevalence (%)	
		Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)
Clinical	39	09 (23.08)	30 (76.92)	15 (38.47)	24 (37.36)
Subclinical	64	18 (28.13)	46 (71.86)	22 (34.38)	42 (65.62)
Total	103	27 (26.21)	76 (73.79)	37 (35.92)	66 (64.07)

Phylogenetic analysis of *blaZ* gene: The gene sequences of present study isolates showed higher similarity with the *blaZ* gene of *S. aureus* from already reported sequences available in GenBank database by Basic local alignment search tool (BLAST). The homology comparison of *blaZ* gene from current study isolates was done with already reported sequences retrieved from the NCBI database. The ClustalW alignment of present study isolates revealed significant differences among themselves and with already reported sequences. Among the present study isolates, the NS sequence from the current study shows substitution at 4 different positions (e.g. 4, 53, 90, and 324th) and also deletion at one position (10th) while NG41 only shows substitution and deletion at 1 place each (e.g. 4th & 10th) to NG7 *blaZ* gene sequence. The comparison with other countries sequences revealed significant similarities and differences. All the sequences shows substitution at common positions (e.g. 4, 10, 12, 53, 54, 78, 90, 112, 118, 130, 133, 135, 168) except sequences from USA (accession number: NG047533) which shows different pattern (Fig. 2). The *ClustalW* aligned sequences were further analyzed by constructing phylogenetic via the Maximum likelihood method at 1,000 replications bootstrapping technique (Fig. 3). The local study isolates showed the highest similarity among themselves while the sequences from Italy (accession number: U58139), China (accession number: FJ809758) and Australia (accession number: KM362525) form different clad and were found less similar with the present study isolates. Moreover, the nucleotide sequences from the USA (accession number: NG047533) and India (accession number: KX181859) were also found connected by the same internal node with previous clad exhibiting less similarity with the local study isolates. However, the gene sequence of the isolate from the USA (accession number: AF086644) forms out-group and found least similar with current study isolates (Fig. 3).

Absolute frequency of the *blaZ* and *mecA* genes: Coagulase-positive *S. aureus* isolates were also tested for the presence of both *mecA* and *blaZ* genes. Out of these 103 isolates, 37/103 (35.92%) and 19/103 (18.45%) of the isolates were found positive towards *blaZ* and *mecA* gene respectively while 13/103 (12.62%) of the isolates were positive for both of them. The frequency of the detection of the *mecA* gene was lower as compared to *blaZ* gene (Table 3).

Antibiogram profile of β-Lactam resistant coagulase positive *S. aureus* isolates: All isolates were subjected to antibiotic susceptibility testing by using antibiotics of various groups for the treatment of mastitis in dairy animals. Out of β-Lactam resistant isolates (n=37), 25 of them were found resistant towards oxytetracycline, 19 against tylosin, followed by 23 towards gentamicin while 17 for both vancomycin and Trimethoprim + Sulphamethoxazole respectively (Table 4). The resistance pattern against the remaining groups was recorded as linezolid (3/37), ciprofloxacin (5/37), levofloxacin (02/37), moxifloxacin (1/37), and chloramphenicol (05/37). The moxifloxacin group showed highest sensitivity pattern (36/37) followed by levofloxacin (35/37) and ciprofloxacin (30/37). The intermediate profile revealed that 11 of the isolates are in between resistant and sensitive towards oxytetracycline, 10 against

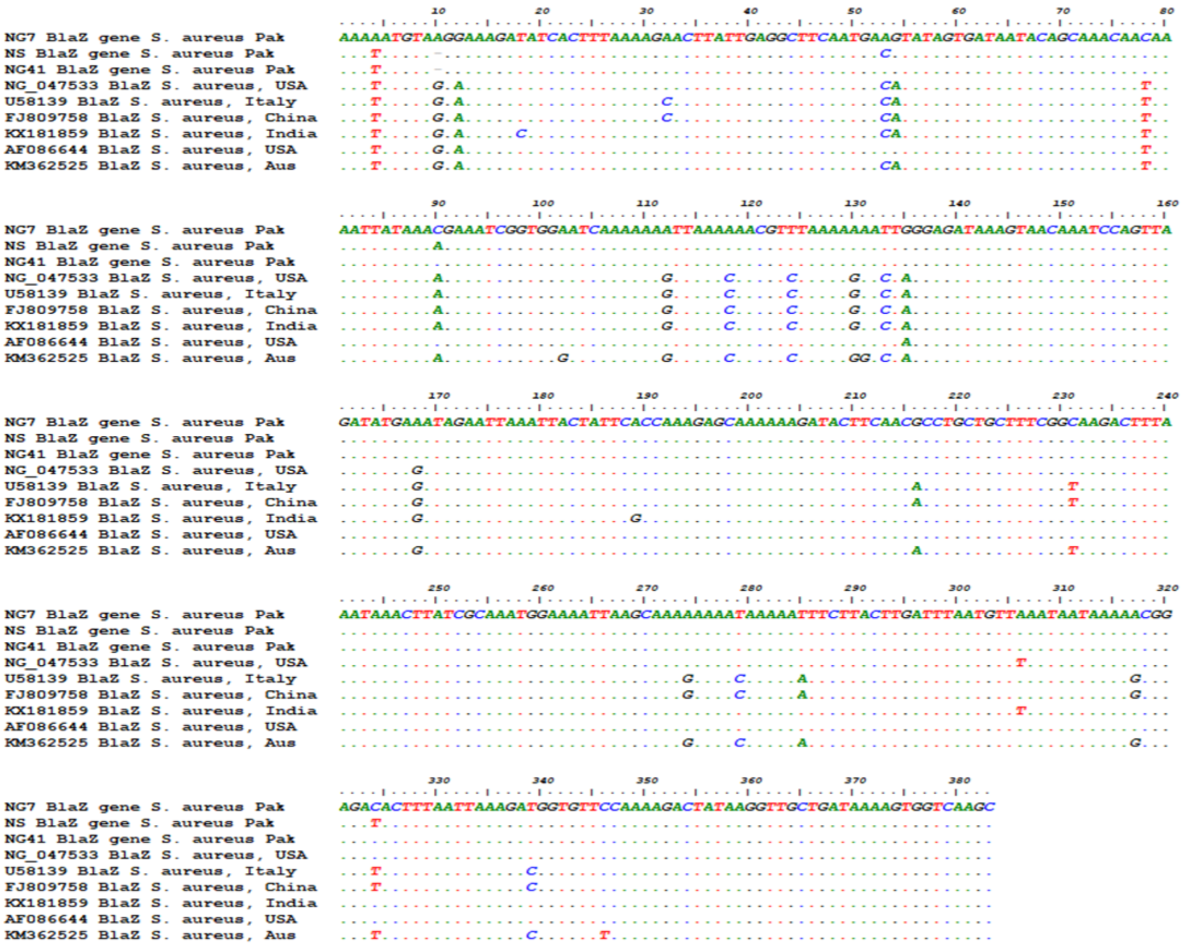

Fig. 2: *Clustal W* Sequence alignment showing the difference of present study isolates with already reported sequences from GenBank database

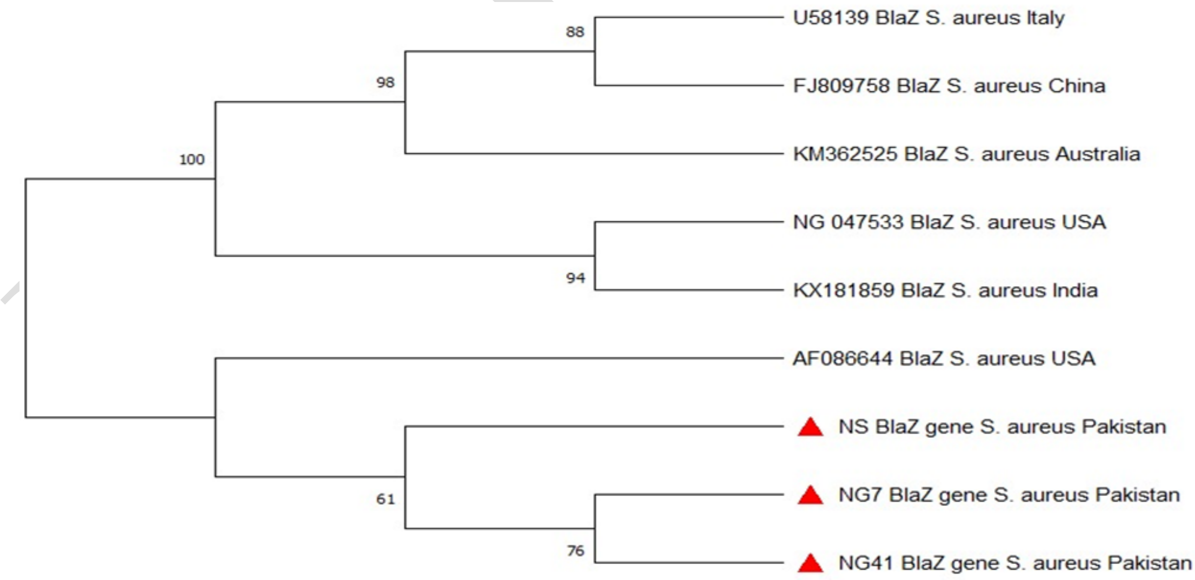

Fig. 3: Phylogenetic tree exhibiting the *blaZ* gene comparison of reported isolates with study isolates

Table 3: Absolute frequency of the *bla*Z and *mecA* genes in Coagulase positive *S. aureus* isolates

Sample type	CPS isolates	Absolute frequency		
		*mec*A	*bla*Z	*bla*Z + *mec*A
Clinical	39	06 (15.39)	15 (38.47)	04 (10.26)
Subclinical	64	13 (20.31)	22 (34.38)	09 (14.06)
Total	103	19 (18.45)	37 (35.92)	13 (12.62)

Table 4: Antibiogram profile of β-Lactam resistant and sensitive Coagulase-positive *Staphylococcus aureus*

Antibiotic	Coagulase positive *S. aureus*					
	β- Lactam resistant (n=37)			β- Lactam sensitive (n=66)		
	Resistant %	Intermediate %	Sensitive %	Resistant %	Intermediate %	Sensitive %
Vancomycin	17 (45.95)	-	20 (54.05)	-	11 (16.67)	55 (83.34)
Amikacin	12 (32.43)	10 (27.02)	15 (40.55)	6 (9.09)	13 (19.70)	47 (71.21)
Linezolid	03 (8.10)	3 (8.10)	31 (83.79)	-	2 (3.03)	64 (96.97)
Gentamicin	23 (62.16)	08 (21.62)	06 (16.21)	3 (4.54)	4 (6.06)	59 (89.40)
Tylosin	19 (51.35)	5 (13.51)	13 (35.13)	4 (6.06)	-	62 (93.94)
Trimethoprim + Sulphamethoxazole	17 (45.95)	06 (16.21)	14 (37.84)	08 (12.12)	-	58 (87.88)
Oxytetracycline	25 (67.57)	11 (29.72)	01 (2.70)	7 (10.60)	2 (3.03)	57 (86.37)
Ciprofloxacin	05 (13.51)	02 (5.40)	30 (81.08)	3 (4.54)	-	63 (95.46)
Levofloxacin	02 (5.40)	-	35 (94.60)	-	-	66 (100.00)
Moxifloxacin	01 (2.70)	-	36 (97.30)	-	-	66 (100.00)
Chloramphenicol	05 (13.51)	4 (10.81)	28 (75.68)	3 (4.54)	11 (16.67)	52 (78.79)

vancomycin, and 8 against gentamicin while the least number of isolates have an intermediate pattern against ciprofloxacin (2/37) and linezolid (3/37) group of antibiotics. By considering the β-lactam sensitive group (n=66), the Trimethoprim + Sulphamethoxazole group (8/66) exhibited more resistance as compared to other groups followed by oxytetracycline (7/66), amikacin (6/66), and tylosin (4/66) groups (Table 4).

DISCUSSION

The resistant strains of *S. aureus* responsible for the causation of clinical and subclinical mastitis in goats can be transmitted to human beings by milk and milk products (Obaidat *et al.*, 2018). The overall prevalence of clinical and subclinical mastitis in goats was recorded to be 44.79% and 55.20%. The findings of SCM was in line with the study conducted by (Hussein *et al.*, 2020; El-Zamkan and Mohamed, 2021) but differs in case of clinical mastitis as per study of (Suman *et al*., 2023) who reported 25.38% prevalence of clinical mastitis in dairy goats. The prevalence in mastitis in goat of beetle breed was 12.6% on basis of CMT, in case of teddy breed it was 29.5% while highest prevalence was recorded in desi breed that was 57.7% as per findings of (Zamin *et al.*, 2010). The present study showed that phenotypic as well as genotypic prevalence of coagulase-positive *S. aureus* associated with mastitis in goats was 55.47% and 26.82% respectively. The current prevalence was in line with the findings of (Cortimiglia *et al.*, 2016) who reported 53.1% isolates positive for the growth of *S. aureus* in goat milk. The prevalence of *S. aureus* in this study was contrary to the outcomes (17.3%) stated in Taiwan (Stegger *et al.*, 2012). The prevalence of *S. aureus* isolated from bovines, equines, ovine, and caprine has also been reported previously in Pakistan at 56.12% and 28.70% (Ijaz *et al*., 2023; Ghumman *et al*., 2023), 44.24% (Rasheed *et al*., 2023), 39.39% (Sabir *et al*., 2024) and 26.82% (Javed *et al*., 2023).

Among the *S. aureus* isolates, 26.21% isolates showed resistance to oxacillin on the disc diffusion test. The genotypic evaluation of beta-lactam resistance of isolates was evaluated by the presence of the *mecA* and *blaZ* genes. The results revealed that 18.45% of isolates showed *mecA* gene in their DNA while 35.92% of samples were positive for *blaZ* gene. The data regarding the beta-lactam-resistant *S. aureus* in dairy goats is scarce (Aras *et al.*, 2012) and a few studies have been conducted regarding this. A study conducted by (Aragão *et al.*, 2019) reported that only 7.4% of *S. aureus* isolates showed resistance to methicillin on disc diffusion test while 42.6% of isolates were found positive to *blaZ* gene but no isolate was found positive to *mecA* gene. Similar findings were also reported by (França *et al.,* 2012) who reported that 40.2% of isolates were positive for the *blaZ* gene but only 15.8% isolates showed phenotypic resistance while no isolate was found positive for the *mecA* gene.

Another study also reported a positive prevalence of 33.3% and 54.5% for phenotypic and *blaZ* positive *S. aureus* isolated from mastitis in goats. Many studies have shown that *S. aureus* isolates from goats lack the *mecA* gene, but some have confirmed the presence of *S. aureus* isolates from goat milk exhibiting *mecA*-associated β-lactam resistance (Titouche *et al.,* 2019). The prevalence of the *mecA* gene in the current study (18.45%) was corroborated by the findings of (Obaidat *et al.*, 2018) who reported an 11.5% prevalence of the *mecA* gene. A study conducted by (Turutoglu *et al.,* 2006) reported a 55.6% prevalence of beta-lactamase-producing *S. aureus*. The discrepancies in the prevalence of *S. aureus* in various studies might be due to the variations in sampling size, farming practices, and hygiene status of study animals.

The phenotypic resistance to oxacillin shown by the study isolates is usually attributed to the presence of *mecA* gene which leads to the production of PBP-2a. Many isolates revealed oxacillin resistance even in the absence of *mecA* gene which might be attributed to the production of the beta-lactamase enzyme by the *S. aureus*. The *S. aureus* shows resistance to oxacillin and other β-lactam antimicrobials either by expression of *mecA* or *mecC* gene (García-Álvarez *et al.,* 2011) or by the production of β-lactamase enzyme (Aragão *et al.,* 2019).

The current study isolates displayed a pronounced resistance pattern towards oxytetracycline (67.57%) with gentamycin (62.16%) followed by tylosin (51.35%), amikacin (45.95%), Trimethoprim + Sulphamethoxazole (45.95%), vancomycin (32.43%), Ciprofloxacin (13.51%), Linezolid (08.10%) and Levofloxacin (5.40%). Similar findings have been reported in the *S. aureus* isolates from goats showing high resistance to Gentamycin, Amikacin, and Oxytetracycline (Javed *et al.,* 2023; Altaf *et al.*, 2019). Antibiotic susceptibility in goat isolates of *S. aureus* also revealed higher resistance to Gentamicin, Oxytetracycline, and Trimethoprim + Sulphamethoxazole (Rajala-Schultz *et al.,* 2004; Turutoglu *et al.,* 2006; Javed *et al*., 2023) . These isolates were highly sensitive towards Linezolid, Ciprofloxacin, and Levofloxacin which were in line with the findings of (Nemeghaire *et al.*, 2014; Altaf *et al.*, 2019). The development of resistance against various antibiotics might be associated with the medicinal practices in farm animals of Pakistan. The imprudent use of antibiotics by the farmers without any professional consultation and the wrong or incomplete treatment of mastitis also play a role in the development of antibiotic resistance.

Conclusion: The current study reported a higher occurrence of coagulase-positive *S. aureus* responsible for causing subclinical mastitis in goats. Before this, coagulase-negative staphylococci were considered prevalent pathogens in dairy goats. Response of antibiotics against this emerging pathogen shows the importance of antimicrobial susceptibility testing along with the identification of resistant genes. Determining the resistance phenotype and preventing the selection of resistant strains are crucial for the treatment of diseased animals.

Conflict of interest: The authors have no conflict of interest in publishing this data.

Authors contribution: The first draft of the manuscript was written by MUJ. AA did sampling and laboratory analysis. MI did conceptualization and write up. HR and MJS performed data analysis AAJ did reviewing and editing of manuscript. All authors read and approved the final manuscript.

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