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Novel capsular diversity and antimicrobial resistance determinants of *Staphylococcus aureus* associated with bovine and bubaline mastitis

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Abstract

In Pakistan, bovine mastitis has been identified as one of the biggest limitations to dairy production, and *Staphylococcus aureus* has been identified as one of the most enduring and economically relevant mastitogens. The current study was conducted to examine the capsular genotype and antimicrobial resistance (AMR) of *S. aureus* isolated from cases of clinical and subclinical mastitis in cows and buffaloes of the Punjab and Sindh provinces. One hundred and fifty *S. aureus* isolates (109 from cows and 41 from buffaloes) were isolated out of 87 dairy herds and verified using *nuc* gene-based PCR. Genotyping of capsular polysaccharide (CP) demonstrated that there were only *cap*5 (56%) and *cap*8 (44%) loci, but no *cap*1 and *cap*2. The *cap*5 was the most common among clinical (20.66%) and subclinical (35.33%) isolates, whereas *cap*8 had a frequency of 12.66% and 31.33% in clinical and subclinical isolates, respectively, suggesting that CP5 and CP8 are the common circulating types of capsular pathogens in the study areas. The antimicrobial susceptibility testing involving 13 routine antimicrobial agents revealed that 92% of isolates were resistant to one or more antimicrobials, and 63.3% of the isolates were multidrug-resistant (MDR). The greatest resistance was found with penicillin (72.66%), then amoxicillin (53.33%), and amoxicillin-clavulanic acid (37.33%). Those resistant to methicillin (3.33%) were *mec*A-positive MRSA, but no isolate was positive for *mec*C. Molecular screening showed that the prevalence of the *bla*Z gene (95.33%) was high and in line with the prevalence of resistance mediated by β-lactamase. The *tet*M (92.10%) and *tet*K (84.21%) were most common among the tetracycline-resistant isolates. The determinants of macrolide resistance were *msr*C (87.5%), *erm*B and *erm*C, and the *aac-aph*D aminoglycoside resistance gene was also present in 17.64% of resistant isolates. Resistance to critically important antimicrobials like vancomycin and linezolid was low, and *optr*A was not identified. Strong genotype-phenotype concordance was shown by correlation analysis to occur in 22 cases where 2 beta-lactam, tetracycline, and macrolide resistance determinants were genotyped and phenotyped, indicating the occurrence of co-selection and possible horizontal gene transfer. This study provides the first comprehensive molecular epidemiological insight in bovine and bubaline *S. aureus* capsular diversity, as well as AMR determinants of *S. aureus*, in Punjab and Sindh. The prevalence of CP5/CP8 is in favor of their inclusion in vaccine development, whereas high rate of MDR burden evidences the urgency of antimicrobial stewardship and long term molecular surveillance within one health paradigm.

Keywords: *Staphylococcus aureus*, bovine and bubaline mastitis, capsular polysaccharide, antimicrobial resistance, multidrug-resistant (MDR) isolates

Introduction

Pakistan is the fifth-largest milk producer, with a total milk production of 72 million tonnes annually (Economic Survey of Pakistan 2024-2025). However, milk production is seriously threatened by mastitis (inflammation of the milk-producing organ), which is undoubtedly an economically important disease (Zigo et al. 2021, Tommasoni et al. 2023, Javed 2025). As a result of mastitis, low production, loss of lactation, premature culling, and antimicrobial cost are among the substantial losses faced by the dairy farmers in developing countries. Among the mastitogens (pathogens causing mastitis), bacteria are the foremost cause of mastitis worldwide. Owing to poor adoption of preventive measures (post-milk teat dipping, maintenance of milking equipment, dry cow therapy, and culling of chronic cases), contagious mastitogens (*Staphylococcus aureus* and *Streptococcus agalactiae*) are quite prevalent in dairy animals (Quadratullah et al. 2022). Of these, *S. aureus* is the main etiologic agent that spreads swiftly from infected to healthy udder during milking, persists in dairy herds, and causes both clinical and sub-clinical mastitis in dairy animals (Rossi et al. 2019, Chen et al. 2020). In Pakistan, losses are undoubtedly underestimated due to the high prevalence of subclinical mastitis in dairy cows and buffaloes (Ghumman et al. 2025).

Staphylococcus aureus is a well-armed pathogen in terms of virulence factors, which include adhesion proteins, exoenzymes, toxins, and capsular polysaccharides (CPs). The CPs are cell wall components that interfere with host defense mechanisms, as capsulated isolates have been more virulent than those of non-capsulated (Gogoi-Tiwari et al. 2015). Above 90% of *S. aureus* strains produce capsular polysaccharides (CPs), and eleven capsular types have been recognized by polyclonal sera (Camussone et al. 2013). However, a subsequent study reported only four CP types (1, 2, 5, and 8) with all other types identified as mutated versions (Grunert et al. 2013, Mohamed et al. 2019). Most of the *S. aureus* of human and animal origin may either belong to CP 5 or CP 8. However, great variation in CP types has been reported geographically. The distribution of capsular serotypes among *S. aureus* isolates from bovine mastitis from different countries shows great variability (Tollersrud et al. 2000, Camussone et al. 2012). Capsular (CP5 & CP8)-CMR conjugate vaccines have been found to be protective in a murine model of staphylococcal infection (Cheng et al. 2017). The knowledge of the capsular types of *S. aureus* population is pivotal for the rational selection of a vaccine candidate for bovine/bubaline mastitis. Nevertheless, the prevalence of existing CP types of *S. aureus* causing mastitis in cows and buffaloes in Pakistan is yet unknown.

Antimicrobial resistance in *S. aureus* has emerged as a result of extensive and irrational use of antimicrobials (Ahmed et al. 2020). Furthermore, antibiotic resistance in *S. aureus* is wreaking havoc on animal and human health. This setback not only restricts therapeutic options but also contributes to the spread of extended-spectrum *S. aureus* genes from contaminated milk to human normal flora (Shoaib et al. 2023). β-lactam antibiotics, such as penicillin and cephalosporins, are commonly used to treat bovine mastitis (Ziesch et al. 2018). Resistance to these β-lactams is based on two distinct mechanisms. One is encoded by the *bla*Z resistance gene that operates by inactivating β-lactamases enzymatically. These β-lactamases can hydrolyze the β-lactam, producing inactive metabolites (Côté-Gravel and Malouin 2019). The methicillin resistance gene (*mec*A/C) encodes the other mechanism, which results in the development of an altered penicillin-binding protein (PBP) to which antimicrobials have a low affinity and so lose efficiency (Wendlandt et al. 2013). Furthermore, the following genes *tet*K/M, *erm*A/B/C, *aac*A-*aph*D, *aad*D, *fus*B, *rpo*B, *van*A, and *ile*S encode resistance to tetracycline, macrolide/lincosamide/streptogramin, gentamicin/tobramycin/kanamycin, kanamycin/meomycin, fusidic acid, rifampicin, vancomycin, and mupirocin, respectively (Jensen and Lyon 2009).

Globally, there are numerous reports from many countries signifying the importance of antibiotic stewardship and revealing the genes for antibiotic resistance in *S. aureus*. To date, there is no comprehensive study available from Pakistan to characterize the genetic nature of antimicrobial resistance among *S. aureus* of bovine mastitis. To close this research gap, the current study was designed with an attempt to provide the landscape of antibiotic resistance in *S. aureus* isolates at the genetic level, originating from mastitic cows and buffaloes from different localities of Pakistan. To the best of our knowledge, this is the first study from Punjab and Sindh to report capsular types and antimicrobial susceptibility and resistance genes representing six different antimicrobial classes in bovine- and bubaline-origin *S. aureus* isolates.

Materials and Methods

In this study, a total of 1800 lactating dairy animals (1100 cows and 700 buffaloes) from 87 dairy herds were initially examined for clinical and subclinical mastitis (Fig. 1). A total of 7200 udder quarters (4,400 in cows and 2,800 in buffaloes) were examined, however, 198 quarters (2.82%) were identified as blind, atrophied, or non-lactating and were therefore excluded

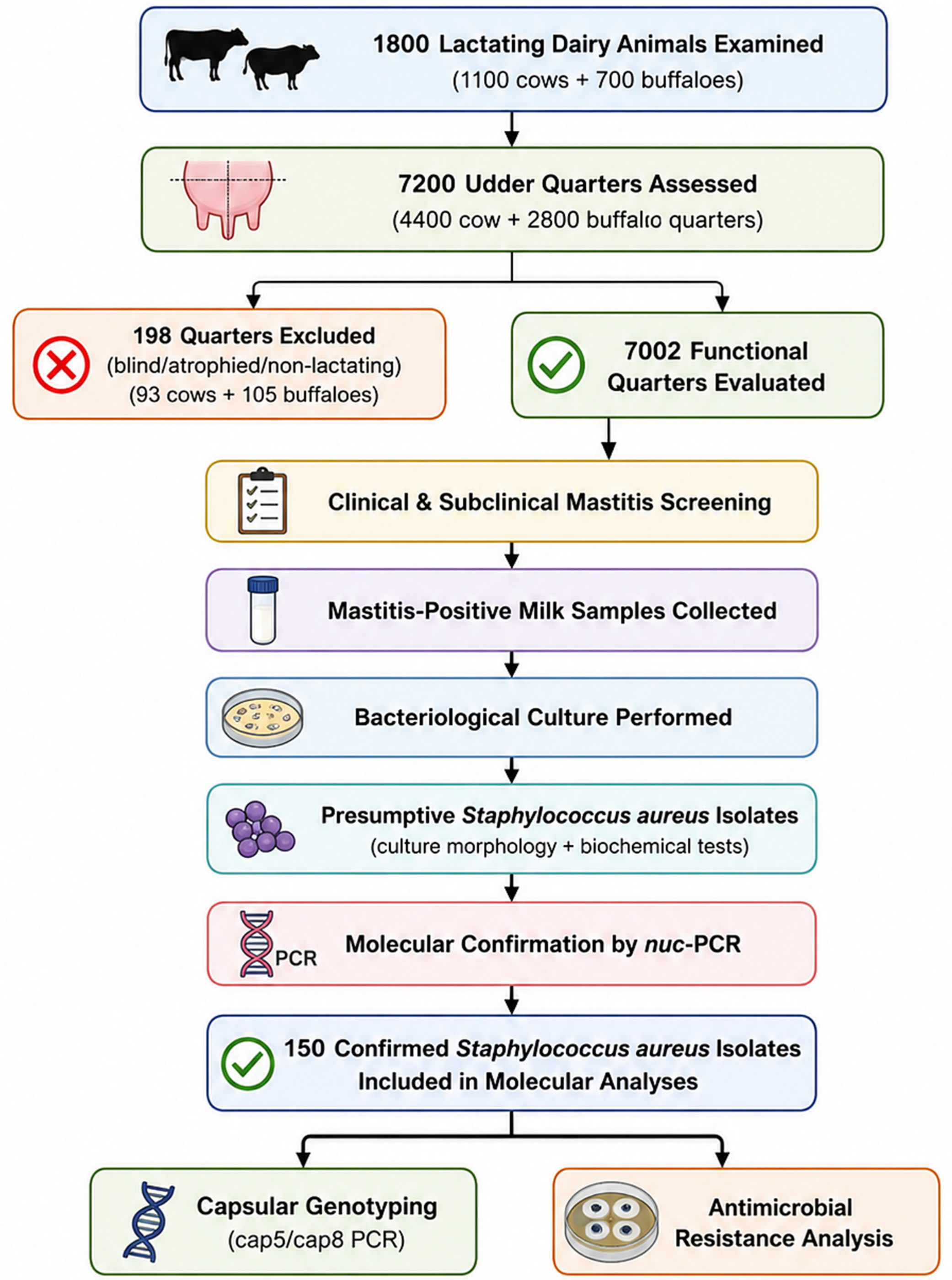

Fig. 1. Sampling flow diagram showing progression from examined dairy cows and buffaloes and udder quarters to mastitis positive samples, bacteriological culture, *nuc*-PCR confirmation of Staphylococcus aureus, and inclusion of isolates in the downstream molecular analysis.

from milk sampling and subsequent analyses. Of these excluded quarters, 93 belonged to cows, and 105 belonged to buffaloes. Consequently, the number of functional quarters available for examination was reduced to 4,307 in cows and 2,695 in buffaloes, giving a total of 7,002 examined quarters. Clinical mastitis cases were identified through physical examination of the udder and milk, whereas subclinical mastitis was screened at the quarter level using Surf Field Mastitis Test. Among these, quarters positive for clinical or subclinical mastitis were subjected to milk sampling and bacteriological culture.

Milk samples yielding bacterial growth suggestive of *S. aureus* were further processed using standard microbiological methods, including colony morphology, Gram staining, catalase, coagulase, and latex agglutination tests. Subsequently, molecular confirmation was performed using the *nuc* gene-based PCR.

From all cultured mastitic milk samples, a total of 150 non-duplicate *S. aureus* isolates were finally confirmed and selected for downstream capsular genotyping and antimicrobial resistance analyses. These included 109 isolates from cows and 41 isolates from buffaloes.

Bacterial isolates

A total of 150 isolates of *S. aureus* recovered from milk samples of clinical and subclinical cases of mastitis in cows (n=109) and buffaloes (n=41) were included in this study. The samples originated from 87 dairy herds located in central-eastern and south-eastern regions of Pakistan (data not shown). The isolates were presumptively identified by colony characteristics on Columbia blood agar, Gram staining, and by the catalase test. Tube coagulase and Prolex Staph XTRA Latex kit (Lot No. C16108, Prolex, Pro-Lab Diagnostics, Wirral, UK) tests were performed for the confirmation (Nordin et al. 2021). The isolates were finally stored at -80˚C in cryo-preservative medium with porous glass beads (Microbank™ Pro-Lab Diagnostics, UK). Based on clinical data, the isolates were designated as clinical and subclinical as defined by the National Mastitis Council, USA.

Antibiotic susceptibility testing

The susceptibility of *S. aureus* to different antimicrobials was determined by the disc diffusion method on Muller-Hinton agar as described elsewhere (CLSI 2020). The panel of antimicrobials used was penicillin (P), amoxycillin (AML), amoxycillin-clavulanic acid (AMC), erythromycin (E), gentamycin (CN), chloramphenicol (C), oxytetracycline (OT), trimethoprim-sulphamethoxazole (SXT), oxacillin (OX), cefoxitin (FOX), ciprofloxacin (CIP), and linzolide (LZD). The ATCC 25923 was used as a quality control organism. The zones of inhibition (mm) were measured according to Clinical Laboratory Standards Institute (CLSI M100). The methicillin resistance was confirmed by oxacillin E-test strips (bioMérieux, France) (Muhammad et al. 2010).

DNA extraction and PCR assays

The frozen stocks of *S. aureus* were re-grown in brain heart infusion broth (BHI, Liofilchem®, Italy) for 24 h at 37°C, and total DNA preparations were made from the broth cultures of S. aureus as per instructions provided with GeneJET Genomic DNA Purification Kit (Thermo Fisher). The concentration and purity of eluted DNA samples were measured by NanoDrop Lite Spectrophotometer (Thermo Fisher). Using Dream*Taq* master mix (Thermo Fisher, USA), uniplex PCR was performed for detection of individual gene targets, including *nuc* and *eap* (for confirmation of *S. aureus*), *cap*1, *cap*2, *cap*5, and *cap*8 loci (for capsular polysaccharide biosynthesis), *mec*A (methicillin resistance), and *blaZ* (beta-lactamase production). Phenotypically non-susceptible isolates (resistant plus intermediate; R+I) were selectively subjected to multiplex PCR for the detection of tetracycline resistance genes (*tet*K, *tet*L, *tet*M, *tet*O) and macrolide/erythromycin resistance genes (*erm*A, *erm*B, *erm*C, *msr*C, and *mef*A). A representative sample of erythromycin non-susceptible isolates was chosen for the macrolide resistance determinants to be sequenced for this unusually high number of intermediately susceptible phenotypes identified by disc diffusion testing. This targeted strategy was adopted to investigate genotype – phenotype concordance while optimizing molecular screening resources. PCR amplifications were carried out in a 50 μL volume containing 50ng of DNA, multiplex buffer, multiplex DNA polymerase, and primer mix according to the manufacturer's (Vazyme Biotech, China) guidelines. The nucleotide sequence of primers and amplicon size are shown in Table 1. The PCR reactions were performed on MyCycler thermocycler (Bio-Rad, USA), and the DNA products were visualized by electrophoresis on 1.2% agarose gel containing fluorescent nucleic acid dye (Ultra GelRed, Vazyme Biotech, China). For confirmation, one amplicon of each target gene was sequenced from a commercial service (Macrogen, Korea). For cap amplifications, the prototype *S. aureus* strains CP5 (Reynolds) and CP8 (Becker) were used as positive controls, cordially provided by the Veterinary Research Center, Oman.

Statistical analyses

The data were entered into Microsoft Excel and analyzed using WinPepi (Abramson, 2011) and Statistix-10 (Data analysis software for researchers). Descriptive statistics were used to summarize the prevalence of capsular genotypes and antimicrobial resistance profiles. Associations between capsular genotypes and mastitis type were evaluated using Pearson's chi-square test or Fisher's exact test. Odds ratios (OR) with 95% confidence intervals (CI) were calculated to estimate the strength of associations. Correlation analysis between phenotypic antimicrobial resistance and corresponding resistance genes was performed using Pearson correlation coefficients. A p-value of <0.05 was considered statistically significant.

Results

Of the 1800 lactating animals (cows n=1100; buffaloes n=700) examined for mastitis, 11% (93 cows and

Table 1. Primer sequences and amplicons for different gene targets used in this study.

	Primer Name	Sequence (5'-3')	Product (bp)
Capsular type	*nuc*-F	TACAGGTGACTGCGGGCTTATC	270
	nuc-R	CTTACCGGGCAATACACTCACTA	
	*cap*1-F	AGGTCTGCTAATTAGTGCAA	550
	*cap*1-R	GAACCCAGTACAGGTATCACC	
	*cap*2-F	AGCAATCTTCGGTTATTGCCGGTG	Variable amplicon size, as previously described in published primer references
	*cap*2-R	ATGACGGTAAGGCATCAAGGTCG	
	*cap*5-F	ATGACGATGAGGATAGCG	881
	*cap*5-R	CTCGGATAACACCTGTTG	
	*cap*8-F	ATGACGATGAGGATAGCG	1148
	*cap*8-R	CACCTAACATAAGGCAAG	
Antibiotic-resistance-associated genes	Primer Name	Sequence (5'-3')	Product (bp)
Methicillin/Oxacillin	*mec*A-F	ACTGCTATCCACCCTCAAAC	163
	*mec*A-R	CTGGTGAAGTTGTAATCTGG	
Penicillin	*blaz*-F	AAGAGATTTGCCTATGCTTC	517
	blaz-R	GCTTGACCACTTTTATCAGC	
Tetracycline	*tet*K-F	TCGATAGGAACAGCAGTA	169
	*tet*K-R	CAGCAGATCCTACTCCTT	
	*tet*L-F	TCGTTAGCGTGCTGTCAT	267
	*tet*L-R	GTATCCCACCAATGTAGCCG	
	*tet*M-F	GTGGACAAAGGTACAACGAG	406
	*tet*M-R	GCGTAAAGTTCGTCACACAC	
	*tet*O-F	AACTTAGGCATTCTGGCTCAC	515
	*tet*O-R	TCCCACTGTTCCATATCGTCA	
Aminoglycosides	*aac-aphD*-F	TAATCCAAGAGCAATAAGGGC	227
	aac-aphD-R	GCCACACTATCATAACCACTA	
Erythromycin	*erm*A-F	TATCTTATCGTTGAGAAGGGATT	138
	*erm*A-R	CTACACTTGGCTTAGGATGAAA	
	*erm*B-F	GAAAAGGTACTCAACCAAATA	639
	*erm*B-R	AGTAACGGTACTTAAATTGTTTAC	
	*erm*C-F	CTTCTTGATCACGATAATTTCC	189
	*erm*C-R	ATCTTTTAGCAAACCCGTATTC	
Macrolides	*msr*C-F	AAGGAATCCTTCTCTCTCCG	342
	*msr*C-R	GTAAACAAAATCGTTCCCG	
	*mef*A-F	AGTATCATTAATCACTAGTGC	500
	*mef*A-R	TTCTTCTGGTACTAAAAGTGG	
Linezolid/Chloramphenicol	*optr*A-F	AGGTGGTCAGCGAACTCA	1400
	*optr*A-R	ATCAACTGTTCCCATTCA	

* The *cap*2 primer set may generate variable amplicon sizes depending on capsular gene polymorphism, as previously reported in the original primer reference.

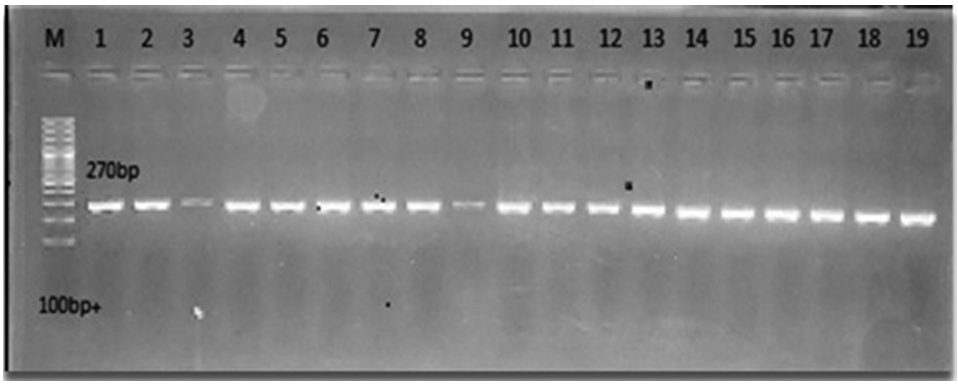

Fig. 2. PCR amplification of species-specific *nuc* gene of *Staphylococcus aureus* with molecular weight of 270bp from milk samples with mastitis. Lane M: 100 bp plus molecular marker (Thermo Scientific, Germany). Lane 1: *Staphylococcus aureus* ATCC 25923 as positive control. Lane 2-19: amplified *nuc* gene product of *S. aureus* field isolates.

Table 2. Number of *nuc* gene-based PCR-positive *Staphylococcus aureus* isolates recovered from milk samples of cows and buffaloes.

Source of samples	Number of S. aureus isolates		Total
	Clinical	Sub-clinical	
Cows	23	86	109
Buffaloes	27	14	41
Total	50	100	150

Among the 109 isolates from cows, 23 were recovered from clinical mastitis and 86 from subclinical cases. Similarly, in buffaloes, of the 41 isolates, 14 originated from sub-clinical, and 27 were from clinical cases of mastitis.

Table 3. Capsular genotype frequencies (absolute and relative) of *S. aureus* isolates from clinical and subclinical mastitis in cows and buffaloes.

Capsular genotype	Absolute frequency	Relative frequency (%)	OR	Fisher's 95% C.I	Pearson chi square(p- value)
cap 5	84/150	56.00	1.27	0.84-1.93	0.230
cap 8	66/150	44.00	-		

105 buffaloes) had clinical mastitis. Approximately 9.4% (405/4307) and 6.67% (180/2695) of quarters from cows and buffaloes, respectively, had subclinical mastitis. Approximately 2.82% (198/7002) quarters were blind or non-lactating teats.

The species-specific gene*, viz., nuc,* validated the identification of all 150 biochemically characterized *S. aureus* isolates of intramammary origin. The PCR assay yielded bands 270 bp (Fig. 2). The number of *nuc* gene-based PCR-positive Staphylococcus aureus isolates recovered from milk samples of cows and buffaloes is mentioned in Table 2.

Capsular polysaccharide genotyping of *S. aureus* isolates

Capsular genotyping of 150 intramammary *S. aureus* isolates with specific primers revealed *cap*5 in 84 isolates (56.0%), including 31 from clinical cases (20.66%) and 53 from sub-clinical cases (35.33%). Similarly, *cap*8 was detected in 66 animals (44.0%), including 19 clinical cases (12.66%) and 47 sub-clinical cases (31.33%). Absolute and relative frequencies of capsular genotyping of *S. aureus* isolates recovered from clinical and subclinically mastitic milk samples of cows and buffaloes are mentioned in Table 3. The genotypes, *cap*1 and *cap*2 loci, could not be detected in any of the isolates. The distribution of capsular genes of *S. aureus* isolates from cows and buffaloes in relation to types of mastitis is shown in Table 4. The length of the PCR amplicons of *cap*5 and *cap*8 genes was 881bp and 1148bp, respectively (Fig. 3A and 3B).

Antimicrobial susceptibility and resistant genes profile

The zone of inhibition of 13 antimicrobial agents for the quality control reference strain (ATCC 25923) was within the acceptable limits of CLSI (CLSI 2020). A majority (138/150; 92.00%) of *S. aureus* isolates

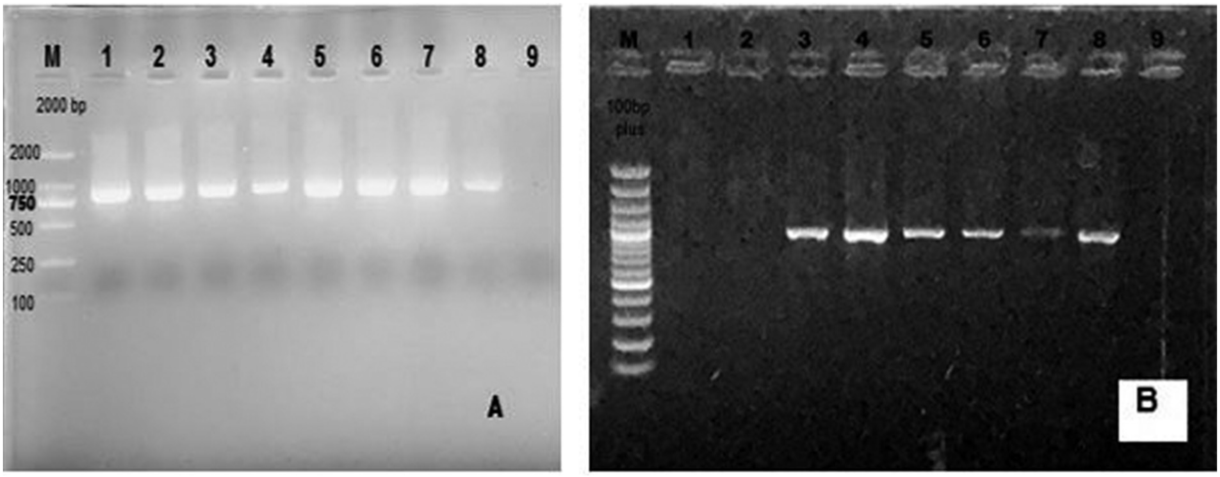

Fig. 3. Agarose gel electrophoresis of capsular polysaccharide genes in *Staphylococcus aureus* isolates. (A) PCR amplification of the cap5 gene showing the expected 881 bp amplicon. Lane M: 2000 bp molecular marker; Lanes 1–8: cap5-positive field isolates; Lane 9: PCR-negative field isolate. (B) PCR amplification of the cap8 gene showing the expected 1148 bp amplicon. Lane M: 100 bp plus molecular marker (Thermo Scientific, Germany); Lanes 2–8: cap8-positive field isolates; Lanes 1 and 9: PCR-negative field isolates. Negative experimental controls (without template DNA) were included separately in each PCR assay but are not shown in the figure.

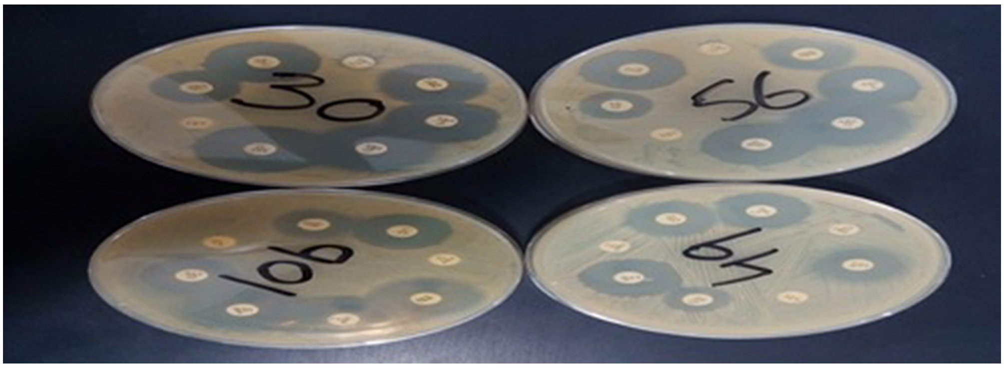

Fig. 4. Antibiotic disc diffusion plates of *Staphylococcus aureus* showing zones of inhibition.

Table 4. Capsular gene distribution of *S. aureus* isolates from cows and buffaloes by mastitis type.

Capsular genes	Cow		Buffalo		Total
	Clinical	Sub-clinical	Clinical	Sub-clinical	
cap5	15	48	16	5	84
cap8	8	38	11	9	66
Total	23	86	27	14	150

exhibited resistance to at least one antimicrobial whilst 8% were entirely susceptible to antibiotic agents (Fig. 4). A high level of resistance (72.66%; 109/150) was noted to penicillin followed by amoxicillin (53.33%; 80/150) and amoxillin-clavulanic acid (37.33%; 56/150) (Table 5). Resistance to anti-staphylococcal beta-lactam markers was 15.33% and 14.00%, respectively, for oxacillin and cefoxitin. For non β-lactam antibiotics, the resistance frequencies were 13.33% for oxytetracycline, 10.66% for ciprofloxacin, 8.66% each for linzolide and gentamicin, 8.0% for trimethoprim-sulphamethoxazole, 6% for erythromycin, 2.66% for chloeamphenicol. The resistance to vancomycin was unusual and was observed in 2 (1.33%) isolates. A significant number of isolates demonstrated intermediate susceptibility to erythromycin (105/150; 70%) and ciprofloxacine (34/150; 22.66%), whereas intermediate resistance responses were infrequent or absent for most of the antimicrobials. A notably high proportion of isolates (70.66%) demonstrated intermediate susceptibility to erythromycin according to CLSI M100 interpretive criteria, whereas only 6% were clas-

Table 5. Antibiogram of *S. aureus* isolates (n=150) recovered from bovine mastitic milk samples.

Sr. No.	Name of antibiotic	Sensitive (S)		Intermediary Sensitive (I)		Resistant (R)	
		N	%	N	%	N	%
1	Amoxicillin	70	46.66	0	0	80	53.33
2	Amoxicillin+clavulanic acid	87	58.0	07	4.66	56	37.33
3	Penicillin G	41	27.33	0	0	109	72.66
4	Ciprofloxacin	100	66.6	34	22.66	16	10.66
5	Gentamicin	133	88.66	04	2.66	13	8.66
6	Vancomycin	148	98.66	0	0	02	1.33
7	Oxacillin	127	84.66	0	0	23	15.33
8	Sulphamethoxazole/ Trimethoprim	132	88.00	06	4.00	12	8.00
9	Oxytetracycline	112	74.66	18	12.00	20	13.33
10	Linzolid	137	91.33	0	0	13	8.66
11	Chloramphenicol	116	77.33	30	20.00	04	2.66
12	Cefoxitin	129	86.00	0	0	21	14.00
13	Erythromycin	35	23.33	106	70.66	09	6.00

sified as fully resistant. Most erythromycin inhibition zone diameters clustered close to CLSI breakpoint values, suggesting reduced susceptibility rather than complete phenotypic resistance. Similarly, although 8.66% of isolates were categorized as resistant to linezolid by disc diffusion testing, the absence of *optr*A indicates that alternative resistance mechanisms or borderline susceptibility patterns may be involved. The resistant phenotypes observed should be interpreted with caution as only disc diffusion was used to detect linezolid resistance based on CLSI interpretive criteria. No confirmatory testing based on MIC was conducted and other transferable oxazolidinone resistance determinants (*cfr* and *poxt*A) were not explored in the present study. Thus, the observed linezolid-resistant phenotypes might be a result of imperfect susceptibility or other types of resistance that need additional molecular and phenotypic identification. Excluding isolates demonstrating intermediate resistance, the resistance pattern analysis indicated that 37 isolates (24.66%) were resistant to one antimicrobial, 30 (20%) to two, 34 (22.66%) to three, 21 (14%) to four, and 13 (8.66%) to five antimicrobials. Extensive resistance was found to be uncommon, and only 2 isolates (1.33%) were resistant to 11 antimicrobials. The most frequent patterns were P alone (14%), P+AML+AMC (11.3%), P+AML (5.3%), and P+AM+AMC+OT (3.3%). Using multidrug resistance (MDR) and extended drug resistance (XDR) criteria, further analysis indicated that 63.3% isolates were MDR [non-susceptible (R+I) to at least one agent in ≥3 antimicrobial classes], and 1.33% (2/150) were classified as XDR, and no isolate could fall in the pan-drug resistance (PDR) category.

Investigation of antimicrobial resistance genes indicated a widespread carriage of penicillinase-assocated resistance as *blaZ* was detected in 95.33% (143/150) of intramammary *S. aureus* isolates (Table 6). The *mecA* gene, which confers the methicillin resistance in *Staphylococcus* species, was detected in 5 isolates (3 from cows and 2 from buffaloes) and were therefore classified as MRSA, while *mec*C was not detected in any of the isolates. Other antimicrobial resistance determinants associated with tetracycline, aminoglycoside, macrolide, and oxazolidinone/chloramphenicol were screened in the phenotypically resistant subset of isolates. The *optrA* gene, which confers resistance to chloramphenicol and linezolide, could not be detected in any of the resistant phenotypes (Table 6). Among gentamicin non-susceptible (R/I) phenotypes, 17.64% (3/17) demonstrated *aac-aph*D gene imparting resistance to aminoglycosides. Tetracycline resistance genes were common among the tetracycline non-susceptible isolates (n=38; R=20, I=18). The PCR revealed *tetM* as the dominant determinant (35/38; 92.10%), followed by *tet*K (32/38; 84.21%) and *tet*O (13/38; 34.21%), whereas *tet*L was uncommon (2/38; 5.26%). Molecular screening for macrolide resistance determinants was carried out purposely on those erythromycin non-susceptible isolates that were selected for testing (n=24; nine erythromycin-resistant and 15 intermediate) and the test results showed that all the isolates harbored macrolide resistance genes, with 95.83% being erythromycin-resistant and 41.66% being macrolide-resistant. This representative subset was selected to test for representative genotype – phenotype concordance and to maximise the use of molecular screening

Table 6. PCR results for the detection of resistance-associated genes of *Staphylococcus aureus*.

Antibiotic Class	Resistance-Associated Genes	Detected/Total tested	Percentage (%)
Penicillin	*bla*Z	143/150	95.33
β-Lactam/MRSA	*mec*A	05/150	3.33
	*mec*C	00/150	0.00
Chloramphinicol	*optrA*	00/34(R=4+I=30)	0.00
Linezolid	*optrA*	00/13 (R=13)	0.00
Aminoglycoside	*aac-aph*D-F	03/17(R=13+I=4)	17.64
Tetracycline	*tet*K	32/38(R=20+I=18)	84.21
	*tet*L	02/38(R=20+I=18)	5.26
	*tet*M	35/38(R=20+I=18)	92.10
	*tet*O	13/38(R=20+I=18)	34.21
Erythromycin/Macrolide	*erm*A	00/24(R=09+I=15)	0.00
	*erm*B	05/24(R=09+I=15)	20.83
	*erm*C	02/24(R=09+I=15)	8.33
	*msr*C	21/24(R=09+I=15)	87.50
	*mef*A	00/24(R=09+I=15)	0.00

resources not to estimate the prevalence of resistance genes in the total collection of erythromycin-intermediate isolates. Erythromycin phenotype-based subset (n=24; R=9, I=15) screened for methylases and efflux pump-encoding genes revealed that *msrC* was the predominant determinant (87.50%) followed by *erm*B (20.83%) and *erm*C (8.33%). However, none of *the ermA and mefA genes could be* detected.

Table 6. PCR detection of antimicrobial resistance-associated genes among phenotypically non-susceptible *Staphylococcus aureus* isolates. Values are presented as the number of gene-positive isolates/the number of isolates tested. The notation R and I represent resistant and intermediate phenotypes, respectively. Only phenotypically non-susceptible isolates (R+I) were subjected to molecular screening for the corresponding resistance determinants.

Correlation analysis between phenotypic antibiotic resistance and corresponding resistance genes revealed distinct co-resistance patterns (Fig. 5). β-lactam antibiotics, including amoxicillin (AML), amoxicillin – clavulanic acid (AMC), and cefoxitin (FOX), showed moderate to strong positive correlations with the *mec*A and *bla*Z genes, indicating *mec*A-mediated methicillin and β-lactam resistance. Tetracycline resistance (OT) exhibited significant positive associations with multiple tetracycline resistance determinants (*tet*K, *tet*M, *tet*L, and *tet*O), which also demonstrated strong inter-gene correlations, suggesting co-selection or horizontal gene transfer. Macrolide resistance, particularly to erythromycin (E), correlated positively with *erm*A, *erm*B, and *erm*C as well as *mef*A, highlighting the predominance of ribosomal target modification and efflux mechanisms. Gentamicin resistance (CN) was moderately associated with the aminoglycoside-modifying enzyme gene *aac*-*aph*D. In contrast, vancomycin (VA) and linezolid (LZD) resistance displayed weak or negligible correlations with the investigated resistance genes, indicating limited dissemination of resistance determinants to these last-line agents. Overall, the analysis underscores the presence of genetically linked multidrug resistance clusters, particularly among β-lactam, tetracycline, and macrolide antibiotics.

Discussion

Bovine mastitis is still among the most economically devastating diseases of dairy animals in the world, and especially in those countries that have a developing dairy industry, such as Pakistan. The current research provides a thorough molecular understanding of *S. aureus*, which causes clinical and subclinical mastitis in cows and buffaloes in Punjab and Sindh, and combines capsular genotyping data with in-depth antimicrobial resistance (AMR) data. As far as we know, this is the first large-scale molecular characterization of these regions that combines all the capsular types with resistance determinants in six antimicrobial classes.

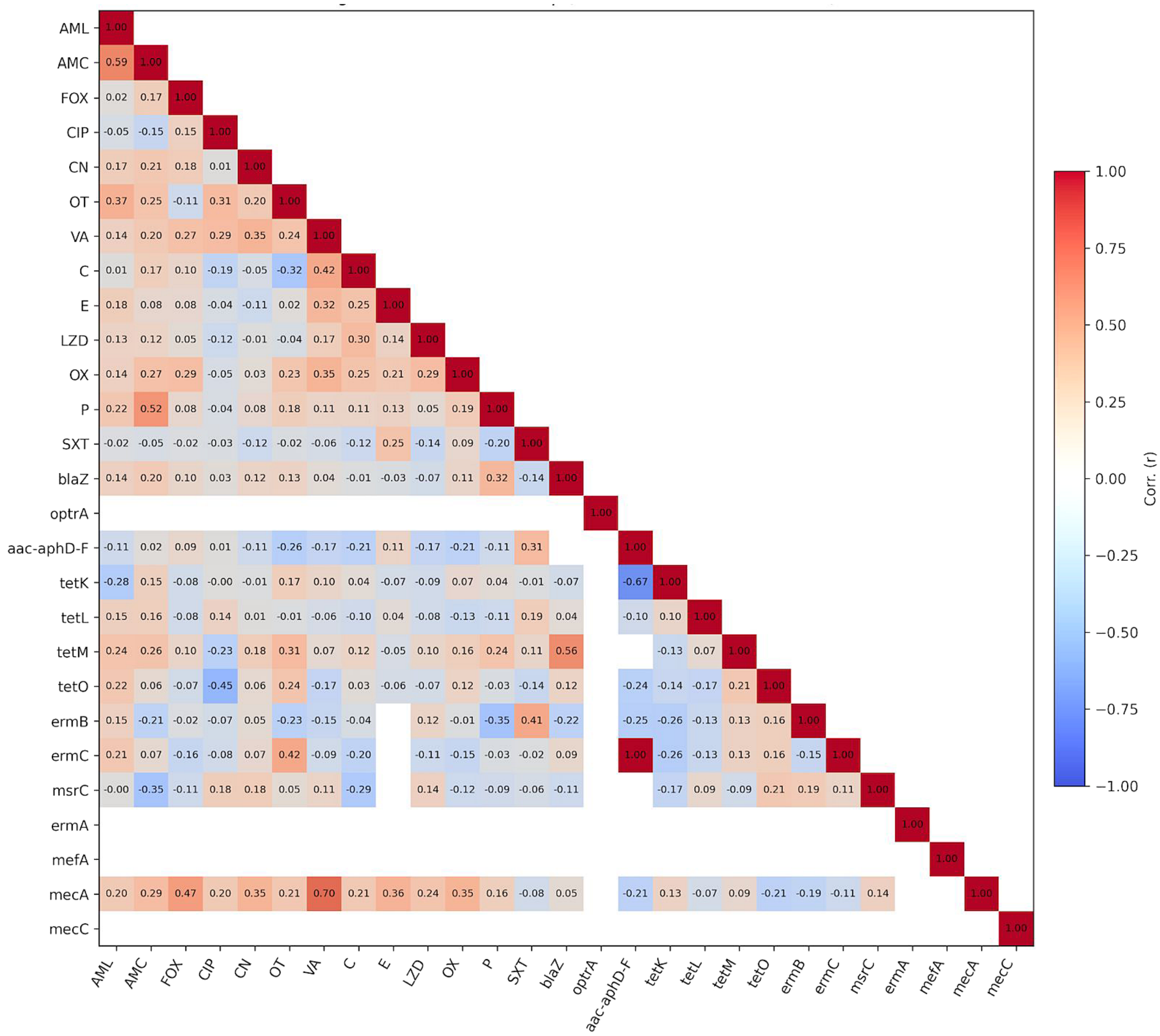

Fig. 5. Correlation analysis between phenotypic antibiotic resistance and corresponding resistance genes.

Prevalence and clinical distribution

The clinical and subclinical mastitis prevalence in the current study correlates with recent regional surveys in South Asia, with subclinical mastitis prevalence exceeding clinical mastitis prevalence (Tommasoni et al. 2023, Javed et al. 2025). The increased prevalence of *S. aureus* in subclinical cases (66.66%) compared with clinical cases (33.33%) is consistent with the well-established capacity of S. aureus to establish long-lasting intramammary infections with low-grade inflammation and intermittent shedding of the bacteria (Zigo et al. 2021). Cases with similar epidemiological patterns have been reported in China (Chen et al. 2020), Italy (Tommasoni et al. 2023), and Pakistan (Ghumman et al. 2025), highlighting the insidious nature of subclinical S. aureus mastitis and its role as a reservoir in the dairy herd.

Capsular genotype distribution (cap5 and cap8)

Capsular polysaccharides (CPs) are key virulence factors that promote immune evasion by inhibiting opsonophagocytosis. Only *cap*5 (56%) and *cap*8 (44%) appeared in the current study, and *cap*1 and *cap*2 were not observed. This distribution supports the general finding that CP5 and CP8 are predominant in bovine and human isolates of *S. aureus* (Côté-Gravel and Malouin 2019, Mohamed et al. 2019). CP5 and CP8 were also identified as the main genotypes among mastitis-related isolates in recent reports in Brazil (Camussone et al. 2013) and Egypt (Ahmed et al. 2020) as shown by results from European dairy herds. The *cap*5 is the primary causative pathogen in both clinical and subclinical cases in our data, as shown by results from European dairy herds, where CP5 tends to have a more persistent character (Salimena et al.

2016, Tommasoni et al. 2023). Nevertheless, geographical diversity is also present; some Asian and Middle Eastern reports have observed the prevalence of cap8 (Mohamed et al. 2019), and this should be approached with a focus on regional surveillance before vaccine development.

A lack of *cap*1 and *cap*2 is not unique. It is consistent with molecular epidemiological data indicating that such types are either not common in modern bovine populations or are mutated variants (Grunert et al. 2013, Mohamed et al. 2019). In terms of vaccinology, the findings in this study showed that CP5/CP8-based conjugate vaccines can be employed in Pakistan, as conjugates of CP5 and CP8 be protective in experimental murine models (Cheng et al. 2017). The design of vaccines needs to be rationalized based on regional *cap* distribution; therefore, the research results address a major epidemiological gap in South Asia.

Antimicrobial susceptibility trends

Ninety-two percent of the isolates had at least one antimicrobial resistance phenotype, and 63.3% met MDR criteria, indicating a high degree of therapeutic pressure in the dairy systems. Similar MDR rates have recently been observed in studies in China (Chen et al. 2020), Egypt (Ahmed et al. 2020), and Italy (Tommasoni et al. 2023), with rates ranging from 45-70%. The prevalent MDR phenotype likely over-the-counter supply reflects uncontrolled antimicrobial use, and the lack of antimicrobial stewardship in emerging dairy industries (Shoaib et al. 2023).

β-lactam resistance

Resistance against penicillin was the highest (72.66%), which is comparable to the rest of the world, where the most commonly used antimicrobials to treat mastitis are the β-lactams (Ziesch et al. 2018, Côté-Gravel and Malouin 2019). The extremely high prevalence of *bla*Z (95.33%) confirms that penicillinase production remains the major resistance mechanism. Bovine isolates detected in Europe and Asia have been found to have similar high *bla*Z carriage (>80%) (Chen et al. 2020, Tommasoni et al. 2023).

A resistance of the 3.33% of isolates was identified as Methicillin resistance (*mec*A-positive MRSA). Even though it is low, this is epidemiologically important because it has zoonotic potential. Similar rates of MRSA between 2-10% have been reported in bovine mastitis in China and North Africa (Ahmed et al. 2020, Chen et al. 2020). The absence of *mec*C aligns with its generally sporadic occurrence in livestock (Cuny et al. 2015).

Tetracycline resistance

Resistance genes were examined in phenotype-selected subsets (non-susceptible; R/I), one that can also influence the interpretation of gene–phenotype associations (Liu et al. 2025). Tetracycline non-susceptibility was primarily associated with *tet*M and *tet*K, consistent with the global predominance of ribosomal protection and efflux mechanisms in *S. aureus* (Mlynarczyk-Bonikowska et al. 2022). The distribution of tetracycline resistance genes, with the highest occurrence among non-susceptible isolates being extensive, with the highest frequencies for *tet*M (92.10%) and *tet*K (84.21%). The dominance of *tet*M has also been similar in bovine isolates in Europe and Asia (Chen et al. 2020, Tommasoni et al. 2023). The *tet*K and *tet*M are co-carried, indicating that horizontal gene transfer is likely mediated by mobile genetic elements. This phenomenon is also becoming common in livestock-associated *S. aureus* (Wendlandt et al. 2013, Côté-Gravel and Malouin 2019). Although this phenotype-guided molecular screening strategy improves the efficiency of detecting clinically relevant resistance determinants, it may also introduce selection bias and overestimate the apparent prevalence of resistance genes compared with whole-population screening. Therefore, the reported frequencies of resistance-associated genes should be interpreted within the context of the selected non-susceptible subsets rather than the entire collection of isolates.

Aminoglycoside and macrolide resistance

Phenotypically, the level of resistance to erythromycin was relatively low (6.00%), but the intermediate susceptibility was high. The high percentage of Erythromycin intermediate (EI) isolates in the present study is probably attributable to the lower susceptibility breakpoints that tended to cluster around the CLSI breakpoints for macrolide resistance, but to stable high-level macrolide resistance. The percentage of macrolide non-susceptible strains with *msr*C, *erm*B and *erm*C detected should be viewed as genotype – phenotype relationships because only a selected subset of erythromycin non-susceptible strains were analysed, and not as true prevalence values. A targeted approach was used to optimise molecular screening resources whilst retaining the ability to characterise the major resistance mechanisms involved with erythromycin non-susceptibility. Molecular analysis revealed that the most common was *msr*C (87.50%), followed by *erm*B and *erm*C. In livestock-associated isolates, efflux-mediated resistance (*msr* genes) has become more common (Chen et al. 2020). The unusually high proportion of erythromycin-intermediate isolates observed in the present study may reflect isolates with borderline susceptibility around CLSI interpretive breakpoints, possibly indicating emerging or low-level macrolide resistance under antimicrobial selection pressure. Such intermediate phenotypes have been described in staphylococci exposed to prolonged macrolide usage and should be interpreted cautiously. The presence of *aac-aph*D in aminoglycoside non-susceptible isolates is in line with its identified contribution to gentamicin resistance in staphylococci worldwide (Côté-Gravel and Malouin 2019).

Last-line agents

Vancomycin (1.33%) and linezolid (8.66%) resistance were infrequent, and *optr*A was not found. This is encouraging and follows previous surveillance data suggesting minimal spread of resistance determinants to last-resort antimicrobials in food animals (Tommasoni et al. 2023). Nevertheless, occasional vancomycin resistance should be further observed, especially because it is of paramount significance in human medicine.

Vancomycin resistance (1.33%) remained uncommon, whereas linezolid resistance (8.66%) was comparatively higher than generally reported for bovine *S. aureus* isolates. However, the *optr*A gene could not be detected among the linezolid-resistant isolates, suggesting that alternative mechanisms such as mutations in 23S rRNA or ribosomal proteins, or other transferable determinants including *cfr* or *poxt*A, may be involved. In addition, disc diffusion testing for linezolid may occasionally yield borderline susceptibility interpretations near CLSI breakpoints. Therefore, further MIC-based confirmation and expanded molecular characterization are warranted before definitive conclusions regarding the epidemiology of linezolid resistance can be drawn. The meaning of "linezolid resistance" in the current study, however, should be viewed with caution. The categorisation of resistance was made only by disc diffusion testing and not from the MIC determination methods (broth microdilution or E-test assays) that are generally used to assess oxazolidinone susceptibility. In addition, other clinically significant transferable resistance determinants such as *cfr* and *poxt*A were not assessed. It is likely, therefore, that the observed linezolid-resistant phenotypes are due to alternate molecular mechanisms, mutations in the ribosomal target sites or borderline interpretation of inhibition zones close to the CLSI breakpoints. Further investigation with molecular screening and MIC confirmation is thus recommended to precisely determine the epidemiology of linezolid resistance in bovine and bubaline staphylococcal isolates.

Correlation between phenotype and genotype

With the molecular mechanisms underlying the observed phenotypes are supported by the positive relationships between β-lactam resistance and *mec*A/*bla*Z, tetracycline resistance and *tet* genes, and erythromycin resistance and *erm*/*msr* genes. Recent molecular epidemiological studies have shown similar genotype-to-phenotype concordance (Ahmed et al. 2020, Chen et al. 2020). The grouping of resistance determinants implies selection together due to antimicrobial pressure and potential coexistence in plasmids or transposons.

One health implications and public health

Discovery of MDR *S. aureus* with zoonotic potential such as MRSA highlights the One Health aspect of mastitis-related pathogenic agents. Contaminated milk can be a source of transmission of resistant strains or resistance genes to humans (Shoaib et al. 2023). In line with global calls for antimicrobial stewardship (WHO 2021), rational antibiotic use, improved herd management, and the implementation of vaccine strategies are urgently required in Pakistan's dairy sector.

Strengths and limitations of the study

One of the strengths of this study is that it has a relatively large sample size, includes both bovine and bubaline isolates, and includes a number of herds in two provinces. Capsular genotyping, combined with extensive AMR gene profiling, provides a complete picture in molecular epidemiology. Nevertheless, it has shortcomings: it cannot study clonal relationships using whole-genome sequencing, spa typing, or MLST, and it lacks virulence gene profiling outside the capsular loci. Further, the present study did not include confirmatory MIC testing and expanded screening of oxazolidinone resistance determinants including, but not limited to, *cfr* and *poxt*A which should be completed in future studies.

Conclusion

This work has shown that CP5 and CP8 are the only capsular types of *S. aureus* among the bovine and bubaline in Punjab and Sindh. A high burden of MDR, particularly β-lactam resistance mediated by *bla*Z, was observed, whereas MRSA prevalence remains comparatively low. The results provide a clear indication of the need to address the problem of antimicrobial stewardship urgently, design regional vaccines according to the prevalence of CP5/CP8, and conduct further molecular surveillance to reduce economic losses and zoonotic threats.

Author Declarations

Ethics approval

Ethical approval was not required, as the samples were collected during routine veterinary diagnostic procedures and no experimental interventions were performed on animals specifically for research purposes.

Use of generative artificial intelligence

This manuscript was prepared without the use of AI tools.

Conflict of interest

The authors declare no conflicts of interest.

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