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Characterization of *Staphylococcus aureus* from Bovine Mastitis: Genetic Markers and Antibiotic Resistance

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Abstract

Staphylococcus aureus is a significant contributor to subclinical bovine mastitis, causing economic losses in the dairy sector. This study aimed to isolate and characterize *S. aureus* from 51 subclinical bovine milk samples. Bacterial colonies were cultured on Mannitol Salt Agar (MSA) using ten-fold serial dilutions, and isolates were identified based on catalase and coagulase tests. Antibiotic resistance screening was conducted on MSA supplemented with 16 µg/mL penicillin G. Molecular characterization employed PCR to detect the *nuc*, *coa*, *clfa*, *mecA*, *blaZ*, and *se* genes. Antibiotic susceptibility testing followed the disc diffusion method, and penicillin G minimum inhibitory concentrations (MICs) were determined using broth microdilution. Sequence and phylogenetic analyses of the *blaZ* gene were performed to investigate genetic variations and their association with resistance. Results showed that 14 isolates harbored resistance genes from milk samples with high somatic cell counts, though the *se* gene was absent. All isolates demonstrated 100 % susceptibility to gentamicin, streptomycin, rifampin, ampicillin, and chloramphenicol, while exhibiting complete resistance to penicillin, cefotetan, and nalidixic acid. The minimum inhibitory concentration (MIC) of penicillin G varied widely, ranging from 16 to 512 µg/mL. Phylogenetic analysis identified three distinct groups; however, no significant correlation was found between *blaZ* variations and resistance levels. These findings emphasizing the importance of robust surveillance and responsible antibiotic use to control bovine mastitis in Thailand.

Keywords: Bovine milk, Subclinical mastitis, Antibiotic resistance, Molecular characterization, Beta-lactamase

Introduction

Bovine mastitis causes significant economic losses in the dairy industry [1-3]. Antimicrobial drugs are the primary tool for controlling mastitis in dairy herds, with β-lactam antibiotics, especially penicillin which inhibits bacterial cell wall synthesis, commonly used. However, high usage frequency and overdose of antimicrobial agents can promote the development of resistance in mastitis-causing pathogens, posing risks to both animal and human health [4-6].

Staphylococcus aureus is a leading cause of bovine mastitis [7], with infected udders serving as the primary reservoir [8]. Its array of virulence factors enables it to evade the host immune system, by interfering with key defense mechanisms such as phagocytosis, complement activation, and neutrophil-mediated responses [9,10]. These immune evasion strategies contribute to chronic infections, local inflammation, and increased resistance to treatment, underscoring the pathogen's impact on dairy health and productivity.

Several studies have reported the isolation of penicillin-resistant *S. aureus* from cow milk with clinical and subclinical mastitis [11,12]. Penicillin resistance in *S. aureus* primarily arises from 2 mechanisms: The production of beta-lactamase (encoded by the *blaZ* gene) and alterations in penicillin-binding proteins (PBPs) [13,14]. The *blaZ* gene provides resistance to penicillin and aminopenicillins by encoding beta-lactamase, which hydrolyzes these antibiotics [15]. In contrast, methicillin-resistant *S. aureus* (MRSA) is defined by the presence of the *mecA* gene, which encodes PBP2a, a low-affinity PBP that confers resistance to nearly all beta-lactams. Most MRSA strains also harbor the *blaZ* gene, further enhancing resistance to penicillin [16]. Interestingly, some isolates carrying the *blaZ* gene may display phenotypic sensitivity to beta-lactams, suggesting variable expression of the resistance mechanism [17].

Understanding the genetic and phenotypic characteristics of *S. aureus*, particularly its resistance profiles, is critical for effective treatment and prevention strategies. This is especially relevant in regions like Southern Thailand, where data on β-lactam-resistant *S. aureus* in bovine mastitis is limited. Antibiotic resistance not only reduces the efficacy of treatment but also poses a risk of zoonotic transmission, endangering public health and the global fight against antimicrobial resistance.

Therefore, this study focuses on isolating β-lactam-resistant *S. aureus* from bovine mastitis milk samples collected from local dairy farms in Southern Thailand. The research investigates antimicrobial susceptibility patterns, detects the presence of the *blaZ* gene, and explores the potential relationship between *blaZ* nucleotide sequence diversity and penicillin G MIC. The findings aim to provide crucial insights for the development of targeted therapeutic strategies and informed antibiotic use in the dairy industry, ultimately contributing to the global effort to mitigate antimicrobial resistance.

Materials and methods

Sample collection and isolation

A total of 51 bovine milk samples were collected from 10 local dairy herds in Phatthalung Province, southern Thailand. The samples were obtained from dairy farms where milk is collected daily by the farmers. Samples with high somatic cell counts, scored as CMT+3 by the California Mastitis Test (CMT), were aseptically collected in 100 mL sterile bottles and immediately stored on ice. A 50 mL aliquot of each sample was used to accurately determine the somatic cell count using an automatic cell counter, the Fossomatic 5000. A 100 µL aliquot of each sample was spread onto Mannitol salt agar (MSA) plates and incubated overnight at 37 °C for 24 h. Round and yellow colonies were selected and re-streaked onto the same medium to obtain single pure cultures for subsequent biochemical identification.

Biochemical identification

The suspected colonies were identified using standard microbiological methods including Gram staining, coagulase, and catalase tests. The coagulase test used a 2.0 McFarland suspension of each isolate mixed with coagulase rabbit plasma and incubated at 37 °C for 6 - 8 h. The catalase test involved mixing bacteria with 0.85 % NaCl and 3 % H_2O_2, observing oxygen bubbles within 5 - 10 s.

Molecular identification

Bacterial gDNA extraction

S. aureus cells were harvested after overnight growth in Nutrient Broth (NB) medium. The cells were pelleted by centrifuging 2 mL of the culture at 5,000 ×g for 5 min. Genomic DNA (gDNA) was then extracted using the TIAamp™ bacterial gDNA extraction kit (China) according to the manufacturer’s instructions. The gDNA was eluted in TE buffer and stored at −20 °C for further analysis.

PCR amplification

Molecular characterization of the isolates was determined by the PCR targeting marker genes (*coa*, *nuc*, *se*, and *claf*) and the antibiotic resistance genes (*blaZ* and *mecA*) using the primers listed in **Table 1**. The PCR reaction was performed in a total volume of 50 µL containing 5X Phusion HF buffer (7.5 mM $MgCl_2$), 0.5 µM of each primer, 200 µM of each dNTP, 1U of Phusion High-Fidelity DNA polymerase (Thermo Scientific, USA), and 10 ng of DNA template. The PCR was carried out in a T100 thermal cycler (Bio-Rad, USA) with initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 60 s, annealing (temperature dependent on Tm of each primer set, **Table 1**) for 45 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. The amplicons were analyzed by 1.5 % agarose gel electrophoresis.

Table 1 Primers and their sequences.

Genes	Sequences	Annealing temp (°C)	Amplicon size (bp)	References
Nuc	Nuc1: CGA TTG ATG GTG ATA CGG TT Nuc2: ACG CAA GCC TTG ACG AAC TAAAGC	52	270	[43]
Coa	Coa1: ATA GAG ATG CTG GTA CAG G Coa2: GCT TCC GAT TGT TCG ATG C	57	640	[44]
Clfa	Clfa1: GGC TTC AGT GCT TGT AGG Clfa2: TTT TCA GGG TCA ATA TAA GC	54	975	[45]
Se	Se1: TTG GAA ACG GTT AAA ACG AA Se2: GAA CCT TCC CAT CAA AAA CA	50	130	[46]
BlaZ	BlaZ1: CAA AGA TGA TAT AGT TGC TTA TTC TCC BlaZ2: TGC TTG ACC ACT TTT ATC AGC	50	480	[47]
MecA	MecA1: AAA ATC GAT GGT AAA GGT TGG MecA2: GCT TCC GAT TGT TCG ATG C	55	533	[48]

Sequence analysis of the *BlaZ* gene in the *S. aureus* strain isolated from bovine mastitis

PCR products of the *blaZ* gene amplification were purified using a PCR product purification kit (Invitrogen, USA) following the manufacturer's instructions, and then sequenced using Sanger's method (1^{st} Base Molecular Biology Service, Malaysia). The 2-directional *blaZ* sequences from each isolate were aligned using BioEdit v 7.2.5 [19] and their closest relative DNA sequences were identified from the NCBI database using the BLAST algorithm. All DNA sequences were translated into amino acid sequences using the Translate tool in the Expasy Proteomic Server. Sequence similarity was aligned with the Clustal Omega program (EMBL-EBI), and a phylogenetic tree was constructed using the MEGA 11 program (Molecular Evolutionary Genetics Analysis v 11) [20].

Antimicrobial susceptibility testing

A total of 293 isolates of *S. aureus* were identified and screened for antibiotic resistance. Pure cultures of *S. aureus* were prepared and spotted on MSA supplemented with 16 µg/mL penicillin G, then incubated at 30 °C for 24 h. Among these, 14 penicillin-resistant *S. aureus* isolates were further tested for antibiotic susceptibility using the disc diffusion method. The following antimicrobial discs (Thermo Scientific, USA) were used: gentamycin (10 µg), kanamycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), rifampin (5 µg), nalidixic acid (30 µg), ampicillin (10 µg), erythromycin (15 µg), amoxicillin (30 µg), cefotetan (30 µg), and methicillin (10 µg). The bacterial density of each isolate was adjusted to a 0.5 McFarland standard using sterile 0.85% NaCl. Cultures were then grown on Mueller-Hinton Agar (MHA) medium at 30 °C. Susceptibility was determined based on the diameter of the clear zone around each disc, following the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. *S. aureus* TISTR 1466 was included as a control for quality assurance.

Minimum inhibitory concentration (MIC) of penicillin G

The MIC of penicillin G was determined by the broth microdilution method (CLSI, 2012). Penicillin G was 2-fold diluted in MSA medium to concentrations ranging from 2 to 1,024 µg/mL. *S. aureus* isolates were prepared to a 0.5 McFarland standard with sterile 0.85 % NaCl. The final reaction volume of 200 µL included 100 µL of bacterial suspension and 100 µL of antibiotic solution. The microplate was incubated at 30 °C for 24 h, with the MIC defined as the lowest concentration producing no visible growth.

Results and discussion

Isolation and characterization of *S. aureus*

Fifty-One subclinical bovine milk samples, with somatic cell counts averaging 2×10^6 to 9×10^6 cells/mL, were analyzed for the presence of *S. aureus* bacteria using the 10-fold serial dilution method. From these samples, 293 *S. aureus* isolates (6.87 %) were identified based on their round, yellow colonies and positive results for coagulase and catalase tests. Among these, 14 isolates exhibited resistance to penicillin G (**Table 2**). The observed prevalence of *S. aureus* (6.87 %) was lower than reported in previous studies conducted in Thailand, including 7.3 % in Khon Kaen Province [21], 8 % in Chiang Mai Province [22], and 10.4 % in central and northeastern provinces [23].

Table 2 Frequency of *S. aureus* isolated from raw milk at different somatic cell count levels.

SCC×1,000 (cell/mL)	**No. milk sample (%)**	**No. *S. aureus* (%)**	**No. penicillin resistant *S. aureus* (%)**	**Isolates**
2,000 - 3,000	16 (31.38)	109 (37.20)	5 (4.59)	SA101, SA102, SA105, SA107, SA108
3,001 - 4,000	12 (23.53)	17 (5.80)	0 (0)	-
4,001 - 5,000	9 (17.65)	36 (12.29)	2 (5.56)	SA304, SA305
5,001 - 6,000	9 (17.65)	46 (15.70)	1 (2.17)	SA303
6,001 - 7,000	3 (5.88)	52 (17.74)	2 (3.85)	SB101, SB105
7,001 - 8,000	2 (3.92)	33 (11.26)	4 (12.12)	SB601, SB602, SB603, SB604
Total	51	293	14 (4.78)	

Molecular charecterization and *BlaZ* gene detection of *S. aureus*

The molecular characterization of the selected *S. aureus* isolates was performed by detecting genetic markers, including the *nuc*, *coa*, *clfA*, *se*, *mecA*, and *blaZ* genes. All isolates harbored the 270 bp *nuc* gene and the 640 bp *coa* gene, while the 975 bp *clfA* gene was detected only in isolate SA303. None of the isolates carried the *se* gene (**Table 3**). The β-lactamase gene, *blaZ*, producing a 480 bp amplicon, was detected in all isolates. **Figure 1(A)** highlights SA303, a representative *S. aureus* strain, which was positive for *nuc*, *coa*, *clfa*, *mecA*, and *blaZ* genes but negative for the *se* gene. The *mecA* gene was also detected in isolates from samples with high somatic cell counts including SA303, SB105, SB603, and SB604 (**Figure 1(B)**). The *blaZ* gene was identified in all isolates (**Figure 1(C)**). As a reference, the *S. aureus* TISTR 1466 contained the *nuc*, *coa*, and *clfa* genes but was negative for the *se* and *blaZ* genes.

All the isolates were confirmed as coagulase-positive *S. aureus*, characterized by the consistent presence of the *nuc* and *coa* genes, which are species-specific markers. The *nuc* gene encodes extracellular thermostable nuclease protein, a hallmark of *S. aureus* [24-26]. The *coa* gene, encoding coagulase - a major virulence factor - facilitates the conversion of fibrinogen to fibrin, providing a protective shield against phagocytosis [24]. Among the isolates, SA303 uniquely carried the *clfa* gene, which encodes clumping factor A. This factor plays a critical role in colonization, invasion, and multiplication within host tissues [27-29]. The interaction between clumping factor A and the platelet GPIIb/IIIa receptor enables invasion and persistence, potentially leading to relapsing infections [27,30]. Furthermore, isolates SA303, SB105, SB603, and SB604 carried the *mecA* gene, classifying them as MRSA strains, consistent with their observed resistance to methicillin. The ubiquitous presence of the *blaZ* gene, which encodes β-lactamase, underscored the widespread potential for penicillin resistance in these isolates. These findings provide valuable insights into the genetic markers and resistance mechanisms of *S. aureus*, emphasizing the importance of monitoring and controlling these pathogens.

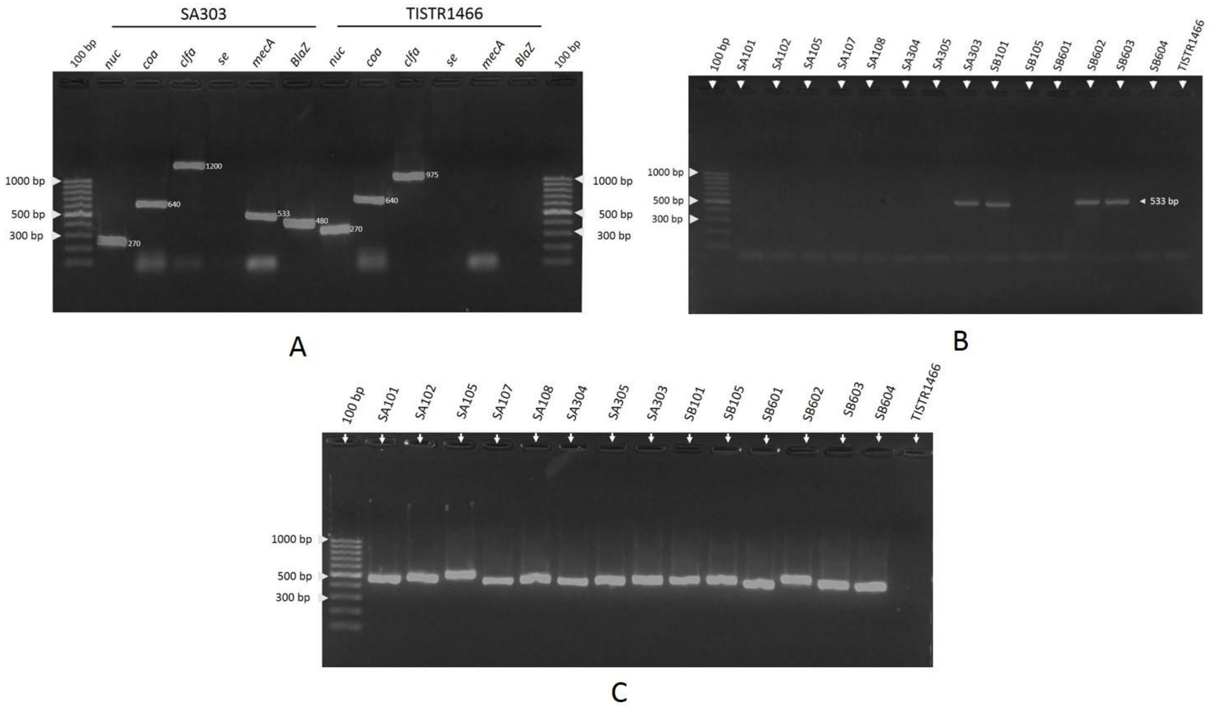

Figure 1 Molecular detection of isolated *S. aureus*. (A) *nuc*, *coa*, *clfa*, *se*, *mec*A and *bla*Z genes, (B) *mec*A and (C) *blaZ* gene. *S. aureus* TISTR 1466 as the reference strain was obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand.

Table 3 Phenotypic and genotypic traits, antibiotic resistance patterns, and penicillin G MIC in isolated *S. aureus*.

Isolates	**Phenotypes**		**Genotypes**						**Antibiograms**	**MIC of Penicillin G (μg/mL)**
	Catalase	**Coagulase**	***Nuc***	***Coa***	***Clfa***	***Se***	***MecA***	***BlaZ***		
SA101	+	+	+	+	-	-	-	+	NA, CTT, K, P	128
SA102	+	+	+	+	-	-	-	+	NA, CTT, K, P	128
SA105	+	+	+	+	-	-	-	+	NA, E, CTT, K, P	128
SA107	+	+	+	+	-	-	-	+	NA, CTT, K, P	128
SA108	+	+	+	+	-	-	-	+	NA, E, CTT, K, P	128
SA303	+	+	+	+	+	-	+	+	NA, CTT, K, P, AMC, MET	256
SA304	+	+	+	+	-	-	-	+	NA, CTT, K, P	64
SA305	+	+	+	+	-	-	-	+	NA, CTT, K, P	64
SB101	+	+	+	+	-	-	+	+	NA, E, CTT, P, TE, AMC, MET	128
SB105	+	+	+	+	-	-	-	+	NA, CTT, K, P	128
SB601	+	+	+	+	-	-	-	+	NA, CTT, K, P	16
SB602	+	+	+	+	-	-	+	+	NA, CTT, K, P, AMC, MET	128
SB603	+	+	+	+	-	-	+	+	NA, E, CTT, K, P, AMC, MET	512
SB604	+	+	+	+	-	-	-	+	NA, E, CTT, K, P	256
TISTR1466*	+	+	+	+	+	-	-	-	-	< 0.25

Antibiotic susceptibility test and MIC of penicillin G

The antibiotic susceptibility and resistance profiles of the *S. aureus* isolates were assessed using the disc diffusion method. All 14 isolates were 100 % susceptible to gentamycin, streptomycin, rifampin, ampicillin, and chloramphenicol. They exhibited varying degrees of susceptibility to other antibiotics including 94.12 % susceptibility to tetracycline, 72.5 % to methicillin, 70.59 % to erythromycin, 28.57 % to amoxicillin, and 17.65 % to kanamycin. Conversely, all isolates were resistant to penicillin, cefotetan, and nalidixic acid (**Table 3**). Five distinct resistance patterns were identified, with the most common NA-CTT-K-P, observed in 50 % (7/14) of the isolates. The other patterns included NA-E-CTT-K-P (21.4 %), NA-CTT-K-P-AMC-MET (14.3 %), NA-E-CTT-P-TE-AMC-MET (7.15 %), and NA-E-CTT-K-P-AMC-MET (7.15 %). These findings indicated that while all isolates were highly susceptible to several antibiotics, they demonstrated complete resistance to penicillin, cefotetan, and nalidixic acid, underscoring the challenges in treating *S. aureus* infections with these agents. The presence of multiple resistance patterns, particularly the NA-CTT-K-P pattern, highlighted the complexity of the resistance mechanisms and the potential for multidrug resistance. Multidrug resistance in *S. aureus* may result from resistance gene acquisition via horizontal gene transfer, antibiotic overuse in agriculture, and the bacterium’s genetic adaptability [31,32]. Genes such as *blaZ* and *mecA* enable beta-lactamase production and target site modifications, emphasizing the need for stringent antibiotic stewardship and monitoring to curb its spread.

The minimum inhibitory concentration (MIC) of penicillin G was determined using the microdilution method, with values ranging from 16 to 512 µg/mL. The distribution of MIC values was 16 µg/mL (7.14 %), 64 µg/mL (21.43 %), 128 µg/mL (42.86 %), 256 µg/mL (21.43 %), and 512 µg/mL (7.14 %). The high MIC values for penicillin G suggested a substantial resistance level, consistent with reports from other studies in Thailand showing a high prevalence of penicillin-resistant *S. aureus* in dairy cows [23], pigs [33], and sport animals [34]. This resistance is likely linked to the widespread use of penicillin in veterinary medicine both in Thailand and globally [35]. The pervasive presence of the *blaZ* gene in all isolates supported this observation because the gene encodes β-lactamase, an enzyme that inactivates penicillin and contributes significantly to resistance [36-38].

BlaZ gene analysis sequence

A 480 bp *blaZ* amplicon was detected in all 14 *S. aureus* isolates using the specific primers BlaZ1 and BlaZ2 (**Figure 1(C)**). The amplicons were sequenced using bidirectional primers, and the resulting sequences were aligned to generate consensus sequences. These sequences were submitted to GenBank with accession numbers KY681022 and KY684200 to KY684212 for isolates SA101, SA102, SA105, SA107, SA108, SA304, SA305, SB101, SB105, SB601, SB602, SB603, SB604, and SA303, respectively. Multiple sequence alignment of the *blaZ* gene was performed using the Clustal Omega program, revealing a high similarity range of 92.1 to 100 % (data not shown). A phylogenetic tree of the nucleotide sequences was constructed using the neighbor-joining method with 1,000 bootstrap replicates and compared with beta-lactamase protein sequences retrieved from GenBank (**Figure 2**). The deduced amino acid sequences, computationally translated using the Expasy Translate tool, consisted of 127 residues with over 98 % identity. Amino acid sequence alignment revealed 14 mutations and 10 unique signature types (**Table 4**).

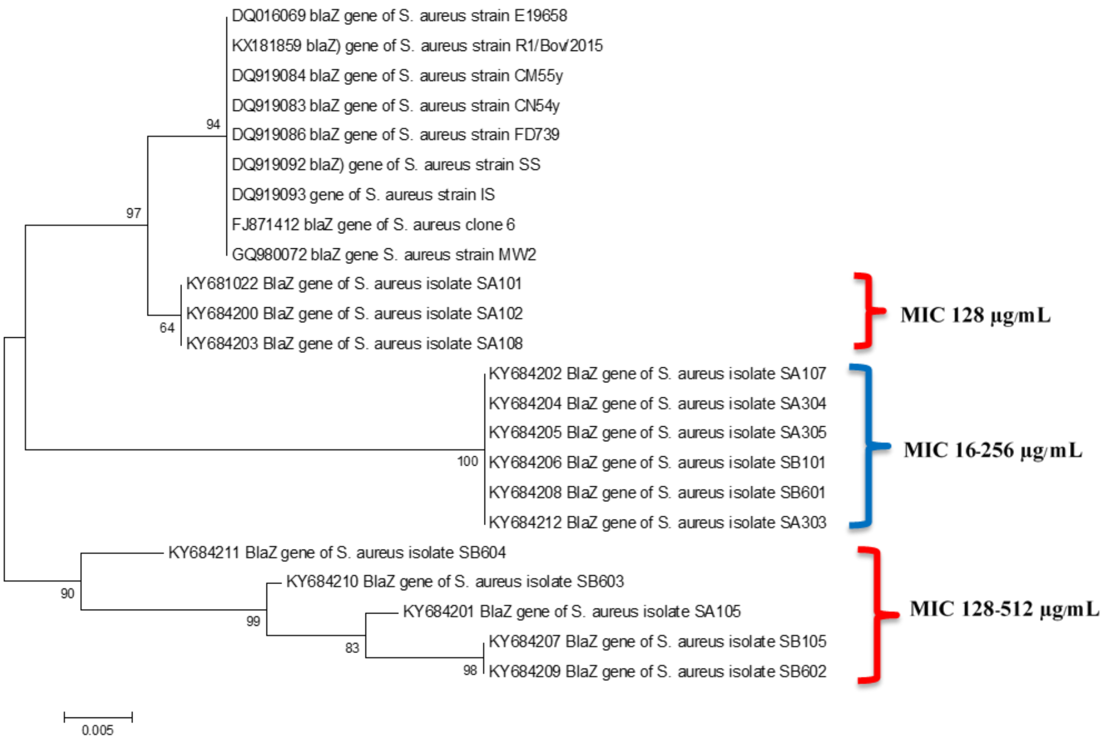

Figure 2 Phylogenetic analysis of nucleotide sequences of the *blaZ* gene in *S. aureus* constructed using the neighbor-joining method in MEGA 10.2, illustrating evolutionary relationships and clustering patterns of isolates based on partial *blaZ* gene sequences.

Table 4 Amino acid variation of proteins encoded by the blaZ gene in isolated *S. aureus*.

Signature type	Protein id	Nucleotide id	MIC of Penicillin G (µg/mL)	BlaZ amino acid variable positions*													
				4	17	29	37	39	43	56	91	94	101	105	114	115	118
I	AVA30207	KY681022	128	K	T	K	V	Q	E	I	K	N	F	N	V	E	Y
	AUP42527	KY684203	128	-	-	-	-	-	-	-	-	-	-	-	-	-	-
II	AUP42524	KY684200	128	E	-	-	-	-	-	-	-	-	-	-	-	-	-
III	AUP42535	KY684211	256	-	-	-	-	-	K	T	-	-	L	-	-	-	-
IV	AUP42526	KY684202	128	-	A	-	-	-	-	-	E	K	L	S	-	S	C
	AUP42536	KY684212	256	-	A	-	-	-	-	-	E	K	L	S	-	S	C
V	AUP42530	KY684206	128	E	A	-	-	-	-	-	E	K	L	S	-	S	C
	AUP42532	KY684208	16	E	A	-	-	-	-	-	E	K	L	S	-	S	C
VI	AUP42534	KY684210	512	-	-	-	I	K		-	-	-	L	-	-	-	-
VII	AUP42528	KY684204	64	-	A	-	I	K	K	-	E	K	L	S	-	S	C
	AUP42529	KY684205	64	-	A	-	I	K	K	-	E	K	L	S	-	S	C
VIII	AUP42525	KY684201	128	E	-	N	I	K	K	-	-	-	L	-	I	-	-
IX	AUP42531	KY684207	64	E	K	N	I	K	K	-	-	-	L	-	I	-	-
X	AUP42533	KY684209	128	-	K	N	I	K	K	-	-	-	L	-	I	-	-

The mutations and unique signatures may reflect evolutionary adaptations affecting protein function, potentially influencing antibiotic resistance and cellular roles [39,40]. The 10 unique signature types highlight protein sequence diversity, suggesting strain-specific variations and environmental adaptations, offering insights into pathogenicity and resistance mechanisms.

Despite minor sequence variations, no significant correlation was observed between the *blaZ* gene sequences and penicillin resistance levels of the isolates. Phylogenetic analysis grouped the isolates into three clusters: one associated with high penicillin resistance (MIC 128 µg/mL), one with varying resistance levels (MIC 16 - 256 µg/mL), and another with very high resistance (MIC 128 - 256 µg/mL).

However, these groupings did not establish a significant relationship between specific *blaZ* sequences and resistance levels, indicating that other factors may influence penicillin resistance. Penicillin resistance in *S. aureus* is a multifactorial phenomenon. In addition to the *blaZ* gene, other contributors included the presence of the *mecA* gene, mutations in penicillin-binding proteins (PBP1, PBP2, PBP3 and PBP4) and the overexpression of staphylococcal beta-lactamase, particularly in *mec*-negative isolates [14,15,41,42].

Conclusions

This study investigated the genetic and phenotypic characteristics of *S. aureus* isolated from subclinical bovine mastitis, focusing on antibiotic resistance profiles and the molecular analysis of resistance genes. All isolates were coagulase-positive and harbored the species-specific *nuc* and *coa* genes, while a subset carried the *clfa* and *mecA* genes indicating virulence and methicillin resistance. Complete resistance to penicillin, cefotetan, and nalidixic acid was observed, with diverse resistance patterns and high MIC for penicillin G (16 - 512 µg/mL), underscoring multidrug resistance challenges. Sequence analysis of the *blaZ* gene revealed minor nucleotide and amino acid variations, and phylogenetic grouping identified clusters associated with varying penicillin resistance levels. However, no direct correlation was found between the *blaZ* gene sequences and penicillin resistance levels, suggesting the involvement of additional factors such as *mecA*, PBP mutations, or beta-lactamase overexpression. These findings highlight the complexity of antibiotic resistance mechanisms in *S. aureus* and emphasize the need for integrated surveillance and antibiotic stewardship strategies to mitigate the impact of resistant pathogens in dairy production systems.

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