Virulence and antimicrobial resistance genes in bovine mastitis causing *Staphylococcus aureus*

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Journal of Livestock Science (ISSN online 2277-6214) 17: 710-715
Received on 18/10/25; Accepted on 4/9/26; Published on 12/9/26
doi. 10.33259/JLivestSci.2026.710-715

Abstract

Mastitis is a most important condition affecting the dairy animals and causes heavy economic losses to the farmers. Mastitis caused by many organisms, among them *Staphylococcus aureus* is considered as a major causative agent. The main objective of the study was to detect virulence and antibiotic resistance genes from *S. aureus* by molecular method. Total of 77 *Staphylococcus* isolates recovered from 140 mastitic milk samples were molecularly confirmed by PCR. Among them 17 isolates were confirmed as a *S. aureus* by PCR. Antibiotic sensitivity test of 17 *S. aureus* isolates revealed highest resistance was observed against penicillin (94.11%) followed by gentamicin (58.82%), cloxacillin, tylosin and moxifloxacin (35.29% each), amoxyclav and methicillin (29.41% each), ceftriaxone, enrofloxacin and levofloxacin (17.64% each), cefoperazone (11.76%), ampicillin/salbactam, oxytetracycline and cefuroxime (5.88% each). Out of 17 *S. aureus* isolates virulence genes, *hlA, hlB, clfA, clfB* were detected in 4 (23.52%), 5 (29.41%), 4 (23.52%) and 13 (76.47%) isolates, respectively. The antibiotic resistance genes *mecA* and *blaZ* were detected in 5 (29.4%) and 7 (41.17%) isolates, respectively.

Keywords: Antibiotic resistance gene, Mastitis, *S. aureus*, Virulence gene

Introduction

Bovine mastitis, the result of complex interactions among the host, environment and infectious agents, is one of the most prevalent diseases of dairy cattle and affects world dairy production, decreasing the quantity and quality of milk produced (Hogeveen and Van Der Voort, 2017). Bovine mastitis is multi-etiological condition mainly caused by environmental and contagious pathogens (Parasana *et al.* 2022). *Staphylococcus aureus* is the most common causative agent of bovine mastitis (Parasana *et al*. 2021).

Antimicrobial treatment is an important tool for controlling intramammary infections in dairy cows in most countries globally (Chauhan *et al.,* 2016, Nobrega *et al.* 2018). However, the efficacy of antimicrobial therapy against mastitis pathogens including *Staphylococcus* species is on the decline (Danilov *et al.,* 2019, Qu *et al.* 2019). Of great concern, is the high level of resistance to beta lactam antibiotics being report among staphylococci species isolated from bovine milk (Yang *et al.* 2020). Multidrug-resistant (MDR) staphylococci, including methicillin-resistant staphylococci (MRS), are an emerging global public health problem (Anjum *et al.* 2019). One of the most common resistance mechanisms is the production of beta lactamases, encoded by the *blaZ* gene, whose transmission is influenced by the selective pressure of antibiotic use (Olsen *et al.* 2006). Resistance to methicillin is conferred by the production of the penicillin-binding protein (PBP2a) encoded by the *mecA* gene, which has a low affinity for β-lactams (Hanssen and Ericson, 2006).

Invasiveness, biofilm formation, toxin-mediated virulence factors, and antibiotic resistance are factors which influence the pathogenicity, cure rate, and ability of these organisms to survive in the host environment. Hemolysins, leukocidins, enterotoxins, and superantigens are virulence factors produced by *S. aureus* that promote intramammary infection (IMI) and enable mastitis-causing bacteria to evade the host immune system (Oliveira *et al.* 2018).

Hemolysins are considered important virulence factors of *S. aureus* that contribute to bacterial invasion and escape from the host immune response, they are of crucial importance in cases of chronic mammary gland infections. *S. aureus* mainly produces α and β hemolysins, encoded by *hla* and *hlb* genes, respectively (Burnside *et al.* 2010). The study was undertaken with objective to detect virulence and antibiotic resistance genes from *S. aureus* by molecular method.

Materials and methods

Sample Collection: The work was carried out using 70 *Staphylococcus* isolates recovered from the 140 milk samples collected from cattle and buffaloes suffering from mastitis in and around Navsari district, Gujarat, India.

***DNA Extraction from Bacterial Culture*:** Loop full of bacterial colony was taken with sterile loop and mixed with nuclease free water (NFW) in 1.5 ml microcentrifuge tube. Stirred with vortex for 20-30 seconds and placed into orbital shaker (Chilling/heating block, Cole- Parmer) and heated for 15 min at 95º C. Then it was held for 1 min at room temperature and placed in the deep freeze (-20°C) till it gets freezed. Then tubes were kept at room temperature until the content get liquified. Tubes were centrifuged in high speed centrifuge machine (Genaxy) at 14000 rpm for 5 min. Then carefully upper aqueous supernatant from the tube was taken into a new sterile microcentrifuge tube and used as template in PCR.

Molecular Confirmation of Staphylococcus Genus and S. aureus: PCR was carried out using primer targeting *16S rRNA* gene of *Staphylococcus* genus and *nuc* gene of *S. aureus* mentioned in Table 1. For PCR, reaction mixture was prepared in 25 µl quantity containing 3.0 µl template DNA, 12.5 µl of 2x PCR master mix, 1.0 µl of forward and 1.0 µl of reverse primer and 7.5 µl sterile nuclease-free water. Cycling condition was set as initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C (*Staphylococcus* genus) and 54°C (*S. aureus*) for 30s and extension at 72°C for 30s with a final extension step at 72 °C for 10 min. PCR product was visualized after electrophoresis using 1.5% agarose gel and with UV Transilluminator (SynGene, UK).

Antibiotic Sensitivity Test: All the *Staphylococcus aureus* isolates were subjected to in vitro antibiotic sensitivity test as per the Kirby-Bauer disc diffusion method as per Clinical and Laboratory Standards Institute (CLSI) standards. In the present study ampicillin/Sulbactam (10/10 mcg), ceftriaxone (30 mcg), amoxyclav (10 mcg), cloxacillin (30 mcg), tylosin (15 mcg), enrofloxacin (10 mcg), methicillin (5 mcg), levofloxacin (5 mcg), moxifloxacin (5 mcg), penicillin (10 units), gentamicin (30 mcg), oxytetracycline (30 mcg), cefuroxime (30 mcg), cefoperazone (75 mcg) (HiMedia) were used.

Detection of virulence and antibiotic resistance genes of Staphylococcus aureus isolates: In current work, four virulence genes *viz, hlA* (Hemolysin A)*, hlB* (Hemolysin B)*, clfA* (Clumping factor A)*, clfB* (Clumping factor B) and two antibiotic resistance genes viz. *mecA* and *blaZ* were targeted. PCR was carried out for molecular detection of virulence and antibiotic resistance genes using primers mentioned in table 2. Cycling condition was set as initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for *hlA* gene, 50°C for *hlB, clfA*, *clfB,* 60°C for *mecA* and 58°C for *blaZ* gene for 30s and extension at 72°C for 30s with a final

extension step at 72 °C for 10 min. PCR product was visualized after electrophoresis using 1.5% agarose gel and with UV Transilluminator (SynGene, UK).

Table 1. *Staphylococcus* Genus (*16S rRNA*) and *S. aureus* (*nuc*) primer sequences

Primer (5' to 3')	Target gene	Product size	Reference
F: GGCCGTGTTGAACGTGGTCAAATCA R: TIACCATTTCAGTACCTTCTGGTAA	*16S rRNA*	370bp	Martineau *et al.* (2001)
F: GTGCTGGCATATGTATGGCAATTGT R: TACGCCGTTATCTGTTTGTGATGC	*nuc*	181bp	Hegde *et al.* (2013)

Table 2 Virulence and antibiotic resistance gene specific primer sequences for *S. aureus*

Primer (5' to 3')	Target gene	Product size	Reference
F: GGTTTAGCCTGGCCTTC R: CATCACGAACTCGTTCG	*hlA*	534 bp	Booth *et al.* (2001)
F: GCCAAAGCCGAATCTAAG R: CGCATATACATCCCATGGC	*hlB*	833 bp	
F: GGCTTCAGTGCTTGTAGG R: TTTTCAGGGTCAATATAAGC	*ClfA*	1000 bp	Stephan *et al.* (2001)
F: ACATCAGTAATAGTAGGGGGCAAC R: TTCGCACTGTTTGTGTTTGCAC	*ClfB*	205 bp	Tristan *et al.* (2003)
F: GGCCAATACAGGAACAGCAT R: CCCAATTTTGATCCATTTGTTG	*mecA*	134 bp	Gonzalez *et al.* (2020)
F: TGACCACTTTTATCAGCAACC R: GCCATTTCAACACCTTCTTTC	*blaZ*	166 bp	Meroni *et al.* (2019)

Results and discussion

Molecular Confirmation of *S. aureus* isolates: In the present investigation, primers targeting *16S rRNA* gene by Martineau *et al.* (2001) was employed for amplification of the genus specific *Staphylococcus* isolates. All the 70 (50 %) isolates were amplified at 370 bp and confirmed as *Staphylococcus*. For confirmation of *Staphylococcus aureus*, species specific *nuc* (thermonuclease) gene primers were used as per Hegde *et al.* (2013). In our investigation, out of 70 *Staphylococcus* 17 (12.14%) isolates were found positive at 181 bp along with reference strain of *Staphylococcus aureus*. Similar result was found by Wang *et al*. (2016) who detected 10.70% *Staphylococcus aureus* by *nuc* gene based PCR. Compared to present study, Hamid *et al*. (2017), Singh *et al.* (2018), Soares *et al*. (2017), Jahan *et al*. (2015), Easaw *et al*. (2022), Lubna *et al.* (2023), Gaddi *et al*. (2016), Hoque *et al*. (2018) and Ewida and Al-Hosary (2020) reported prevalence of 22.5%, 23.38%, 25%, 25.53%, 35.2%, 37%, 52.21%, 72.7% and 95.83%, respectively by the *nuc* gene-based PCR.

Antibiotic sensitivity test: In the present study, different levels of antibiotic resistance were observed in the tested isolates and highest resistance was observed against penicillin i.e., 94.11%. Similar to present findings highest resistance against penicillin was reported by Hamid *et al*. (2017) as 94.4 % while Jahan *et al*. (2015) observed 100 % resistance for penicillin. Highest resistance against penicillin as compared to other antibiotics also reported by Mubarack *et al*. (2012), Mehmeti *et al*. (2016) and Cheng *et al*. (2019) as 83.3 %, 65 % and 66 % respectively. Other antibiotics showed resistance as gentamicin (58.82%), cloxacillin, tylosin and moxifloxacin (35.29% each), amoxyclav and methicillin (29.41% each), ceftriaxone, enrofloxacin and levofloxacin (17.64% each) and cefoperazone 11.76%. While ampicillin/salbactam, oxytetracycline and cefuroxime were least resistant as (5.88% each).

Molecular detection of virulence genes: Out of the 17 *Staphylococcus aureus* isolates 4 (23.52%) isolates were yielded 534 bp amplicon of *hlA* gene. Compared to this, Ali *et al*. (2018), Parth *et al*. (2016) and Elsayed *et al*. (2015) who observed 33.33%, 33.96% and 34.40% *hlA* gene in *Staphylococcus aureus* isolates respectively. While Salasia *et al*. (2011), Wang *et al*. (2016) observed higher frequency of (100%, 94.30%) *hlA* gene in *Staphylococcus aureus* isolated from clinical mastitis, respectively. Out of the 17 *Staphylococcus aureus* isolates 5 (29.41%) isolates were yielded 833 bp amplicon of *hlB* gene. Compared to this lower incidence was reported by Ali *et al*. (2018) as 16.66%. In other studies, higher percentages of 84%, 71% and 79.10% *hlB* gene in *Staphylococcus aureus* isolates were reported by Salasia *et al*. (2011), Memon *et al*. (2013) and Wang *et al*. (2016) respectively. Out of the 17

Staphylococcus aureus isolates 4 (23.52%) isolates were yielded 1000bp amplicon of *clfA* gene. The results were in accordance with reports of Tegegne *et al.* (2021) who found 22% of the isolates containing *clfA* gene. While, higher frequency of *clfA* genes was recorded by Munive Nunez *et al*. (2023), Ibrahim *et al*. (2022) as 80.7%, 89.5% respectively. Out of the 17 *Staphylococcus aureus* isolates 13 (76.47%) isolates were yielded 205bp amplicon of *clfB* gene. Similarly Munive Nunez *et al*. (2023) reported *clfB* gene in 73.7% isolates. Out of the 17 isolates, 13 isolates produced a single amplicon of *clfB* gene for each strain of *Staphylococcus aureus*. This showed overall 76.47 % virulence gene involvement for bovine mastitis. Four isolates (23.52 %) showed positivity for all the virulence genes. One (5.88%) isolate was found positive for two genes *hlB* and *clfB,* while four isolates (23.52 %) were found negative for all the targeted virulence genes.

Molecular detection of antibiotic resistance genes: Out of the 17 *Staphylococcus aureus* isolates 5 (29.4%) isolates were yielded 131bp amplicon of *mecA* gene. Similarly, results were also found by Shahzad *et al*. (2024) 21%, Khasapane *et al.* (2024) 16% and Rodriguez *et al*. (2023) 12.1%. Out of the 17 *Staphylococcus aureus* isolates 7 (41.17%) isolates were yielded 750bp amplicon of *blaZ* gene similarly, Mbindyo *et al.* (2021) detected *blaZ* gene in 41.1% isolates, While Contrary Rodriguez *et al.* (2023) detected *blaZ* gene in 83.9% isolates.

This study revealed a high phenotypic resistance against penicillin among *S. aureus*. In addition, detection of antimicrobial resistance genes and virulence genes in these strains signifies a public health concern and a serious challenge to bovine mastitis therapy. Therefore, there is a need to control the emergence and spread of antimicrobial resistance in dairy farms.

Acknowledgement

The authors thanks to laboratory technicians of Department of Veterinary Microbiology, Kamdhenu University, Navsari for their help.

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