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Original Article

Virulence genes and antibiotic resistance profiles of *Staphylococcus aureus* isolated from bovine mastitis milk samples

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Abstract

Background: The increasing importance of antibiotic resistance shows the need for determining indices of the epidemiology of infection. **Aims:** This study aimed to determine the virulence genes and antibiotic resistance profiles of *Staphylococcus aureus* isolated from bovine mastitis cases. **Methods:** A total of 200 cattle were selected based on California Mastitis Test (CMT) results, and the samples were cultured in the laboratory. Grown colonies were examined by conventional phenotypic methods and confirmed using PCR amplification of *16S rRNA* gene. The prevalence of the virulence genes was also defined. The results of phenotypic and molecular tests were compared using SPSS software by McNemar test. Then, the confirmed isolates were tested for antibiotic susceptibility using the disc diffusion method. **Results:** Of the 200 positive CMT cattle, 24 animals were positive for *S. aureus* and confirmed using *16S rRNA* gene amplification. Statistical analysis showed that the phenotypic and genotypic tests of hemolysin genes were not significantly different (P>0.01). PCR analysis revealed the presence of *coa* and *clfa* genes in more than half of the cases. Overall, nine genetic profiles of virulence factors were found among *S. aureus* isolates. The highest and lowest resistance rates were against penicillin and gentamicin, respectively. **Conclusion:** Our findings showed a high rate of antibiotic resistance. So, accurate and fast diagnosis and antimicrobial susceptibility tests should be considered before prescribing the drugs.

Key words: Antibiotic resistance, Mastitis, *Staphylococcus aureus*, Virulence genes

Introduction

Bovine mastitis is one of the most important diseases of dairy cattle, which is an infection of the breast tissue that occurs frequently during breast-feeding (Tarazona-Manrique *et al*., 2019). Bovine mastitis can appear in two forms, subclinical and clinical. The subclinical form of bovine mastitis occurs more often than the clinical form, generally without visible changes in breast or milk. In the clinical form of the disease, visible changes are observed in milk or the udder (Kibebew, 2017; Campos *et al*., 2022). Bacteria remain in the mammary glands and mammary lesions of cattle, and spread during breast-feeding through the teat canal, milking hands, and the milking machine without disinfectant (Petersson-Wolfe *et al*., 2010). Additionally, heifers infected during gestation may be an issue of propagation to the herd. *Staphylococcus aureus* is the most commonly found micro-organism in bovine mastitis cases (Jena, 2020). *S. aureus* produces several virulence factors, which are often detected in disease cases, including hemolysin (encoded by *hla*, and *hlb* genes) (Ariyanti *et al*., 2011) that can disrupt cell membranes and increase the adhesion factor in the host breast cells (Xiaohong and Yanjun, 2011). Alpha hemolysin has a cytotoxicity effect while beta-hemolysin is a sphingomyelinase (Schmitt *et al*., 2012). Other virulence factors can be mentioned as coagulase and clumping factors. The clumping factor, fixed to the bacterial cellular wall, induces adherence to fibrin clots and facilitates *S. aureus* colonization (Hair *et al*., 2010). On the other hand, *Staphylococcus* is also the cause of food-borne diseases in humans with the consumption of raw milk or raw milk products derived from dairy animals with mastitis (Käppeli *et al*., 2019).

Antibiotics are widely used for control and treatment of bovine mastitis. Antimicrobial susceptibility

(antibiogram) is one of the tests used to determine the appropriate treatment. In recent years, antibiotic resistance has been increased in *S. aureus* isolates from bovine mastitis cases due to the intensive use of drugs without prior drug susceptibility testing, or extensive use of ointments containing antibiotics (Kumar *et al*., 2010). Finding the phenotypic and genotypic patterns can provide a better understanding of the distribution of *S. aureus* isolates in clinical and subclinical bovine mastitis cases. There is little data available in Iran on the prevalence of virulence genes and antibiotic resistance profile of *S. aureus* isolated from bovine mastitis cases. The epidemiological status of bovine mastitis is not clear in Iran, and this study would be a worthwhile contribution to shed light on the antibiotic-resistance management mechanisms (Hosseinzadeh and Saei, 2014), especially during the treatment process and drug prescription. The purpose of this study was to characterize *S. aureus* from bovine mastitis milk samples as well as to detect virulence factors including alpha-hemolysin (Hla), beta-hemolysin (Hlb), CLFA, and Coa. Also, it was designed to find the association between the phenotype and genotype of virulence genes, and the antibiotic resistance profile in bovine mastitis milk samples.

Materials and Methods

Ethics statement

The current study was performed in compliance with the animal welfare act and regulations following the ARRIVE 2.0 guidelines. Before conducting the study, the purposes, expected results, and benefits of the study were clarified to the farmers, and the needed permission was gained from cattle owners for the use of their animals.

Study design and sampling

A cross-sectional study was performed on 200 milk samples randomly collected from cows in Alborz, Iran, during 2018-2019. Cattle were selected based on the results of the California Mastitis Test (CMT) (screening test). For this purpose, 15 ml of milk samples were collected into sterile screw-capped bottles. Milk samples were immediately transported to the laboratory in an ice container. Each sample was divided into two microtubes: the first was used fresh for bacteriological and biochemical tests and the second was stored at -20°C until use.

Bacteriological and biochemical test

Milk samples were centrifuged at 850 g for 5 min. The sediment was streaked on blood agar (BA) and mannitol salt agar (MSA) (Oxoid, UK). Then, the cultured plates were incubated at 37°C for 24 to 48 h. Yellowish-white colonies on BA and golden-yellow colonies on MSA were regarded as presumptive *S. aureus.* Colonies were recognized as *S. aureus* using microbiological methods such as colony morphology, Gram-staining, catalase test and coagulase tube reactions (rabbit plasma). The coagulase and catalase-positive samples were classified as *S. aureus* species.

Hemolysin phenotyping

The positive samples were streaked on BA containing sheep blood and incubated at 37°C for 24 h. The BA plates were then incubated at 4°C for 4 h and hemolysis profiles were observed and analysed. The *S. aureus* ATCC 29213 strain was also used as a control. The colonies were then confirmed by the PCR amplification of *16S rRNA* gene as described below (Monday and Bohach, 1999).

Antibiotic susceptibility test

The isolates were inoculated into the brain-heart infusion medium with turbidity adjusted to 0.5 McFarland tube. The antibiotic susceptibility test was then conducted for a group of antibiotics selected based on their high number of prescriptions and more availability rate, by disc diffusion method in Müller-Hinton agar with the following antibiotics: penicillin (10 units), ampicillin (10 µg), tetracycline (30 µg), gentamicin (10 µg), oxytetracycline (30 µg), sulfamethoxazole (trimethoprim) (23.75 µg/1.25 µg), and streptomycin (10 µg). *S. aureus* ATCC 29213 strain was used as a control for antibiotic resistance. The diameter of the inhibition zone was measured and reported on the basis of the CLSI (Huys *et al*., 2005; CLSI, 2020).

DNA extraction and PCR assay

In brief, samples were centrifuged at 18620 g and the precipitates were washed 2-3 times with Tris-EDTA solution (Tris-HCL 10 mM, EDTA 1 mM, pH 8.8). The precipitates were then washed with PCR buffer (10X buffer: Tris-HCl 100 mM, KCl 500 mM, pH 8.8) and re-suspended. Lysozyme (2 mg/ml) was added and incubated for 20 min at room temperature (RT). Proteinase K was added (40 mg/ml) and incubated at 56°C for 1 h. Thereafter, the samples were centrifuged for 45 s at 18620 g. The supernatant was separated, 70% ethanol was added and incubated at -20°C for 1 h and then, centrifuged (Meiri-Bendek *et al*., 2002). DNA precipitate was dissolved in purified water and the quality of DNA was measured using the NanoDrop (Nano-Drop Technologies, Wilmington, DE, USA).

The final volume of PCR reaction was 30 µL including 1.8 µL of 25 mM $MgCl_2$, 3 µL of 10X PCR buffer, 0.6 µL of 10 mM dNTPs, 0.5 µL of *Taq* DNA polymerase (5 units/ml) (Fermentase, Germany), 2.5 µL of DNA template (100 ng/µL), 1 µL of each primer (10 µM) (CinnaGen, Iran), and 20 µL of double-distilled water. Double-distilled water and *S. aureus* ATCC 29213 strain were used as negative and positive controls, respectively. The *16S rRNA*, α-hemolysin (*hla*), beta-hemolysin (*hlb*), coagulase (*coa*), and clumping factor A (*clfA*) primers as well as the PCR conditions are listed in Table 1. After amplification, 5 µL of each PCR product was mixed with 1 µL of 6X gel loading dye. Gel electrophoresis was done using 1% agarose gel, which was then, stained with ethidium bromide (0.5 µg/ml) for

Table 1: Primer sets for the detection of virulence genes in *S. aureus* isolates

Locus	Nucleotide sequence (5′→3′)	Size (bp)	PCR condition	Reference
16S rRNA	F: AACTCTGTTATTAGGGAAGAACA R: CCACCTTCCTCCGGTTTGTCA	756	Initial denaturation: 94°C, 5 min 30 cycles: 94°C, 45 s, 68°C, 45 s, 72°C, 90 s Final extension: 72°C, 10 min	Ciftci *et al*. (2009)
coa	F: ATAGAGATGCTGGTACAGG R: GCTTCCGATTGTTCGATGC	710	Initial denaturation: 94°C, 5 min 30 cycles: 94°C, 40 s, 58°C, 60 s, 72°C, 60 s Final extension: 72°C, 10 min	Kalorey *et al*. (2007)
clfa	F: GGCTTCAGTGCTTGTAGG R: TTTTCAGGGTCAATATAAGC	1042	Initial denaturation: 94°C, 5 min 35 cycles: 94°C, 60 s, 57°C, 60 s, 72°C, 60 s Final extension: 72°C, 10 min	Kalorey *et al*. (2007)
hla	F: GAAGTCTGGTGAAAACCCTGA R: TGAATCCTGTCGCTAATGCC	704	Initial denaturation: 94°C, 3 min 30 cycles: 94°C, 1 min, 60°C, 45 s, 72°C, 1 min Final extension: 72°C, 10 min	Xiaohong and Yanjun (2011)
hlb	F: CAATAGTGCCAAAGCCGAAT R: TCCAGCACCACAACGAGAAT	496	Initial denaturation: 94°C, 3 min 30 cycles: 94°C, 1 min, 60°C, 45 s, 72°C, 1 min Final extension: 72°C, 10 min	Xiaohong and Yanjun (2011)

15 min, and visualized by UV-light transilluminator (GelDoc, BioRad, USA).

Statistical analysis

Data were analyzed using Chi-square McNemar, and a 99% confidence level was considered to determine the statistical significance. Moreover, the agreement between the tests was measured using the kappa factor by the SPSS software (version 16.0, SPSS Inc., Chicago, USA).

Results

Prevalence of *S. aureus* in milk samples

Of 200 positive CMT cattle, forty specimens were positive for *Staphylococcus* spp. using Gram-staining, culture on BA and MSA, and catalase tests, and only twenty-four cases (60%) were positive for tube coagulase test, considered as *S. aureus*. All of the positive coagulase samples were confirmed by the amplification of the *16S rRNA* gene (Fig. 1).

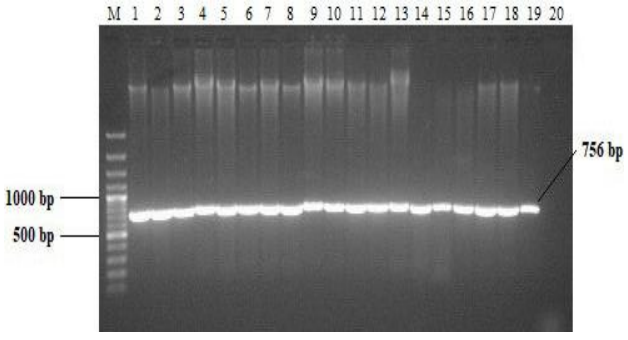

Fig. 1: PCR amplification of *16S rRNA* gene. Lane M: 100 bp Plus DNA ladder. Lanes 1-18: Field isolates, Lane 19: Positive control, and Lane 20: Negative control

Phenotypic and genotypic profiles of *S. aureus*

The results of the hemolysin test on sheep BA plates revealed that eight isolates (33.3%) produced α-hemolysin, six isolates (25%) produced β-hemolysin, four isolates (16.7%) produced double hemolysin, and six isolates (25%) were non-hemolytic. By PCR amplification of the genes encoding hemolysin with specific primers, *hla*, and *hlb* genes were amplified in 10 (41.7%) and four isolates (16.7%), respectively. Additionally, combined amplification of *hla* and *hlb* genes was found in six (25%) isolates and four (16.7%) non-hemolytic isolates (Fig. 2).

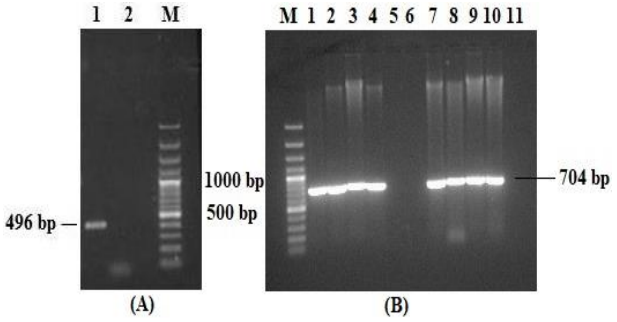

Fig. 2: PCR amplification of *hlb* and *hla* gene. (**A**) Lane 1: *hlb* positive control, Lane 2: Negative control, and Lane M: 100 bp Plus DNA ladder. (**B**) Lane M: 100 bp Plus DNA ladder, Lanes 1-9: Field isolates, Lane 10: *hla* positive control, and Lane 11: Negative control

Based on the findings of this study, α-hemolysis was more frequently observed than other hemolysis cases. The *hla* coding gene was found in all strains with α-hemolysis on BA media. Statistical analysis showed that the difference between phenotypic and genotypic tests of hemolysin genes was not significant (P>0.01). There was a good agreement between phenotypes and genotypes of hemolysin genes based on the kappa factor (kappa = 0.77).

Through PCR amplification of the *S. aureus coa* and *clfa* coding genes with specific primers, *coa* and *clfa* genes were detected in 12 (50%) and 16 (66.66%) isolates, respectively (Fig. 3).

The most frequent virulence gene was *clfa* in Iranian *S. aureus* isolates. The genetic profile of virulence factors in *S. aureus* isolates collected from suspected cattle with mastitis is shown in Table 2.

Antibiotic resistance profiles of *S. aureus*

In vitro antimicrobial sensitivity results indicated that all *S. aureus* isolates were resistant to penicillin followed by ampicillin (81.1%), tetracycline (83.3%), neomycin (45.8%), streptomycin (16.5%), oxytetracycline and trimethoprim (8.3%), respectively. Antimicrobial sensitivity patterns showed that all isolates were sensitive to gentamicin and overall; six antibiotic resistance patterns were found in *S. aureus* isolates in Alborz, Iran.

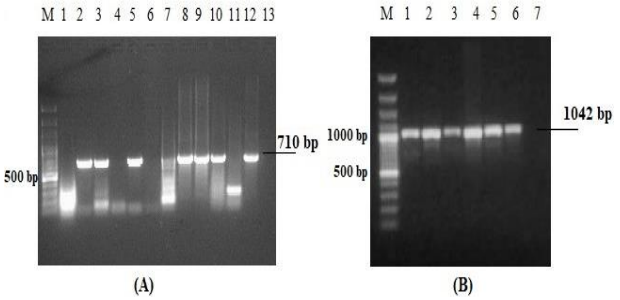

Fig. 3: PCR amplification of *coa* and *clfa* genes. (**A**) Lane M: 100 bp Plus DNA ladder, Lanes 1-11: Field isolates, Lane 12: *coa* positive control, and Lane 13: Negative control. (**B**) Lane M: 100 bp Plus DNA ladder, Lanes 1-5: Field isolates, Lane 6: *clfa* positive control, and Lane 7: negative control

Table 2: Genetic profile of virulence factors among *S. aureus* isolates in Alborz province

Genetic profile	Number (%)
Clfa coa	4/24 (16.6%)
Clfa coa hla hlb	3/24 (12.5%)
Clfa hla hlb	2/24 (8.33%)
Clfa hla	4/24 (16.6%)
Clfa hlb	3/24 (12.5%)
coa hla	5/24 (20.83%)
hlb	1/24 (4.16%)
hla	1/24 (4.16%)
hla hlb	1/24 (4.16%)

Discussion

Staphylococcus aureus is one of the most important causes of bovine mastitis worldwide (Leitner *et al*., 2011; Gonçalves *et al*., 2018). Bovine mastitis results in serious losses in milk quantity/quality, animal reproductive failure, and the culling of infected animals. Epidemiological data are of great value for a better understanding of mastitis among the dairy animal population for the management and control of the disease. The prevalence of the disease varies across geographic zones worldwide. A high frequency of mastitis infections is reported in some countries and dairy herds. For example, the frequency of subclinical mastitis was reported at 52.4%, 62.9%, and 75.9% in Uruguay, Ethiopia, and Tanzania, respectively (Gianneechini *et al*., 2002; Kerro Dego and Tareke, 2003; Karimuribo *et al*., 2008). Previous studies have shown a high prevalence of subclinical mastitis in the Fars, Semnan, and Isfahan provinces of Iran (Hashemi *et al*., 2011). Furthermore, antibiotic resistance especially to penicillin and tetracycline has been seen as a problem in Iran (Bahrami *et al*., 2016). However, the epidemiological status of bovine mastitis is not clear In Iran (Hosseinzadeh and Saei, 2014). Mastitis is also mentioned as a zoonotic disease, transmitted by the consumption of infected milk and meat from bovine to humans (Girma and Tamir, 2022). Hence, the accurate and fast detection of the disease is very important to the cattle industry. CMT is a simple test, which has been used to detect prior to the use of conventional methods. CMT has had a valuable role in the dairy flock surveillance programs as a subclinical mastitis test or intramammary infections (IMI) due to the principal pathogens (Sargeant *et al*., 2001). The results of this study showed that 12% of cows carried *S. aureus* based on culture, biochemical, and coagulase tests. For the detection of *S. aureus*, conventional tests including Gram-staining, catalase, and oxidase are very important, and the tube coagulase test is considered a gold test (Rusenova *et al*., 2013). Based on culture results, Gram-staining and, biochemical tests, 24 isolates were identified as *S. aureus*, and confirmed through PCR amplification of the *16S rRNA* gene. PCR-based methods provide a useful option for quick identification, rather than conventional methods (Gao *et al*., 2011). Further investigation was performed by PCR amplification of hemolysin, coagulase, and clumping factor genes. Based on our results, the *hla* and *hlb* genes were observed more genotypically than phenotypically. So, this gene may be silent and cannot be expressed and therefore, the hemolysin phenotype cannot appear on the medium. Many factors can influence the phenotypic patterns of *S. aureus* isolates including the type of blood cells, incubation temperature, and the age of the sheep red cells. For example, sheep blood cells show an alpha, beta, or gamma hemolysis while horse blood cells show a delta hemolysis characterization on BA plates (Da Silva, 2005). The temperature plays a key role, especially in the beta hemolysis pattern. The age of the sheep is another factor. Older sheep contain higher concentrations of hemoglobin than younger animals. So, hemolysis around colonies on old-sheep BA plate may be not clear which causes confusion in the phenotypic characterization (Ariyanti *et al*., 2011). Based on our result, alpha hemolysis phenotype was seen more than beta hemolysis patterns on sheep BA plates, similar to the results of Xiaohong and Yanjun (2011). Although, Ariyanti *et al*. (2011) have reported vice versa for *S. aureus* isolates from animal sources. In this study, the most prevalent hemolysis gene was *hla*, which is in agreement with previous reports from Iran and other countries, such as the United States, Indonesia, and Uganda (Ariyanti *et al*., 2011; Kateete *et al*., 2011; Tabor *et al*., 2016; Sedaghat *et al*., 2017; Sedaghat *et al*., 2018). High levels of α-hemolysin can be associated with more severe cases of disease (Tong *et al*., 2015). Franco *et al*. (2008) have reported that most of the isolates produced β-hemolysin in clinical or subclinical cases; even α-hemolysin production was dependent on the *β-hemolysin* gene. Although, Xiaohong and Yanjun (2011) have

reported the presence of *hla* and *hlb* genes in 34.8% and 42.6% of the isolates. Statistical analysis showed that the difference between phenotypic and genotypic tests of hemolysin genes was not significant (P>0.01). Both *Ariyanti et al*. (2011), and Xiaohong and Yanjun (2011) have reported that there was no strict correspondence between hemolytic genotype and phenotype. The outcome of our PCR analysis of *clfa* genes showed the prevalence of these genes in more than half of the cases, similar to the previous studies (Franco *et al*., 2008; Momtaz *et al*., 2010). Based on our results, simplex PCR was successful in the detection of *Coa* in 50% of samples. These findings are in agreement with the previous studies (Franco *et al*., 2008; Momtaz *et al*., 2010) and in contrast with the results reported by Elsayed and Dawoud (2015). This research showed a high rate of antibiotic resistance, which was consistent with the results of Brînda *et al*. (2010). This increased resistance may be due to long-term drug use (Kumar *et al*., 2010). In accordance with the previous studies, our results showed that most of the isolates were resistant to penicillin and ampicillin which are frequently used for the treatment of mastitis (Lalita *et al*., 2015). Several studies conducted on bovine mastitis in different geographic areas of the world have shown different rates of resistance to penicillin. Franco *et al*. (2008), and Abrahmsén *et al*. (2014) have found that 68% of isolates were resistant to penicillin in Mexico, which was less than the resistance rate of our study. One of the reasons for penicillin resistance is the relation with beta-lactamase production (Güler *et al*., 2005). Beta-lactamases are a class of enzymes, that break the beta-lactam ring, resulting in the inactivation of the beta-lactam antibiotic and increased antibiotic resistance. In this study, the highest resistance after penicillin was observed to tetracycline, a broad-spectrum antibiotic, which was consistent with the finding of Bahrami *et al*. (2016), but was lower than the percentage reported by some other studies (Mubarack *et al*., 2012; Oliveira *et al*., 2012). The high resistance rate against tetracycline is due to its extensive use in dairy farms (Chopra and Roberts, 2001). All isolates were susceptible to gentamicin, similar to previous studies (Güler *et al*., 2005; Moroni *et al*., 2006; Sahebekhtiari *et al*., 2011; Mubarack *et al*., 2012; Hashemi, 2015). Most of the isolates were also susceptible to oxytetracycline and streptomycin, in accordance with the study of Franco *et al*. (2008).

In recent years, increased antibiotic resistance has been reported in *S. aureus* isolates from bovine mastitis cases. In general, the level of antibiotic resistance can be due to the intensive use without prior drug susceptibility testing or the use of inappropriate doses, prevention from elimination of resistant cattle, and extensive use of ointments containing antibiotics. This emphasizes the need for antimicrobial susceptibility test before prescribing the drug and the completion of treatment. Due to the prevalence rate of bovine mastitis, using accurate and fast diagnostic methods all over the country is suggested. Moreover, it is necessary to do an antimicrobial sensitivity test (especially β-lactamase test) before prescribing the drug and train the farmers, particularly in subclinical mastitis to help the disease control and management.

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Conflict of interest

The authors declare no conflict of interest.

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