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Screening of vancomycin resistance-associated genes in methicillin-resistant *Staphylococcus aureus* isolates from cattle, sheep and goats in northwestern Iran

Leila Maleki, Amir Tukmechi*

Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

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Abstract

Staphylococcus aureus is an important pathogen causing a wide range of diseases in both humans and animals. The aim of this research was to screen the vancomycin resistance-associated genes in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from animals. A total of 400 nasal swab samples were collected from cattle, goats and sheep between February and August 2022 from both industrial and traditional livestock farms in West Azerbaijan province, Iran. Then, nasal swabs were cultured on mannitol salt agar and molecular analysis was performed after bacteriological examination to confirm the presence of *S. aureus*. The *MecA* gene was used to detect MRSA isolates, and two important vancomycin resistance-associated genes, namely *vanA* and *vanB*, were searched in the isolates. Out of 400 nasal swabs, 69 samples had *S. aureus*; of which seven isolates were resistant against methicillin. No vancomycin resistance-associated genes were detected in the MRSA isolates. Based on these findings, vancomycin could be used to treat infections caused by this bacterium.

Introduction

Staphylococcus aureus is an immotile, anaerobic and catalase-positive Gram-positive coccus with a cluster arrangement. Coagulase-positive *S. aureus* is an important pathogen affecting both humans and domestic animals. In livestock, *S. aureus* can cause diseases such as bovine staphylococcal mastitis and tick-borne pyemia.[1] In humans, *S. aureus* is the second most common cause of hospital infections and responsible for 80.00% of purulent and skin infections.[2] It is a leading cause of invasive or complicated infections such as bacteremia, pneumonia, skin infections, endocarditis, osteoarticular infections and osteomyelitis.[3,4]

The term livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) is used to distinguish MRSA strains of animal origin from those isolated from humans (acquired from hospital or community). The LA-MRSA strains have the potential to cause disease in humans and often show multi-drug resistance profiles.[5]

In the 1960s, methicillin and β-lactam antibiotics were used to combat penicillin-resistant strains of *S. aureus.* However, MRSA strains quickly emerged.[2,6] Currently, 30.00 - 50.00% of *S. aureus* strains are resistant to β-lactamase such as nafcillin, cloxacillin and methicillin. All β-lactamase expression *mecA* genes and penicillin binding protein 2a (PBP2a) are involved in this resistance.[7] The MRSA is responsible for 50.00% of deaths[8,9] and it's resistance is mediated by the *mecA* gene.[10] This gene is acquired through horizontal transfer of the staphylococcal cassette chromosome *mec*.[11] The mechanism of resistance to methicillin in *S. aureus* involves the production of PBP2a, having a low affinity to β-lactams. The production of PBP2a is related to the *mec* genes in the bacterial chromosome.[12]

In the 1950s, vancomycin was introduced as an effective antibiotic for treating infections caused by MRSA. Currently, in some countries vancomycin is used as a feed additive and prophylactic antibiotic in livestock; however, the use of it and other antibiotics needs to be minimized. However, after three decades of use, reports of staphylococcal resistance to vancomycin emerged.[2,6] The first vancomycin resistant *S. aureus* (VRSA) was reported in the United States in 2002.[13] The most common type of vancomycin resistance is related to *vanA* and *vanB* genes, being located on the Tn156 and Tn1547 transposons. These genes can be located on both plasmids and chromosomes and can be transmitted through

***Correspondence:**

Amir Tukmechi. DVM, PhD
Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: a.tukmachi@urmia.ac.ir

conjugation. In addition to being able to transfer between different species of *Staphylococcus*, this plasmid can be transferred to other bacteria such as *Enterococcus* leading to the emergence of resistant *Staphylococci.* The *vanA, vanH* and *vanX* genes together are essential for the vancomycin resistance phenotype, with the genotype *VanA* being the most important.[14-16]

The aim of this study was to determine the prevalence of MRSA in nasal swabs of cattle, sheep and goats in northwestern of Iran. Also, the presence of *vanA* and *vanB* genes as vancomycin resistance-associated genes was searched between the isolates.

Materials and Methods

Sampling and bacterial culture. This cross-sectional study was conducted from February to August 2022 in West Azerbaijan province, Iran. A total of 400 nasal swab samples were collected from cattle, goats (150 samples *per* animal) and sheep (100 samples). The number of samples was equal for industrial and traditional livestock (each 200). The samples were collected by sterile swabs from the nasal cavity of each animal and transferred to the laboratory in the test tubes containing normal saline (0.98%) on ice. The samples were cultured on mannitol salt agar (Merck, Darmstadt, Germany) and incubated aerobically at 37.00 ˚C for 24 - 48 hr. Suspected *S. aureus* colonies were investigated by Gram staining, catalase, oxidase and coagulase tests. Colonies of *Staphylococci* are usually white and opaque with a diameter of around 4.00 mm. However, the colonies of human and bovine origins are typically golden yellow in color.[17]

The DNA extraction. To prepare the DNA for analysis, all *S. aureus* isolates were grown on mannitol salt agar at 37.00 ˚C for 24 hr under aerobic condition. A single bacterial colony from each sample was picked and suspended in 200 µL deionized distilled water. The DNA was extracted by a commercial DNA extraction kit (Pouya Gene Azma, Tehran, Iran). The quantity and quality of the extracted DNA were determined by spectrophotometry *via* Nanodrop (Thermo Scientific, Waltham, USA) and the DNA was stored at – 80.00 ˚C until use.

Polymerase chain reaction (PCR). Molecular confirmation was performed by amplifying the *S. aureus*-specific *16s rRNA* gene using primers designed to detect a 257 bp fragment (5'-ACGGTCTTGCTG TCACTTATA-3' and 5'-TACACATATGTTCTTCCCTAAT AA-3'). For each PCR reaction, 12.50 µL of 2.00x master mix (Pishgam Biotech, Tehran, Iran), 1.00 µL of each primer and 6.00 µL of bacterial DNA were mixed, and nuclease-free water was added to make a final volume of 25.00 µL. The reaction mixtures were placed in a thermocycler (Quanta Biotech, London, UK) and subjected to the following cycle: An initial denaturation step at 94.00 ˚C for 5 min, 30 cycles at 94.00 ˚C for 30 sec, annealing at 54.00 ˚C for 1 min and extension at 72.00 ˚C for 2 min. The final extension step was performed at 72.00 ˚C for 7 min. The PCR products were analyzed by 1.50% agarose gel electrophoresis.[18]

Identification of MRSA strains by *mecA* gene. The *mecA* gene was detected using the PCR method with the primers and reaction conditions being listed in Tables 1 and 2. For each PCR reaction, 12.50 µL of 2.00X master mix (Pishgam Biotech), 1.00 µL of each primer and 4.00 µL of bacterial DNA were mixed, and nuclease-free water was added to make a final volume of 25.00 µL. The PCR product was separated by electrophoresis on a 1.00% agarose gel and visualized under ultra-violet light. Amplification of *mecA* gene produced a segment with a size of 1,339 bp.[19]

Screening of *VanA* and *VanB* genes. The primers and PCR reaction conditions used for the detection of *vanA* and *vanB* genes are summarized in Tables 1 and 2. The *vanA* positive control strain was vancomycin-resistant *Enterococcus faecium* ATCC 51559, and the *vanB* positive control strain was *Enterococcus faecalis* ATCC 51299.[20]

Table 1. The primer sequences used for the amplification of *mecA*, *vanA* and *vanB* genes.

Genes	Primer	Sequence (5'-3')	Size (bp)
mecA	Forward	GTGGAATTGGCCAATACAGG	1,339
	Reverse	TGAGTTCTGCAGTACCGGAT	
vanA	Forward	ATCAAGCCCTCAATCGTTC	713
	Reverse	GGCAAGTCAGGTGAAGATG	
vanB	Forward	CCGCCATCCTCCTGCAAAAAA	430
	Reverse	GTGACAAACCGGAGGCGAGGA	

Table 2. The cycling conditions for *mecA*, *vanA* and *vanB* amplification.

Gene		*mecA*	*vanA*	*vanB*
Initial denaturation		94.00 ˚C for 5 min	94.00 ˚C for 5 min	94.00 ˚C for 10 min
Cycle*	Denaturation	94.00 ˚C for 30 sec	94.00 ˚C for 1 min	94.00 ˚C for 30 sec
	Annealing	55.00 ˚C for 30 sec	55.00 ˚C for 1 min	50.00 ˚C for 45 sec
	Extension	72.00 ˚C for 2 min	72.00 ˚C for 2 min	72.00 ˚C for 30 sec
Final extension		72.00 ˚C for 7 min	72.00 ˚C for 5 min	72.00 ˚C for 10 min

* The cycle numbers were set at 30, 40 and 30 for *mecA*, *vanA* and *vanB* genes, respectively.

Anti-microbial susceptibility test. The disc diffusion technique was used to determine the antibiotic resistance profile for isolates. The test was conducted on Mueller Hinton agar medium (Merck) using a bacterial suspension equal to 1.50×10^8 colony forming unit *per* mL according to the Clinical and Laboratory Standards Institute[21] standard for penicillin, vancomycin, tetracycline, kanamycin, ofloxacin, gentamicin, nitrofurantoin and cefixime discs. Bacterial isolates were cultured separately on Mueller Hinton agar medium and antibiotic discs were placed on them. After 24 hr incubation at 37.00 ˚C, the diameter of inhibition zone was measured by a digital caliper.[22]

Results

Out of 400 nasal swab samples, 69 samples (17.25%) were identified as *S. aureus* based on the morphology, biochemical tests and amplification of *16S rRNA* gene (Fig. 1). Samples collected from cattle (12.00%) had higher contamination; while, samples collected from sheep (0.75%) had lower contamination. Samples obtained from goats had 4.25% *S. aureus*. The presence of *mecA* gene was searched in all 69 isolates; only seven (10.14%) were found to have this gene and identified as MRSA (Fig. 2). Interestingly, these seven isolates were from cattle-collected samples.

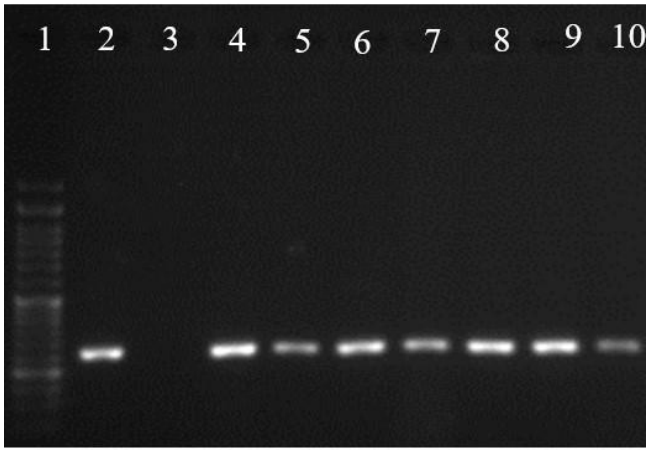

Fig. 1. Agarose gel image of amplified fragment of *16s rRNA* of *Staphylococcus aureus* (257 bp). Lane 1: 50-bp DNA ladder; Lane 2: Positive control; Lane 3: Negative control; Lanes 4-10: Positive samples for *16s rRNA*.

Anti-microbial susceptibility test showed that all MRSA isolates were resistant to cefixime, vancomycin and penicillin (Table 3). The isolates had moderate or complete sensitivity to other antibiotics discs (Fig. 3). Additionally, the results showed that none of the isolates were positive for both *vanA* and *vanB* genes.

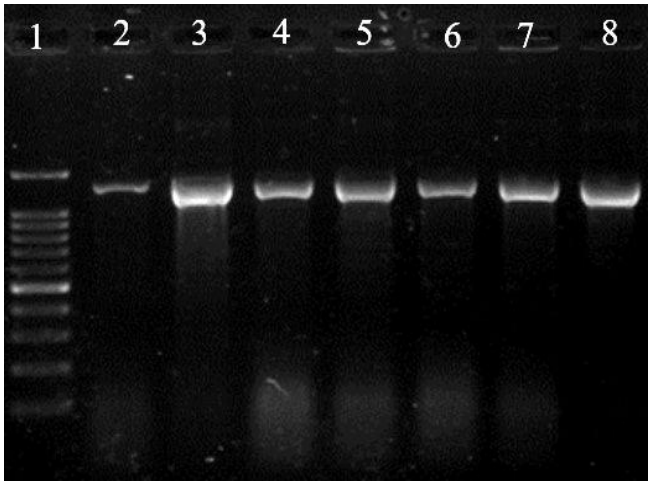

Fig. 2. The amplification of the *mecA* gene produced in *Staphylococcus aureus* by single polymerase chain reaction (1,333 bp). Lane 1: 100 bp DNA ladder; Lanes 2 - 8: Positive samples for the *mecA* gene.

Discussion

Staphylococcus aureus is a Gram-positive bacterium belonging to the *Micrococcaceae* family and is one of the most important bacterial pathogens in humans and animals. This bacterium is known to cause multiple infections and is considered as a potential pathogen. Epidemiological studies on *S. aureus* demonstrated the emergence of resistant strains in veterinary and human medicine, particularly due to their zoonotic potential. With the emergence of resistance to penicillin, a new generation of antibiotics became common. Methicillin, which is resistant to penicillinase was introduced in 1960. However, after some time, the first case of staphylococcal resistance to methicillin was observed leading to the emergence of MRSA. Vancomycin is now the last choice antibiotic for the treatment of MRSA, and its use in humans and animals is limited.[23]

Table 3. The results of anti-microbial susceptibility test for all *Staphylococcus aureus* isolates (n = 69).

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
Amikacin	100	0.00	0.00
Cefixime	0.00	0.00	100
Gentamycin	100	0.00	0.00
Kanamycin	100	0.00	0.00
Methicillin	90.00	0.00	10.00
Penicillin	85.70	0.00	14.30
Vancomycin	71.50	0.00	28.50
Tetracycline	70.00	25.00	5.00
Nitrofurantoin	40.00	45.00	15.00

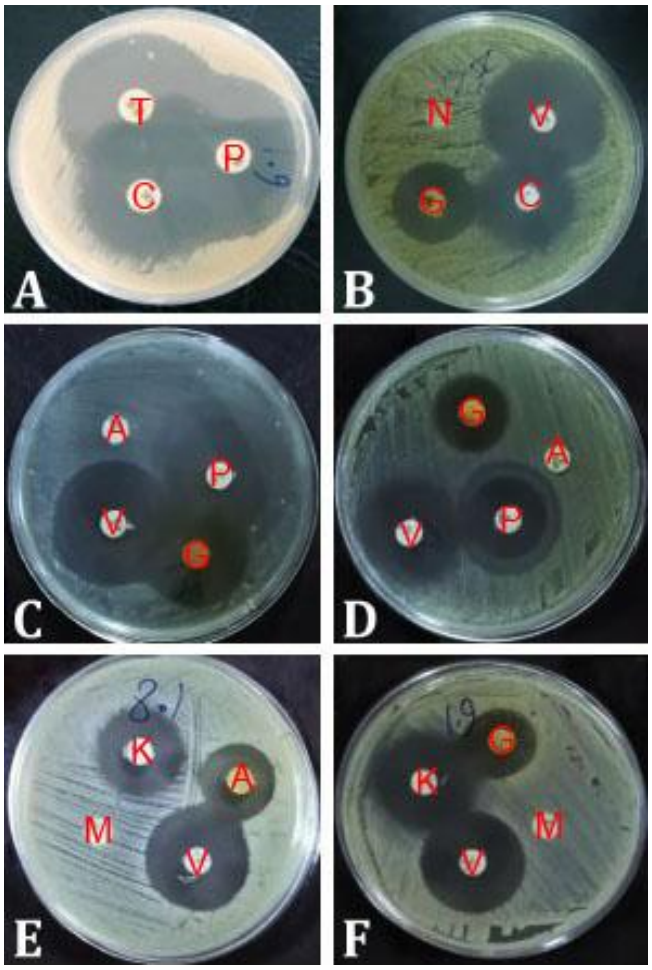

Fig. 3. The results of anti-microbial susceptibility test against some isolates of *Staphylococcus aureus*. **A** and **B)** Isolates from cattle; **C** and **D)** Isolates from sheep; **E** and **F)** Isolates from goats. A: Amikacin; C: Cefixime; G: Gentamicin; K: Kanamycin; M: Methicillin; N: Nitrofurantoin; P: Penicillin; T: Tetracycline; V: Vancomycin.

Considering the problems of drug resistance of bacterial isolates and the impact on treatment, as well as the continuation of previous studies on the resistance of *S. aureus* to methicillin, the resistance of MRSA isolates to the vancomycin was investigated as it is a valuable antibiotic for treatment of infections caused by *Staphylococcus*.[5]

Vancomycin is one of the most effective antibiotics for treating infections caused by *S. aureus.* However, VRSA is now one of the causes of hospital-acquired infections and has acquired multiple resistance to a wide range of antibiotics.[5,24]

In this study, a high rate of *S. aureus* isolation (17.25%) was observed among the 400 nasal samples collected from cattle, goats and sheep. In this regard, in a study conducted in Iran, it was found that 28.13% of hemodialysis patients carried *S. aureus* in the nose which was of hospital origin.[24] The overall prevalence of *S. aureus* in this study was lower than that reported from samples from camels in Nigeria (20.70%).[18] In a study conducted in Italy (2013 - 2015), five MRSA isolates were identified out of 12 *S. aureus* from 14 swab specimens (nasal, oral and skin) from sheep.[25]

In 2009, Thati *et al*. have reported seven VRSA samples out of 356 *S. aureus* samples.[26] In 2015, Abdelgadeir and Elhassan have studied 123 MRSA isolates and found that 6.50% of them are VRSA.[27] These results differ from those of our study, as the rates of nasal *S. aureus,* MRSA and VRSA were lower compared to the other studies. This difference may be due to the presence of *S. aureus* in the exposed environment, geographical residence, racial features and differences between the isolation methods. In a study conducted in 2005 in Türkiye by Sencak *et al*., VRSA samples were not found.[28] Similarly, in another study conducted in Tabriz, Iran, in 2006, VRSA was not detected.[29] Therefore, recent studies are in agreement with the findings of our study.

Staphylococcus aureus can be isolated from various body regions of the healthy ruminants and it can cause mastitis, abscess, abortion, impetigo, vaginal infections, rhinosinusitis and osteomyelitis.[30] The nasal region is the most common site for the initial colonization of *S. aureus* in the ruminants and serves as a primary source of infections.[31]

El-Deeb *et al*., have reported that antibiotic resistance rate of MRSA strains isolated from nasal samples of the healthy animals in Saudi Arabia against penicillin, oxacillin and cefoxitin is 100%.[32] This is similar to our study, where the highest resistance among the seven MRSA isolates from nasal samples was observed against cefixime (57.10%), vancomycin (28.60%) and penicillin (14.20%), respectively. These findings highlight the importance of performing screening tests since nasal strains of *S. aureus* exhibit different antibiotic resistance rates.

In our study, MRSA was detected in 1.75% of the nasal samples (7 of 400), and all of the MRSA isolates were from cattle samples. Several studies have used *mecA* gene as a target for MRSA identification, and in our study, MRSA isolates were identified using a set of primers for *mecA*.

Since the initial report of VRSA in 2002, 52 isolates have been reported worldwide.[33] In our study, none of the 69 *S. aureus* strains in cattle, goats and sheep were resistant to vancomycin. Therefore, it was determined that there is no risk of VRSA transmission to humans through sheep and goats in the region of our study.

In this study, seven samples were found to be resistant to methicillin, indicating the presence of *mecA* gene in their chromosome. Although these isolates were not resistant to vancomycin, it is possible that they possess other vancomycin-resistant genes such as *vanH*, *vanS*, *vanX*, etc. Our findings showed that the prevalence of MRSA in goats and sheep was lower than cattle.

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Conflict of interest

The authors declare that they have no conflict of interest.

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