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# Study on Isolated *Staphylococcus aureus* from Bovine Milk with Mastitis Containing Methicillin and Panton-Valentine Leukocidin Gene

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## Abstract

Nowadays, bovine mastitis has been recorded as an inflammation of the mammary gland and *Staphylococcus aureus* is the main pathogens responsible for contagious mastitis in ruminants. Presented study is looking for to assess the existence of methicillin-resistant and PVL positive *Staphylococcus aureus* from bovine milk and if there is any relation among the detected genes and antibiotic resistance or not? For this purpose, 100 milk samples were collected from cows affected with mastitis from four dairy farms in Fars province. The samples were transferred to MSA medium and incubated at 37 °C for 24 h. The pure colonies were identified by biochemical as well as molecular techniques using *mecA*, *mecC* and PVL gene. Then, the antibiotic pattern of the isolates was analyzed based on CLSI 2019 and Whonet program. Finally, the results were analyzed using statistical analysis. Totally, 6 *S. aureus* strains were detected which was confirmed by biochemical as well as molecular techniques. The isolates showed different antibiotic susceptibility patterns, most of the isolates were multidrug resistant, while, the strain No. 5 was the most sensitive one. Our results indicated presence of *mecA* gene in strains 1, 2 and 3, *mecC* in isolates 4, 5 and 6, and the PVL gene only in strain 4. Hence, it could be concluded that although different phages transporting PVL genes to the *S. aureus* isolate, in this special research we didn't find any relationship between presence of *mecA*, *mecC*, and PVL genes with antibiotics resistant pattern.

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Mastitis
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PVL gene
*Staphylococcus aureus*

## Introduction

Currently, the global population is rising and it is necessary to increase amounts of food (Foresight, 2011). Since milk is a good nutrient for all age groups, the health of this nutrient is very important. Indeed, in dairy cows, mastitis is the major causes of economic problems which occures microorganisms (Halasa, Huijps, Østerås, & Hogeveen, 2007; Seegers, Fourichon, & Beaudeau, 2003). Indeed, contagious mastitis is mainly caused by *Staphylococcus aureus* and *Streptococcus agalactiae*, and as they could be common

source of infected udders of the milking machine. furthermore, contaminated udder washcloths, remaining milk in teat cups, and inadequate milking equipments are also other routs for the transmission of bacterium. Hence, programs designed for the control of contagious mastitis involve improvements in hygiene and disinfection (Patterson, 2017). On the other hand, there are methods which could be used to eradicate the contagious mastitis from the herd including antibiotic therapy and removing the chronically infected cows.

Among different types of virulence factors for pathogenicity of *Staphylococcus aureus*, Panton-Valentine leukocidin (PVL) is one of the most important ones (Holmes *et al*., 2005). This cytotoxin is related with tissue necrosis and also causes destruction of leukocyte membranes (Shrestha, Singh, Raj, Pokhrel, & Mohapatra, 2014). Furthermore, PVL toxin has been linked with community-acquired methicillin-resistant *Staphylococcus aureus* strains (CA-MRSA), which is more dangerous (del Giudice *et al*., 2009; Unal & Cinar, 2012).

Although, effective vaccines are available to reduce the effect of environmental coliform mastitis, vaccine progress for gram-positive bacteria in recent decades has not been promising. Therefore, it seems that the most important tools for prevention of mastitis is developing better technical and biological tools for managing contagious mastitis, which should be accompanied by appropriate incentives and communication strategies for farmers and veterinarians (Klaas & Zadoks, 2018). So, the present study tried to find and screen *Staphylococcus aureus* from bovine milk samples with mastitis based on Penton valentine leukocidin and methicillin resistant genes to evaluate is there any relationship between these two genes and antibiotic patterns or not?

## Materials and methods

### Isolation, purification and characterization

Totally, 100 milk samples were collected from bovine milk with mastitis, which is confirmed using California mastitis tests. The samples were collected in sterile bottles and they were transferred to the microbiology laboratory using a cold box. Then, 0.1 mL of milk samples was inoculated on Mannitol Salt Agar medium and the plates were incubated at 37 °C for 24 h. Then, golden-yellow colonies were purified for further analysis (Fitzgerald *et al*., 2001). To identify the isolates, the purified colonies were analyzed by gram staining and other biochemical tests including: catalase, oxidase, urease, nitrate reduction, gelatinase, glucose utilization, DNAse, coagulase and hemolysis tests (Preethirani *et al*., 2015).

### Molecular identification

DNA was extracted using the Yekta tajhiz nucleic acid extraction Kit following the manufacturer's instructions. The *Staphylococcus* specific *mecA*, *mecC* and PVL genes were amplified by monoplex Polymerase chain reaction (PCR). The PCR was performed in a 25 $\mu$L volume. The PCR mix contained 12.5 $\mu$L master mix (PCR buffer, $Mgcl_2$, dNTP, Taq), 1 $\mu$L of each primer (Table 1), 3 $\mu$L DNA and 7.5 $\mu$L distilled water. Amplification was carried out using the following conditions: 1 cycle of primary denaturation at 95 °C, 30 cycles of amplification at 95 °C for 30 s, 54, 55, and 53.5 °C for 20 s respectively for PVL, *mecC* and *mecA* genes, and 72 °C for 20 s, which was followed by 4 min of an additional extension at 72 °C. The PCR products were analyzed by agarose gel electrophoresis (1.5%) (E 705, Fanavaran Akhtareyan, Iran), ethidium bromide staining and UV trans illumination.

**Table 1.** Primers used for PCR (Pajić *et al*., 2014)

| Gene Target | Primer | Sequence (5' to 3') | Product size (bp) |
|---|---|---|---|
| PVL | F | GCTGGACAAAACTTCTTGGAATAT | 83bp |
| | R | GATAGGACACCAATAAATTCTGGATTG | |
| *mecC* | F | GAAAAAAAGGCTTAGAACGCCTC | 138bp |
| | R | GAAGATCTTTTCCGTTTTCAGC | |
| *mecA* | F | TCCAGATTACAACTTCACCAGG | 162bp |
| | R | CCACTTCATATCTTGTAACG | |

**Antibiotic susceptibility test**

For evaluation of antibiotic susceptibility of the isolates, disk diffusion method was used. In this regard, 12 commercially available antimicrobial sensitivity discs was tested on the isolates and the sensitivity/resistance was interpreted based on the zone of inhibition, following the guidelines of the Clinical Laboratory Standards Institute 2019 and analyzed using Whonet program (Król *et al*., 2016; Seyoum, Kefyalew, Abera, & Abdela, 2018).

**Statistical analysis**

SPSS Statistics Version 24.0 was used for statistical analysis. ANOVA and Duncan's test were used to analyze the data sorted by antibiotic resistance and the probability value ($P$<0.05) was considered as different significantly.

## Results and discussion

Out of 100 milk samples, 6 strains of *Staphylococcus aureus* were isolated at a frequency of 6% based on biochemical tests. Among them, 2 were coagulase positive and 4 were coagulase negative. Since presence of *mec* genes could be pointed as resistance of the organism to methicillin, the present study wanted to determine the relationship between the presence of *mec* and PVL resistance genes. As shown in Fig. (1), isolated bacteria (1, 2 and 3) contained the *mecA* gene (162 bp), isolates 4, 5 and 6 contained the *mecC* gene (138 bp) and isolate 4 was the only isolate containing the PVL gene. This isolate also showed resistance to cloxacillin, erythromycin as well as Trimethoprim/Sulfamethoxazole. Therefore, since isolate 4 also contains the *mecC* gene, maybe there is a correlation between the presence of these two genes and its resistance. The results obtained from the determination of the antimicrobial susceptibility pattern of the isolates are presented in Table (2). Among the strains, Number 1 was the most resistant and number 5 isolate was the most sensitive isolate. Furthermore, all isolates were resistant to cloxacillin antibiotics (Fig. 1) and the data was confirmed using whonet 2019 (Table 3).

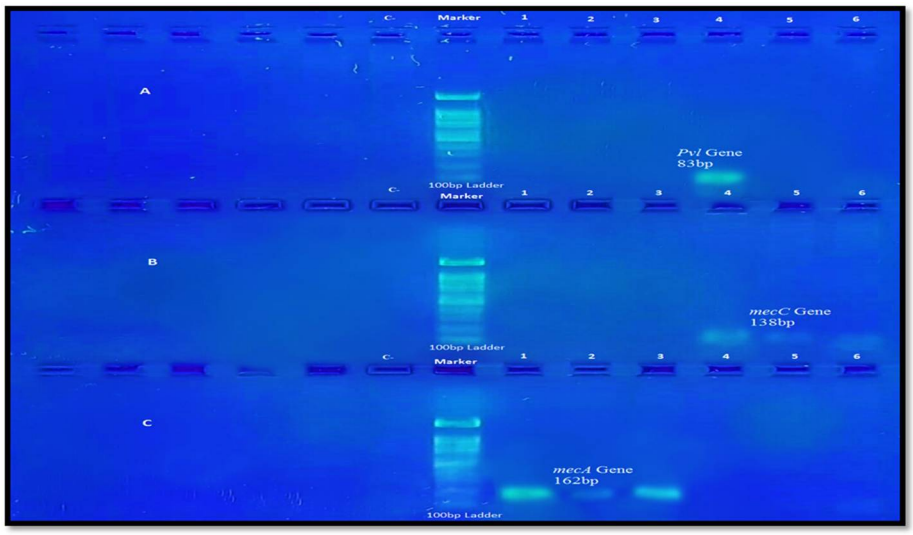


**Fig. 1.** PCR for genus specific *mecA*, *mecC* and PVL genes. Lane designation: M: 100bp ladder; 1-6 Staphylococcus isolates, C-: negative control

**Table 2.** Frequency of resistance and sensitivity of antibiotics (mm)

| Isolates | PEN | VAN | KAN | ERY | CHL | CLO | AMC | DAN | SXT | TCY | NEO | NOV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | S<br>40.00±1.00 | S<br>25.66±4.00 | S<br>21.66±5.77 | I<br>17.00±5.29 | S<br>34.33±5.13 | R<br>6.66±11.54 | R<br>16.00±27.71 | R<br>9.00±15.58 | R<br>00.00 | I<br>15.00±25.98 | R<br>7.33±12.7 | R<br>7.00±12.12 |
| 2 | S<br>38.00±2.64 | S<br>25.33±4.50 | S<br>22.33±7.00 | R<br>10.00±10 | S<br>33.33±3.51 | R<br>00.00 | S<br>40.33±3.51 | S<br>23.00±2.64 | R<br>00.00 | S<br>36.66±2.88 | S<br>22.00±1.73 | S<br>20.00 ±1.00 |
| 3 | S<br>32.66±2.51 | S<br>23.66±3.21 | S<br>20.66±5.85 | R<br>3.66±6.35 | S<br>31.66±1.52 | R<br>00.00 | S<br>39.00±5.29 | S<br>24.00±2.29 | R<br>00.00 | S<br>37.66±6.42 | S<br>20.00±2.64 | S<br>18.00±3.60 |
| 4 | S<br>45.00±1.00 | S<br>23.33±3.21 | S<br>24.00±3.6 | R<br>13.33±5.85 | S<br>44.66±2.51 | R<br>6.33±5.50 | S<br>44.33±4.00 | S<br>36.00±1.73 | R<br>00.00 | S<br>36.00±3.60 | S<br>25.33±0.57 | S<br>26.33±1.15 |
| 5 | S<br>42.00±2.00 | S<br>19.66±2.51 | S<br>21.33±7.00 | S<br>26.00±7.81 | S<br>29.33±2.00 | R<br>15.33±4.50 | S<br>42.66±2.30 | S<br>33.00±5.19 | S<br>27.66±6.42 | S<br>30.00±7.81 | S<br>19.00±2.00 | S<br>31.33±4.93 |
| 6 | R<br>23.33±2.88 | R<br>13.33±11.15 | S<br>21.00±4.00 | I<br>21.66±1.52 | S<br>22.33±6.42 | R<br>6.66±5.85 | S<br>29.66±0.57 | S<br>26.00±1.00 | S<br>25.00±1.00 | S<br>23.66±4.72 | S<br>18.66±1.15 | R<br>6.00±5.19 |

R: Resistant; S: Sensitive;
PEN: Penicillin, VAN: Vancomycin, KAN: Kanamycin, ERY: Erythromycin, CHL: Chloramphenicol, CLO: Cloxacillin, AMC: Amoxicillin_clavulanic acid, DAN: Danofloxacin, SXT: Trimethoprim sulfamethoxazole, TCY: Tetracyclines, NEO: Neomycin, and NOV: Novobiocin

**Table 3.** Analyzed antibiotic pattern using Whonet 2019

| Code | Antibiotic name | Antibiotic class | Breakpoint type | Site of infection | Host | Breakpoints | Number | %R | %I | %S |
|---|---|---|---|---|---|---|---|---|---|---|
| AMC_TD20 | Amoxicillin/Clavulanic acid | Beta-lactam+Inhibitors | Animal | Breast milk | Cattle | 14-17 | 6 | 0.0 | 16.7 | 83.3 |
| CHL_TD30 | Chloramphenicol | Phenicols | Animal | Breast milk | Cattle | 13-17 | 6 | 0.0 | 0.0 | 100.0 |
| CLO_ND5 | Cloxacillin | Penicillins | Animal | Breast milk | Cattle | 23-24 | 6 | 100.0 | 0.0 | 0.0 |
| DAN_TD5 | Danofloxacin | Quinolones | Animal | Breast milk | Cattle | 17-18 | 6 | 16.7 | 0.0 | 92.3 |
| ERY_TD15 | Erythromycin | Macrolides | Animal | Breast milk | Cattle | 14-22 | 6 | 50.0 | 33.3 | 16.7 |
| KAN_ND30 | Kanamycin | Aminoglycosides | Animal | Breast milk | Cattle | 14-17 | 6 | 0.0 | 0.0 | 100.0 |
| NEO_ND30 | Neomycin | Aminoglycosides | Animal | Breast milk | Cattle | 15-16 | 6 | 16.7 | 0.0 | 83.3 |
| NOV_ND5 | Novobiocin | Polypeptides | Animal | Breast milk | Cattle | 16-17 | 6 | 33.3 | 0.0 | 66.7 |
| PEN_TD10 | Penicillin G | Penicillins | Animal | Breast milk | Cattle | S>=29 | 6 | 16.7 | 0.0 | 83.3 |
| SXT_TD1.2 | Trimethoprim/Sulfamethoxazole | Folate pathway inhibitors | Animal | Breast milk | Cattle | 11-15 | 6 | 66.7 | 0.0 | 33.3 |
| TCY_TD30 | Tetracycline | Tetracyclines | Animal | Breast milk | Cattle | 12-14 | 6 | 0.0 | 0.0 | 100.0 |
| VAN_TD30 | Vancomycin | Glycopeptides | Animal | Breast milk | Cattle | 10-12 | 6 | 0.0 | 0.0 | 100.0 |

%I: Intermediate; S: Sensitive; %R: Resistant

**Table 4.** Statistical analysis of data using ANOVA test

| COUNT | | | | | |
|---|---|---|---|---|---|
| | Sum of Squares | df | Mean Square | F | Sig. |
| Between Groups | 6788.530 | 11 | 617.139 | 8.817 | 0.000 |
| Within Groups | 4199.824 | 60 | 69.997 | | |
| Total | 10988.355 | 71 | | | |

**Table 5.** Confirmation of data by Duncan test

| Duncan[a] | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Subset for alpha = 0.05 | | | | | |
| AB | N | 1 | 2 | 3 | 4 | 5 | 6 |
| CX | 6 | 5.8300 | | | | | |
| SXT | 6 | 8.7667 | 8.7667 | | | | |
| E | 6 | 15.2750 | 15.2750 | 15.2750 | | | |
| NB | 6 | | 18.1100 | 18.1100 | | | |
| N | 6 | | 18.7200 | 18.7200 | | | |
| V | 6 | | | 21.8283 | 21.8283 | | |
| K | 6 | | | 21.8300 | 21.8300 | | |
| DFX | 6 | | | 25.1667 | 25.1667 | 25.1667 | |
| TE | 6 | | | | 29.8300 | 29.8300 | 29.8300 |
| C | 6 | | | | | 32.6067 | 32.6067 |
| P | 6 | | | | | | 36.8317 |
| AMC | 6 | | | | | | 36.9967 |
| Sig. | | 0.069 | 0.063 | 0.077 | 0.136 | 0.151 | 0.182 |

In addition, the results of statistical analysis using ANOVA and Duncan tests on antibiotics indicated that, there is a significant difference between group and antibiotics ($P$<0.05) (Tables 4 and 5).

*Staphylococcus aureus* is the most prominent pathogen in humans with different virulence factors (DeVries *et al*., 2011; Ramezani *et al*., 2019). In addition, highly contagious strains of leukocidin-positive pantone valentine (PVL) and methicillin-resistant *Staphylococcus aureus* (PVL) have been recognized as a globally important health problem. Indeed, epidemiologic information of PVL has been suggesting that as an important virulence factor in *Staphylococcus aureus* necrosis infections (Berube & Bubeck Wardenburg, 2013).

Nowadays, bovine mastitis is documented as the most common and expensive disease affecting dairy industires. Indeed, the organism by attacking the mammary glands and multipllicaiton in the milk producing tissues, causes enormous financial losses. Therefore, researchers believe that the best form of treatment for bovine mastitis is the intra mammary injection of antibiotics (Mushtaq *et al*., 2018).

Türkyilmaz, Tekbiyik, Oryasin, & Bozdogan (2010) showed that among 93 *S. aureus* strains isolated from bovine milk with mastitis, 16 were resistant to methicillin (17.2.%) (Türkyilmaz *et al*., 2010), which is more than the recieved results from our study (6%). In addition they presented that the MRSA strains were multi-drug resistant, which is parallel to our results.

As de Freitas Guimarães *et al*. (2013) showed in their research, the occurrence of MRSA isolates among milk samples of cow with mastitis was relatively low (6.2%), they showed that they were not able to isolate coagulase positive *Staphylococcus aureus*. While, in this project we were able to isolate 2 coagulase positive and 4 coagulase negative.

On the other hand, Liu *et al*. (2017) worked on occurrence of *Staphylococcus aureus* strains isolated from raw milk in northern China, and then tried to describe antimicrobial susceptibility of the strains

and their key virulence genes. They showed that out of 195 samples, 54 (27.7%) were positive for *Staphylococcus aureus* and 16 strains were recognized as methicillin-resistant *Staphylococcus aureus*. In addition, their results illustrated that the strains showed high percentages of resistance to penicillin G (85.2%), ampicillin (79.6%), and erythromycin (46.3%) with presence of different gene patterns (Liu *et al.*, 2017). While, our results showed that all of the isolates, recognized as methicillin-resistant *Staphylococcus aureus* and they were presented one or two genes. Also Obaidat, Bani Salman, & Roess (2018) worked on the occurrence and antimicrobial resistance of *mecA* and *mecC* methicillin-resistant *Staphylococcus aureus* (MRSA) in cattle, sheep, and goat dairy farms in Jordan. They have collected their samples from 117 dairy farms. They showed that none of the tested bulk milk samples were positive for *mecC* while, 26% (95% CI 20-32%) were positive for *mecA* MRSA and most of the isolates were multi drug resistant. Our investigation demonstrated that 50% of the isolates at least had one of the gene: *mecA* or *mecC*, and most of the isolates were multi drug resistant which is near to the results gained by Obaidat *et al*. (2018).

Furthermore in Argentina, researchers tried to isolate *Staphylococcus aureus* isolates from 829 mastitis milk samples from 21 farms with isolation rate of 28.1%, and all isolates were negative for the *mecA*, *mecC* and PVL genes (Srednik *et al.*, 2018). While in our study, although number of the samples were lower, 50% of the isolates presented presence of *mecA* and *mecC* gene and one isolates showed presence of *mec*C as well as PVL gene.

Besides, Dekker *et al*. (2016) by collection of blood culture samples from febrile patients admitted in hospitals showed that out of 9834 samples 0.6% of the isolates were positive for *Staphylococcus aureus* and among the isolates 35.7% were uulti drug resistant (MDR). They showed that of all isolates 75% carried the PVL gene, and PVL gene was detected in all isolates. Furthermore, they showed that the frequency of genetically diverse and PVL-positive methicillin-sensitive *Staphylococcus aureus* (MSSA) was high and could represent a reservoir for the emergence of virulent PVL-positive MRSA clones.

## Conclusions

Mastitis is the inflammation of mammary gland and udder tissue, which is occurring in human as well as cattle. The disease is documented by physical, chemical, and bacteriological changes in the milk and pathological changes in the glandular tissues, which could be prevented by keeping udder hygiene, sanitation of the cow barn, cleaning the food and water supplement. In this study, the PVL-positive *Staphylococcus aureus* was detected from bovine milk with mastitis, although the PVL gene is transforming by the specific bacteriophages to the *Staphylococcus aureus*, the presence of such strains close to the consumer is of great public health concern. Hence, detection of MDR isolates from dairy products could treat animal as well as human public health. Our results showed different antibiotic resistance patteren for the isolates. Furthermore, all of the isolates were resistant to the cloaxicillin and the most resistant isolate was No 1. Which is presented *mecA* gene. In addition, only one of the isolates showed PVL gene (No. 4) which is sensitive to the all evaluated antibiotics except cloaxicillin. This isolate also showed resistance to cloxacillin, erythromycin as well as Trimethoprim/Sulfamethoxazole.

Therefore, since isolate 4 also contains the *mecC* gene, maybe there is a correlation between the presence of these two genes and its resistance. Hence, according to the results obtained from this study it could be concluded that there was no relation to the antibiotic resistant and presence of the Pvl gene with the presence of *mecA* and *mecC* genes. However, it could be concluded that monitoring of such isolates from dairy products and its environment is necessary. Also, specific and synergic effective antibiotics should be used for the treatment of mastitis to prevent an increasing resistance problem to antimicrobials all over the world.

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# مطالعه روی *استافیلوکوکوس اورئوس* جداشده از شیر گاو مبتلابه ماستیت حاوی ژن‌های متی‌سیلین و پنتون-والنتین لکوسیدین

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## چکیده

امروزه، ماستیت گاوی به‌عنوان التهاب غدد پستانی تشریح شده است که *استافیلوکوکوس اورئوس* یکی از مهم‌ترین پاتوژن‌های معمول و مسئول ماستیت مسری در بین نشخوارکنندگان می‌باشد. تحقیق حاضر به‌دنبال ارزیابی حضور سویه‌های مثبت ازنظر ژن مقاومت به متی‌سیلین و پنتون-والنتین لکوسیدین از شیر گاو آلوده و ارتباط بین حضور ژن‌ها و مقاومت آنتی‌بیوتیکی است. براین‌اساس 100 نمونه شیر از گاوهای آلوده به ماستیت از دامداری‌های اطراف فارس جمع‌آوری شد. نمونه‌ها به محیط مانیتول سالت آگار منتقل شد و در دمای 37 درجهٔ سانتی‌گراد برای 24 ساعت نگهداری شد. کلنی‌های خالص به کمک آزمون‌های بیوشیمیایی و تکنیک‌های مولکولی براساس ژن‌های *mecA*، *mecC* و PVL شناسایی شد. سپس الگوی آنتی‌بیوتیکی جدایه‌ها براساس CLSI 2019 و نرم‌افزار هونت ارزیابی شد و درنهایت آنالیز آماری گردید. در مجموع 6 ایزولهٔ *استافیلوکوکوس اورئوس* جدا گردید که به کمک آزمون‌های بیوشیمیایی و مولکولی تأیید گردید. جدایه‌ها الگوی مقاومت آنتی‌بیوتیکی متفاوتی را نشان دادند و اغلب جدایه به داروها مقاوم بودند، درحالی‌که سویهٔ شمارهٔ 5 حساس‌ترین جدایه شناسایی شد. نتایج بیانگر حضور ژن *mecA* در استرین‌های 1، 2 و 3 *mecC* در ایزوله‌های 4، 5 و 6 و PVL تنها در استرین 4 مشاهده گردید. براساس نتایج به‌دست‌آمده می‌توان نتیجه گرفت که گرچه فاژهای مختلف ژن PVL را به *استافیلوکوکوس اورئوس* منتقل می‌نمایند در تحقیق حاضر ارتباطی بین حضور ژن‌های *mecA*، *mecC* و PVL با الگوی آنتی‌بیوتیکی مشاهده نگردید.

**واژه‌های کلیدی**: *استافیلوکوکوس اورئوس*، ماستیت، *mecA*، *mecC*، PVL