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# Molecular Detection of Methicillin-Resistant *Staphylococcus aureus* Isolated from Subclinical Mastitic Cows in Basrah Province

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## Abstract

The study collected 200 milk samples from apparently normal cows in Basrah province from 21 November 2023 to 6 February 2024. 95 (48) % were positive for the CMT test. This study used various techniques to detect the presence of *Staphylococcus aureus*, including conventional microbiological assays (Mannitol Salts Agar and CHROMagar™ *Staph aureus*) and Molecular Methods (amplifying the *nuc* gene using polymerase chain reaction ). The percentage of *S. aureus* isolates was 38 ( 19 % ). From 38 (19 %) *S. aureus* isolates, 27 (71%) carried the *mecA* gene, and 6 (16%) carried *pvl.* However, none of the isolates had the *mecC* gene.

**keywords**: Mastitis, Subclinical mastitis, Cows, MRSA, CMT, *mec A.*

## Introduction

Bovine mastitis is among the most common diseases impacting dairy cows globally (1, 2). Visible abnormalities, such as redness, swollen udder, and fever, readily identify clinical bovine mastitis in dairy cows. The cow's milk appears watery with flakes and clots (3).

Contrary to clinical mastitis, sub-clinical mastitis has no apparent abnormalities in the udder or milk; it is associated with less milk production and an elevation in somatic cell count (SCC). (4).

*Staphylococcus aureus* is frequently associated with subclinical mastitis and causes significant financial losses due to decreased milk quantity and quality (5). *S. aureus* produces several virulence factors, such as enterotoxins and leukocidins, that impart immune system resistance and facilitate adaptability. These substances help the bacterium avoid the host immune system and form an intramural infection.

Therefore, *S. aureus*'s diversity of virulence factors significantly influences how animal infections develop (6, 7). Two significant public health issues are the discovery of bacteria resistant to antibiotics for bovine mastitis and the possibility that unpasteurized dairy products could spread to humans.

Another danger in the community is antibiotic residues in milk, and the abuse of antibiotics to treat mastitis can lead to resistance (8). Since β-Lactam antibiotics have long been misused on dairy farms to treat mastitis, the rise of resistant bacteria and veterinary medications in milk poses serious public health problems. (9).

As a result, MRSA linked to livestock poses a global risk to both humans and animals. A primary reason for methicillin resistance in staphylococci is the expression of the *mecA* gene or its homolog *mecC*. It contains various staphylococcal cassette chromosomes, known as SCCmec, which are based on a mobile genetic element (10). A trans-peptidase that is only 63% identical to PBP2a, encoded by *mecA*, is encoded by the novel genetic determinant known as *mecC*, defined as mutations in *mecA*.

MRSA isolates with *mecA* positive are seen in cattle and other animals and also spread from other Staphylococci or livestock-associated MRSA to human MRSA. (11). For instance, cytotoxin, known as Panton-Valentine Leukocidin (*PVL*), encoded by the *pvl* gene, a virulence feature, is implicated in leucocyte destruction and necrosis of the tissues. (12). This study aimed to identify subclinical mastitis in cows caused by Staphylococci. (in particular *S. aureus*). Moreover, to determine the virulence genes of MRSA among *S. aureus* isolates.

## Materials and Methods

### Samples collection

Out of 200 milk samples were collected from raw milk cows from different parts of Basrah province. The sample collection started on 21 November 2023 and ended on 6 February 2024. The raw milk samples were submitted to CMT.

### Microbiological techniques

**Isolation and identification of bacteria:** All samples were primarily submitted to the California mastitis test (CMT) and categorized by CMT scores (13). The positive CMT samples were transported immediately to the Central Research Unit in the Veterinary College using an icebox. Upon laboratory arrival, the milk sample was inoculated in Brain Heart Infusion broth (BHI) and incubated at 37°C overnight. The preincubated samples were subcultured on Mannitol Salt Agar (Himedia, India) and CHROMagar™ *Staph aureus*. (Chromogenic Media "pioneer/ France). The plates were incubated aerobically for 24 hours at 37°C. Gram's stain identified the suspected colonies on CHROMagar™ *Staph aureus*.

### Molecular techniques

**Genomic DNA extraction:** According to the manufacturer's recommendations, a DNA kit (Geneaid, USA) was used to extract the genomic DNA of probable *S. aureus* isolates.

**Detection of the *nuc* gene:** Table (1) shows the primer of the *nuc* gene. The

amplification conditions were implemented in (14).

**Molecular detection of MRSA:**Detecting the *mecA, mecC*, and *pvl* genes for the molecular confirmation of MRSA isolates. Table (1) Provided primer sequences. The PCR heat cycling protocol comprised based on (15).

**Table (1) Sequence of primer pair for amplifying *nuc, mecA, mecC,* and *pvl* genes.**

| Gene | Primer sequences (5 → 3) | Length | Product size | References |
|---|---|---|---|---|
| ***nuc*** | F: 5′- GCTTGCTATGATTGTGGTAGCC 3' | 22 | 423 bp | 14 |
| | R: 5'- TCTCTAGCAAGTCCCTTTTCCA 3' | 22 | | |
| ***mecA*** | F:5'-TCCAGATTACAACTTCACCAGG-3' | 22 | 162bp | |
| | R:5'-CCACTTCATATCTTGTAACG-3 | 20 | | |
| ***mecC*** | F:5'-GAAAAAAAGGCTTAGAACGCCTC-3' | 23 | 138 bp | |
| | R:5'-GAAGATCTTTTCCGTTTTCAGC-3' | 22 | | 15 |
| ***Pvl*** | F: 5' – GCTGG;ACAAAACTTCTTGGAATAT – 3 | 24 | 85bp | |
| | R:5'– GATAGGACACCAATAAATTCTGGATTG – 3' | 27 | | |

## Results

**Subclinical mastitis:** Ninety-five (48%) percent of the California Mastitis Test indicated positive for CMT. (Table 2) illustrated the percentage according to CMT results. Figure (1). Using conventional microbiological techniques, The rate at which suspected *S. aureus* isolates using Mannitol Salts Agar and CHROMagar™ *Staph aureus* was 90 (45% ) and 70 ( 35%), respectively, as shown in Table (3). All suspected isolates were subjected to PCR using the *nuc* gene Table (3), Figure (2), and Figure (3). The molecular assay results were 38 (19%) characterized as *S. aureus*. It was determined that the difference between these outcomes was statistically significant ($p<0.05$).

**Detection of MRSA isolates:** Methicillin-resistant *S. aureus* (MRSA) multiplex PCR virulence gene results for *mec A* and *pvl* genes revealed that 71% of the isolates were *mecA* and 16% were *pvl*, but none had *mecC*. Table (4), figure figure (4) A and B.

**Table (2): Results of the CMT test based on the degree of gel formation**

| Total number | Positive results (%) | Trace (%) | Weak (%) | Distinct (%) | Strong (%) |
|---|---|---|---|---|---|
| 200 | 95 (48%) | 6 (3%) | 39( 20%) | 35 (18%) | 15 ( 8%) |

**Chi-square: 126,632 degrees of freedom: 3, p-value: 0,001**


**Figure (1): Results of California Mastitis test based on the degree of gel formation**

**Table (3) Shows the number of *S. aureus* isolates identified by cultural and traditional microbiological techniques and molecular detection of the *nuc* gene**.

| Total number of milk samples | Number of suspected isolates of *Staphylococcus aureus* | | | | Confirmed isolates by Molecular detection of *nuc* gene | |
|---|---|---|---|---|---|---|
| | Mannitol Salts Agar | | CHROMagar™ *Staph aureus* | | | |
| No | No | ٪ | No | ٪ | NO | ٪ |
| 200 | 90 | 45 | 70 | 35 | 38 | 19 |

**Chi-square: 20,848 degrees of freedom: 2, p-value: 0,001**

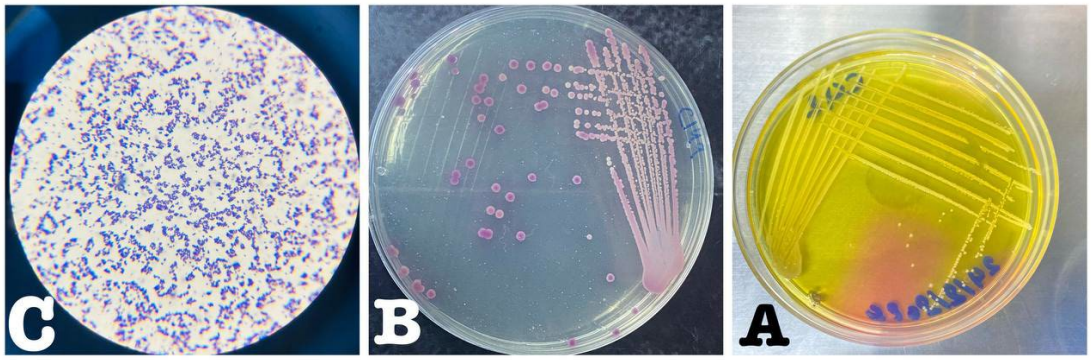


**Figure (2): A: *Staphylococcus aureus* appeared as golden yellow colonies on Mannitol Salts Agar, B: Chromogenic agar: colonies seemed mauve (pink), C: Gram-positive cocci.**

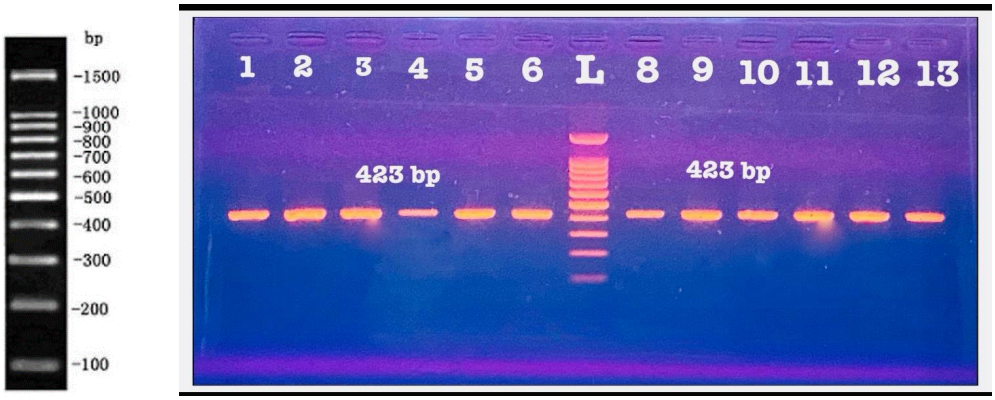


**Figure (3): Electropherogram of *nuc* gene amplification.** The PCR product was run on 1 % agarose gel, stained with ethidium bromide. L: mean Ladder and *nuc* gene product size approximately (423 bp).

**Table (4): Showed percentage of ( *mec A*, *mec C*, and *pvl* ) genes among *S. aureus* isolates.**

| No. of *S. aureus* | No. of *mec A* | | No. of *mec C* | | No. of *pvl* | |
|---|---|---|---|---|---|---|
| No | No | % | NO | ٪ | No | % |
| 38 | 27 | 71 | 0 | 0 | 6 | 16 |

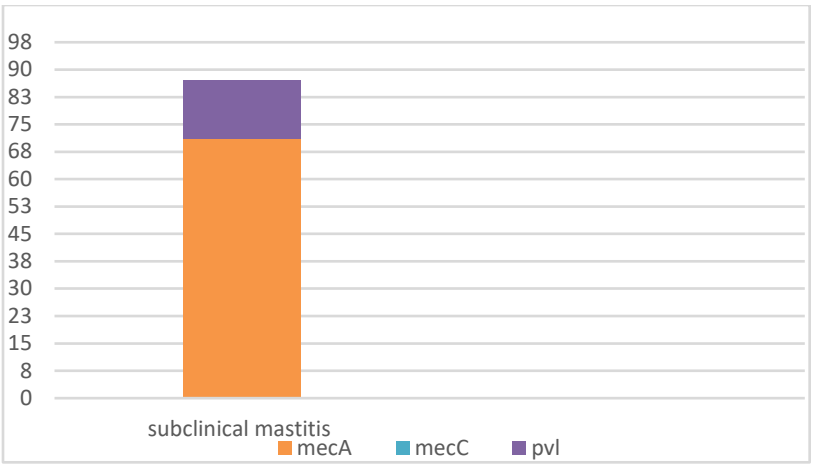


**Figure (4) A: Percentage of ( *mec A, mec C*, and *pvl* ) genes in *S. aureus* isolates**

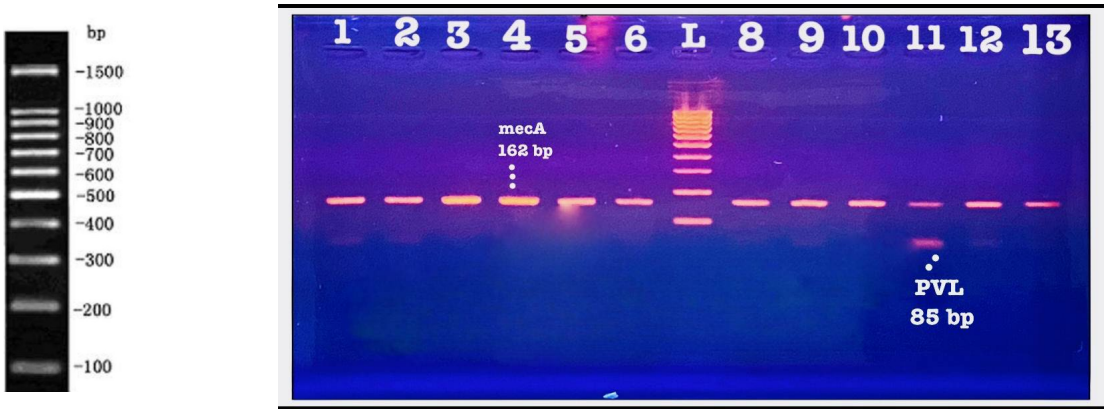


**Figure (4) B: Electropherogram of amplification of *mecA,mecC, and pvl* genes. The PCR products were run on a 1 % agarose gel stained with ethidium bromide. L: mean Ladder, *mecA* (162 bp, *pvl*: 85 bp ), whereas none of the isolates carried *mec C*.**

## Discussion

The dairy business suffers the most significant financial losses globally due to bovine mastitis, which also risks consumers' health (16). However, subclinical mastitis in dairy cattle is a significant and quiet issue that causes farmers to suffer more economic losses. In addition, it leads to low milk yield and quality, considered the first disease that causes significant loss to owners (17).

In this study, the California mastitis test results revealed that 46 % of the samples tested positive for CMT. This result agrees with (18)., who found that the frequency of subclinical mastitis was 42.5 %. In Iraq, studies like (19). found that 38.89% of the samples in Al Sulaimaniyah Province had

subclinical mastitis. In other studies, CMT varied from 38% to 56.6% compared to other research in Basrah (20, 21)**.** *Staphylococcus aureus* (*S. aureus*) is considered among the most essential udder pathogens, causing significant economic losses worldwide (22). The isolation rate of *Staphylococcus aureus* from SCM was 19 %. This incidence was consistent with a study conducted in Nepal that found that 15.2% of milk tested positive for CMT had *S. aureus* (23). However, our study's *S. aureus* prevalence is more than that of a study by (24)., in which 6 (2.8%) cows with SCM had *S. aureus*.

This incidence was consistent with a study conducted in Nepal that found that 15.2% of milk tested positive for CMT had *S. aureus* contamination (23).. The prevalence of S. aureus is higher than what was found in a study by (24)., where 6 (2.8%) cows with SCM had S. aureus contamination.

The difference in *S. aureus* prevalence between this study and previous research could be explained by variations in isolation technique, geographic areas, and sample characteristics (size, season, and type).

*mecA* is the most significant approach to MRSA isolation (25)**..** The lowest β-lactam potential altering protein (PBP2a) on SCCmec-resistant genomes is encoded by the *mecA*. (26)**..** PCR results indicated that 71% of MRSA isolates carried the *mecA* gene. This rate agrees with (27)**.,** who reported that the *mecA* percentage was 86.66%. Moreover, Hammadi and Yousif announced that 88% of *S. aureus* cases were MRSA (28).; in contrast, (29) observed that MRSA is found in just 10% of *S. aureus* infections. Meanwhile, the *mecC* gene was not found in any of the isolates in this study. This result agrees with (30, 31), who reported that none of the isolates carried the *mecC* gene. These findings disagree with (32) who reported that 3 (12.5%) were positive for *mecC*.One of *S. aureus's* main virulence factors is *PVL*. It makes it more harmful by hastening apoptosis and eliminating mononuclear and polymorphonuclear cells (33). The occurrence of the *pvl* gene in MRSA isolates has not been extensively studied. According to earlier research, the *pvl* gene's predominance ranges from 2% to 35%. (34, 35). Conversely, this study's findings for the *pvl* gene were 6 (16%), near the lower limit from other studies. Ultimately, this study showed that MRSA was common in cows with subclinical mastitis. This major public health issue should make veterinarians more knowledgeable about antibiotics.

## Conflicts of interest

The authors declare that there is no conflict of interest.

## Ethical Clearance

This work is approved by The Research Ethical Committee.

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# الكشف الجزيئي عن المكورات العنقودية الذهبية المقاومة للميثيسيلين المعزولة من الابقار المصابة بالتهاب الضرع تحت السريري في محافظة البصرة

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## الخلاصة

تم جمع (٢٠٠) عينة حليب من ابقار سليمة ظاهريا من اماكن مختلفة في محافظة البصرة للفترة من ٢١ تشرين الثاني ٢٠٢٣ لغاية ٦ شباط ٢٠٢٤. تم تشخيص التهاب الضرع تحت السريري (SCM) ٩٥( ٤٨%) من العينات التي تم فحصها باستخدام اختبار التهاب الضرع كاليفورينا ( CMT). تم استخدام تقنيات مختلفة للكشف عن وجود المكورات العنقودية الذهبية وهذه التقنيات شملت الاختبارات البكتيريولوجية التقليدية والتي تتضمن الزرع على اوساط تفرقية وانتخابية والتقنيات الجزيئية. تشير نتائج الاختبارات البكتيريولوجية التقليدية الى تحديد ٧٠(٣٥%) عزلة تم اعتبارها *S.aureus* بينما بالنسبة للطرق الجزيئية باستخدام البادئ الخاص بتفاعل البلمرة لجين (*nuc*) لتاكيد العزلات, وكانت نتيجة هذه التقنية تأكيد ٣٨ (١٩%) من العزلات على انها *S. aureus* تم تقيم المقاومة الوراثية للميثيسيلين . خضعت هذه العزلات لتفاعل البلمرة للكشف عن الجينات المقاومة للميثسيلين ( *mecA,mecC* ) و(*pvl*) . نتائج البلمرة اكدت أن ٢٧( ٧١% ) عزلة تمتلك الجين المقاوم للميثيسيلين ( *mecA* gene ) في حين أن النسبة الأقل كانت ٦( ١٦% ) تمتلك (*pvl* gene ).

**الكلمات المفتاحية :** التهاب الضرع , التهاب الضرع تحت السريري ,الابقار , المكورات العنقودية الذهبية المقاومة للميثيسيلين , اختبارا كاليفورنيا لالتهاب الضرع , جين البادئ *mecA*.