MOLECULAR DETECTION OF METHICILLIN-RESISTANT
Staphylococcus aureus
ISOLATED FROM MILK AND CHEESE OF COW AND BUFFALOES
IN BASRAH CITY

Weam Abd Ali Aboud and Bassam Yasein Khudaier*
Department of Microbiology, College of Veterinary Medicine, University of Basrah,
Basrah, Iraq.
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Corresponding: Bassamy10@yahoo.com

ABSTRACT
In the present study, 135 samples were collected from different animal's including: 75 samples were from milk and 60 samples were from cheese, 54 (40%) sample were found to be harbored with Staphylococcus aureus. The rate of S. aureus isolates was 50% in buffalo's cheese, 40.54% in buffalo's milk, 36.8% in cow's milk, and 33.33% in cow's cheese. 100% strains were Methicillin Resistance Staphylococcus aureus. The antibiotic sensitivity test was determined against 8 common antibiotics by the agar disc diffusion method on Muller-Hinton agar. These antibiotics were amoxicillin (25mcg), ampicillin (10mcg), Oxacillin (1mcg), chloramphenicol (30mcg), erythromycin (15mcg), gentamycin (10mcg), methicillin (5mcg), and tetracycline (30mcg). S. aureus strains were screened by PCR for 16S rRNA and nuc genes. 49 out of 54 S. aureus isolates were yielded products with molecular weight approximately (228 bp) corresponding to 16S rRNA gene, 42 out of 54 isolates were give products with molecular weight approximately (270bp) corresponding to nuc gene, 22 and 4 out of 30 S. aureus isolates were give products with molecular weight approximately (310bp and 509bp) corresponding to mecA and femA genes, respectively.

INTRODUCTION
The genus Staphylococcus includes over 30 species and subspecies, the most important species for the human and veterinary medicine is S. aureus which is among the most frequent causal organisms in human and animal's bacterial infections. S. aureus is one of the leading causes
of foodborne disease outbreaks due to its ability to produce staphylococcal enterotoxins [1,2].

Antibiotics Methicillin resistant *Staphylococcus aureus* (MRSA) is important because, in addition to being methicillin resistant, most strains are also resistant to other β-lactam antibiotic, with the exception of glycopeptide [3]. The genus *Staphylococcus* is Gram-positive bacteria, and particularly *S. aureus* is one of the most harmful species of staphylococci encountered. It is extremely a major important pathogens, and it’s one of the most common causes of nosocomial infections, especially pneumonia, surgical site infections and blood stream infections, it’s also the leading cause of bacteremia, myocarditis, acute endocarditis, pericarditis, osteomyelitis, encephalitis, meningitis, chorioamnionitis, mastitis in dairy animals, and scalded skin syndrome and continues to be a major cause of community-acquired infections [4].

More recently, Methicillin Resistance *Staphylococcus aureus* (MRSA) has been isolated from most animals and foods of animal origin. MRSA strains have been isolated from cows’ or small ruminants’ milk and various dairy products in many countries. The MRSA prevalence in milk and dairy products reported from different countries or even regions of the same country differs significantly [5,6,7,8,9]. Depending on growth conditions, the colony pigmentation varies from grey, grey white with yellowish to orange shades and a typical β-haemolysis on the blood agar [10]. The methicillin resistance mechanism is well understood in MRSA strains [11]. It is caused by the production of a novel penicillin-binding protein, PBP-2a, with a decreased binding affinity for β-lactams [12]. This process is encoded by the chromosomal gene *mecA* that is found in the mec region. The sequence of *mecA* gene is conserved in all methicillin-resistant strains of *S. aureus* [13]. The β-lactam antibiotics damage bacteria by inactivating penicillin-binding proteins that are essential in the assembly of the bacterial cell wall [14]. The aim of this study is to evaluation of the occurrence of *S. aureus* in milk, and cheese from cows and buffalos in Basrah city, antimicrobial-resistance pattern, and detection of the important genes 16S rRNA and *nuc* genes (*S. aureus* species specific determinants), and *mecA* and *femA* genes (methicillin resistance determinant).

**MATERIALS AND METHODS**

**Collection of Samples and Identification of *S. aureus***:

A total of 135 samples were collected; included 75 samples of raw milk: 38 cows’ milk samples and 37 buffalo’s milk samples, and 60 samples of cheese: 30 samples were from each of cows’ cheese and buffalo’s cheese). Samples were collected during the period from October 2017 to February 2018 from different area of Basrah City. All samples were
transported in an ice box to laboratory of microbiology. Milk samples were immediately cultured onto mannitol salt agar (MSA), and incubated at 37°C for 24-48 h. Mannitol fermenting colonies (i.e. those that were yellow or gold) were selected from the MSA and sub-cultured on Blood agar (BA). The colonies were subjected to Gram’s staining, catalase test, coagulase test, oxidase test, and DNase test. [15]. Cheese samples were processed depending on [16].

**Antimicrobial Susceptibility Testing**

Antimicrobial susceptibility test for isolated *S. aureus* were determined by the agar disk diffusion method [17].in Mueller-Hinton agar (MHA) plate. The turbidity of the actively growing broth culture was adjusted to the 0.5 McFarland standard [18].

Antimicrobial susceptibility performed for 8 common antibiotics, amoxicillin (25mcg), ampicillin (10 mcg), Oxacillin (1mcg), chloramphenicol, (30mcg), erythromycin (15mcg), gentamicin (10mcg), methecillin (5mcg), and tetracycline (30mcg). The results were recorded after 24h incubation at 35°C according to the guidelines of the National Committee of Clinical Laboratory Standards guidelines [18].

**DNA Extraction and PCR Assay**

DNA template was extracted using the presto Mini g DNA Bacteria Kit (Geneaid/korea). A PCR reaction with specific primers was performed to identify (16S rRNA), *nuc*, *mecA*, and *femA* in each isolate. A total PCR reaction mixture was 25µl contained: 12.5µl Green Go *Taq* Master Mix pH 8 (Promega, USA) contained [(50unit/ml) of Go *Taq* DNA polymerase, (400Mm) of each dNTPs and (3mM) of MgCl₂], 1 µl for each primers, 5µl DNA template and the mixture was complete to 25µl by adding 5.5µl deionized distilled water. The oligonucleotide primers that used for generated bands for the genes detection are illustrated in the table (1) and reaction to each PCR reaction was listed in table 2.
Table 1: The oligonucleotide primer sets used for the genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Product size (bp)</th>
<th>Sources</th>
</tr>
</thead>
</table>
| 16SrRNA | F:GTAGGGTGGCAAGCGTTATCC  
R:CGCACATCAGCGTCAG | (228) | [20] |
| Nuc | F:GCCTTGTGGTACGCGTT  
R:AGCCAGGCTTGACGAACTAAAGC | (270) | [20] |
| mecA | F:GTATAATGACTGAACGTCCGATGA  
R:CCAATCTACATGTTGATCTAA | (310) | [21] |
| femA | F:AGACAAATGAGGTAAATGAT  
R:AAATCTACACTGAGTGATA | (509) | [19] |

Table 2: Primer conditions sets used for the genes.

<table>
<thead>
<tr>
<th>Primer</th>
<th>The condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SrRNA</td>
<td>Initial denaturation 94°C for 2min, 35 cycle (94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec) and final extension 72°C for 4 min.</td>
</tr>
<tr>
<td>Nuc</td>
<td>Initial denaturation 95°C for 2min, 35 cycle (94°C for 1min, 50°C for 50sec, 72°C for 2min) and final extension 72°C for 5min.</td>
</tr>
<tr>
<td>mecA</td>
<td>Initial denaturation 95°C for 4min, 30 cycle (94°C for 30sec, 53°C for 45sec, 72°C for 4min) and final extension 72°C for 4 min.</td>
</tr>
<tr>
<td>femA</td>
<td>Initial denaturation 94°C for 5min, 30 cycle (94°C for 30sec, 45°C for 1 min, 72°C for 45sec) and final extension 72°C for 10 min.</td>
</tr>
</tbody>
</table>
RESULTS

Isolation and Identification

In the present study, 135 samples were tested (cows’ milk, buffalo’s milk, cows’ cheese and buffalo’s cheese). These samples included 75 milk’s samples and 60 cheese’s samples. *S. aureus* isolated from 54 samples (40%). The highest rate of *S. aureus* isolation was observed in buffalo’s cheese 15/30 (50%), followed by buffalo’s milk 15/37 (40.54%), then cows’ milk 14/38 (36.8%), and cows’ cheese 10/30 (33.33%). Table (3).

<table>
<thead>
<tr>
<th>Sample source of animal</th>
<th>No. of sample</th>
<th>No. of <em>S. aureus</em></th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows’ milk</td>
<td>38</td>
<td>14</td>
<td>(36.8)</td>
</tr>
<tr>
<td>Buffalo’s milk</td>
<td>37</td>
<td>15</td>
<td>(40.54)</td>
</tr>
<tr>
<td>Cows’ cheese</td>
<td>30</td>
<td>10</td>
<td>(33.33)</td>
</tr>
<tr>
<td>buffalo’s cheese</td>
<td>30</td>
<td>15</td>
<td>(50)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>135</strong></td>
<td><strong>54</strong></td>
<td></td>
</tr>
</tbody>
</table>

*p*> 0.05 no significant

Screening for Methicillin Resistance *S. aureus* (MRSA)

By using agar disc diffusion method, 30 randomly selected *S. aureus* isolates were subjected for susceptibility toward methicillin. Total of the 30 (100%) *S. aureus* strains were MRSA which were isolated in this study.

Antimicrobial Susceptibility

Table (4) provides antimicrobial susceptibility results by agar disc diffusion test. Interestingly, 30 *S. aureus* isolates exhibited the Multi-Drug Resistance (MDR) trait against 8 commonly used antibiotics; the isolates were completely resistant (100%) for amoxicillin, ampicillin, Oxacillin, and methicillin, and (92%) of them were sensitive to chloramphenicol and gentamycin and (8%) resistant, while all the tested isolates were completely sensitive to tetracycline (100%), and (72.%) sensitive for erythromycin and 26% resistant (2% intermediate) *p* > 0.05, Figure (1).
Figure 1: Disc diffusion method with inhibition zones for antibiotics against *S. aureus*.

Table 4: Antimicrobial Susceptibility pattern of MRSA isolated from milk and dairy products.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration (mcg)</th>
<th>Resistance (%)</th>
<th>Intermediate (%)</th>
<th>Sensitive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxillin</td>
<td>(25)</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>(10)</td>
<td>(100)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>(1)</td>
<td>(100)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>(30)</td>
<td>-</td>
<td>(8)</td>
<td>(92)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>(15)</td>
<td>(26)</td>
<td>(2)</td>
<td>(72)</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>(10)</td>
<td>(8)</td>
<td>-</td>
<td>(92)</td>
</tr>
<tr>
<td>Methicillin</td>
<td>(5)</td>
<td>(100)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>(30)</td>
<td>-</td>
<td>-</td>
<td>(100)</td>
</tr>
</tbody>
</table>
Detection of the 16S rRNA, nuc, mecA and femA Genes by PCR Assay

Results of PCR assay revealed that 49 (90.7%) isolates from total 54 isolated S. aureus were yielded products with molecular weight approximately (228 bp) corresponding to 16S rRNA gene, as showed in Figure (2), 42 isolates of them (54 isolates) were gave products with molecular weight approximately (270bp) corresponding to nuc gene, Figure (3).

While 22 out of 30 S. aureus isolates were gave products with molecular weight approximately (310 bp) corresponding to mecA gene, Figure (4), and only 4 out of 30 S. aureus isolates were yielded products with molecular weight approximately (509 bp) corresponding to femA gene p> 0.05, Figure (5).

Figure 2: Agarose gel electrophoresis of PCR-amplified for 16S rRNA gene and nuc gene of S. aureus isolates. LineM: DNA marker. Lines. 1-3: nuc gene 270bp and 1-4 16s rRNA gene 228bp and line C: Negative control.

Figure 5: Agarose gel electrophoresis of PCR-amplified femA gene of *S. aureus* isolates. Line. M: DNA marker. Lines. 1-5: femA gene 509bp line C: Negative control.
DISCUSSION

The results of bacterial isolation and identification from milk and cheese of cows and buffaloes revealed that 54 out of 135 (40%) samples were implicated with *S. aureus*. Among 135 samples, 75 milk's samples showed (40.54%) buffalo milk and (36.8%) cow milk, and 60 cheese samples showed (50%) buffalo cheese and (33.33%) cow cheese. The percentage of *S. aureus* infection in the present study agreed with the study conducted in Iraq [5]. The present study results are in line with study of [22]. who reported that the percentage of *S. aureus* was (48.57%) from bovine’s milk. But it's not in agreement with the results that recorded by [6, 23].which were (10.23%) and (21.25%), respectively. Several studies have demonstrated that pathogenic bacteria like *S. aureus* may responsible for vomiting, abdominal cramps and diarrhea like diseases in humans [24,25,26,27,28]

Thirty *S. aureus* isolates were subjected towards different antibiotics. The percentage of susceptibility toward methicillin and oxacillin was (100%). The present study is along with the study of Nusrat [23]. who found that coagulase positive *S. aureus* was found to be highly resistant against oxacillin (100%). In addition to that occurrence of methicillin resistant *S. aureus* in food samples has been a major concern worldwide [29]. In developing countries more than 70% of infecting bacteria have been accounted as multi drug resistant strain (MDR) [30,31]. noticed the least effective drugs were tetracycline, and amoxicillin with bacterial resistance percentages of 65.2%, and 55.6%, respectively.

*S. aureus* isolates were analyzed by PCR assay. DNA from *S. aureus* isolates of milk and cheese were extracted by purification kit to detect the presence of 16S rRNA, *nuc*, *mecA*, and *femA* genes. In the present study, 90.7% *S. aureus* isolates that identified were confirmed by amplification of the 16S rRNA gene [32] confirmed that 100% of *S. aureus* isolates from cow and buffaloes were produced 16S rRNA gene. The present study agreed with the study of Al-Ashmawy [31] found that all recovered *S. aureus* isolates were genetically verified as MRSA strains by molecular detection of the *mecA* gene.
CONCLUSION

The high percentage of methicillin resistant *S. aureus* isolates which also resistant to the most of the antibiotics used, which may be milk and milk products the main source of transmission to humans and these are a high risk to human health.
REFERENCES


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