Molecular and phylogenetic analysis of methicillin resistant *Staphylococcus aureus* isolated from subclinical mastitis in lactating ewes

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**Abstract**

This study was conducted for estimation of subclinical mastitis prevalence caused by *Staphylococcus aureus* (*S. aureus*) in apparent normal lactating ewes in the areas around Baghdad (Al-Rasheed, Al-Yousifya, Al-Latifya). 400 milk samples from 200 ewes were collected and examined by California mastitis test (CMT), Bacteriological examination was done by culturing on Blood agar, Mannitol salt agar, Staph-110 agar and Chrome agar then bacteria examined by Gram stain and biochemical test. Antibiotic sensitivity test and molecular assay by PCR were done to detect methicillin- resistant strains with the phylogenetic study. The results showed that 49/400 samples gave positive to CMT in the field and positive to *S. aureus* isolation. The percentage of subclinical mastitis in ewes was 12.25% in this study. All isolated bacteria were resistant to methicillin 100%. The result of PCR revealed that 16.32 % (8/49) of the MRSA were possess mecA gene, while no isolate carried the mecC gene. PCR-product for 8 positive samples of mecA target gene were sequenced, analyzed, and reported in Genbank-NCBI and obtained accession number and became a reference to Iraq and the Middle East and the world. Seven samples that match the global results by 100%, and one sample contains a mutation at the site 1539, Nucleotide. Our conclusion revealed that detection of subclinical mastitis in sheep very important caused by *S. aureus* and all *S. aureus* was resistant to methicillin and some strains possess mecA gene. However, absence of mec A in other MRSA isolates requires detecting the alternative genetic possibilities related to the resistance profile and fragment of gene used.

**Introduction**

Mastitis defined as a mammary gland infection characterized by physical, chemical, and usually bacteriological changes in milk and glandular tissue abnormalities (1). It is an important disease economically and its subclinical mastitis is difficult in the diagnosis, which makes it mitigation more challenging (2,3). *Staphylococcus aureus* is Gram-positive, non-spore-forming, facultative anaerobes that grow by aerobic or by fermentation, non-motile, arranged as irregular “grape-like clusters cocci, pairs or tetrads. It is characterized by production of golden pigment. Typical biochemical identification test includes positive catalase to differentiate from Streptococci and Enterococci. Coagulate test is a guide to differentiate between *Staphylococcus aureus* from coagulase-negative *Staphylococcus* spp and ferment Mannitol (4). There are numerous studies in Iraq on mastitis in sheep, a survey study by (5) revealed the prevalence of *Staphylococcus* subclinical ovine mastitis at a percentage 26.9%. Hassan and Yousif (6) found the 12.22% of subclinical mastitis in ewes in Al Anbar Province. Many articles showed that *S. aureus* is one of the most important bacteria causing mastitis in dairy animals including sheep (7,8). California mastitis test used as a quick field assay to determine subclinical mastitis of ewes and other animals, its
reagent lyses the membrane of somatic cells including WBCs in the milk and precipitate the protein and DNA as a gel, the amount of gel formation reflects the numbers of WBCs in the milk sample (9). Resistance of S. aureus to beta-lactam antibiotics was firstly seen in penicillin by the production of penicillinase which hydrolyzed the beta-lactam ring in penicillin. However, methicillin was introduced to substitute penicillin resistance, therefore, methicillin-resistant S. aureus (MRSA) strains developed and this has been mediated by the mecA gene (10).

There are several techniques were developed for the detection of MRSA including phenotypic (disk diffusion test) (11) and genotypic assay, such as mecA and mecC genes detection by Polymerase chain reaction (PCR), PCR is a reliable, accurate and confirmatory technique for the identification of pathogens especially S. aureus recovered from mastitic milk samples from animals and the genes mecA and mecC was the code for methicillin-resistant Staphylococcus aureus (12,13). Phylogenetic analysis was used to determine the evolutionary history of the species, the genes or proteins. Understanding the phylogenetic relationships between the organisms is a prerequisite of almost any evolutionary studies, as contemporary spp. all share a history through their ancestry. Moreover, Phylogenetic analysis is important because of its wide range of applications that includes understanding genomic organization, epidemiological studies, protein functions, and deciding genes to be analyzed in comparative studies (14, 15).

This study aimed to isolate MRSA from Subclinical mastitis in lactating ewes and molecular study of these isolates with recorded phylogenetic properties of mecA gene of the isolates to compare it with strains in the world.

Materials and methods

Animals and samples collection

This study was conducted on 200 lactating ewes located around Baghdad (Al-Rasheed, Al-Yousifya, Al-Latifya) during a period between December, 2019 to December, 2020.

The Ewes were examined clinically, the udders were washed with clean water then dried by clean tool, later the teat disinfectant with 70% ethanol, the foremilk was discarded and then the milk samples (400) were collected from each half separately in Labeled sterile test tubes under aseptic condition. Then California mastitis test was done on all collected samples at the field (4).

The samples were transported immediately to the laboratory of the College of Veterinary Medicine/Department of Internal and Preventive Medicine in Baghdad by ice box. Milk samples were examined physically to record the changes in color, odor and consistency (4).

Bacteriological examination

All bacterial examination was done according to (4). 400 milk samples were cultured on blood agar and Mannitol salt agar, then incubated at 37ºC / 24 hours. Identification of S. aureus was done according to colony morphology and Gram stain finally suspected colony sub-cultured on different selective media, Staph-110 agar and Chrome agar (Biomedia company). Then Biochemical tests were done (Catalase test, Oxidase test, Urease test, Coagulase test, Gelatin medium and O/F glucose test) were done to confirm the diagnosis of S. aureus.

Antibiotic sensitivity test

Isolated S. aureus isolates were examined for detection of sensitivity to methicillin by using disc diffusion test (4), the antibiotic inhibition zone estimated as mention by the Clinical and Laboratory Standards Institute (11).

DNA Extraction

The isolation of DNA genomic from bacterial growth done according to ABI pure protocol extraction. The purity and concentration of the final template DNA after extraction were measured by Quantus Fluoro meter.

Primers

Two primers were used mecA and mecC were used in this study (Table 1). These primers were supplied by (Macrogen Company) in a lyophilized form. Dissolved in a nuclease-free water, to give a final concentration of (100 pmol/μl) as a stock. The working solution of primers was prepared by adding 10μl of primer stock solution which (stored at freezer -20º C) to 90μl of nuclease-free water to get a working primer solution of 10pmol/μl.

Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Size</th>
</tr>
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<tbody>
<tr>
<td>mecA</td>
<td>F AAAAGATAAATCTTGGGGGTG&lt;br&gt;R CCTTGTTCATYTTGAGTTC</td>
<td>525</td>
</tr>
<tr>
<td>mecC</td>
<td>F CAGCCAGATTCATTGTGACC&lt;br&gt;R AACATCGTCAGATGGGGTAC</td>
<td>486</td>
</tr>
</tbody>
</table>

PCR Program

Steps of PCR cycle/s were recorded in tables 2 for different primers (mecA, mecC). Only one difference in the annealing time at 55ºC for primer mecC. After PCR amplification -Agarose gel electrophoresis was adopted to know the presence of amplification, PCR was dependent on the extracted DNA criteria completely.

Standard Sequencing

PCR products were sent for (Sanger sequencing) using ABI3730XL, the automated DNA sequences, by (Macrogen Corporation)/ Korea. The results were received by email then, analyzed using geneious software 9.
Table 2: Steps of PCR cycles for the two primers

<table>
<thead>
<tr>
<th>Steps</th>
<th>ºC / Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 / 5 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 / 30 second</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>55/50 second</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72 / 30 second</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72 / 7 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Hold</td>
<td>10 minutes</td>
<td></td>
</tr>
</tbody>
</table>

DNA loading
PCR products were loaded. 10μl was loaded in well. Ethidium bromide has stained the bands in gel were visualized using camera.

Results
Bacteriological examination
The cultural results showed different morphological shapes and color of s. spp. on different media, on blood agar the colonies appeared round and opaque colonies with β-hemolysis. On Staph 110, the colonies were convex, round, mucoid and white in color, while on mannitol salt agar appeared mucoid, round, convex and change the color of media to yellow. On the selective Chrome agar S. aureus produce green colonies. On microscopic examination the bacteria appeared as Gram-positive cocci, irregular clusters either pairs or tetra, these bacteria were non spore-forming and non-motile.

Percentage of subclinical mastitis
A total of 400 milk samples were examined for detection of a percentage of subclinical mastitis by CMT and bacteriology, the results showed that 49 samples were positive for both S. aureus isolation and CMT. The distribution of subclinical mastitis on ewes and halves basis were 14.5% and 12.25% respectively (Table 3). In addition, all isolates of S. aureus (49 samples) were resistant to Methicillin antibiotic.

DNA extraction
The 49 isolates of S. aureus were successfully extracted to obtain genomic DNA and this DNA which loaded on the agarose 1.2%, produced sharp, clear and pure bands (Figure 1).

Confirmatory detection of MRSA by PCR
Results of amplification on the extracted DNA by PCR by using two types of primers mecA, and mecC revealed that the 8 out of 49 MRSA gave positive results to mecA (16.32%) (Figure 2) while mecC gene not detected in any isolates of S. aureus (Figure 3).
Sequencing

Eight samples positive to mecA were taken for gene analysis, and the result was seven samples that match the global results by 100%, and one sample contains a mutation at the site 1539, Nucleotide A/G, Nucleotide change ATA/GTA, Amino acid change Isoleucine/ Valine and Predicted Effect Missense; the second at the site 1626, nucleotide A/G, nucleotide change AAA/AAG, Amino acid change Lysine/ Lysine and Predicted Effect Silent. The evolutionary tree was drawn using mega and NCBI program, and it appeared when compared with the world that there were seven samples that matched the results of the mentioned countries by 100%, while the similarity of one Iraqi isolate with Russia- Kazan was 99% (Figure 4).

Discussion

Mastitis is one of the important diseases of sheep which reduces the productivity of dairy animals. The dominant form of mastitis was Subclinical in sheep, which resulted in decreased milk yield, which lead to growth retardation and a higher mortality rate among suckling kid and lambs (2,16). In this study, the prevalence of Subclinical mastitis was 12.25%, according to CMT and bacterial isolation. Similar results were recorded by (6) who found a percentage 12% of SCM in ewes. In contrast, our finding was lower than those reported by (17) who found the prevalence of subclinical mastitis in ewes 51.1%. Gebrewahid and colleagues (17) reported a high prevalence of SCM reached 28.14% in sheep by using CMT positive milk samples and bacterial isolation. *Staphylococcus aureus* isolates were found in 49 out of 400 (12.25%). A Similar isolation rate was also recorded 12.22% previously (6). Cortimiglia and coworkers (18) found the most prevalent species was *Staphylococcus aureus* 20.14% in sheep milk and isolated *S. aureus* prevalence 43% in bulk tank milk in sheep, while (5) found that the isolated *Staph. aureus* bacteria a higher percentage 26% of SCM in sheep.

Our conclusion this difference in the prevalence of SCM in ewes may be due to many factors such as management especially in Iraq they used an open system of living for sheep, also environmental condition which was not for long time of year and ewes were not highly producing milk. In this study, all isolated *S. aureus* strains were conducted to methicillin antibiotic sensitivity test, and for detection of mecA and mecC genes using PCR assay which are considered a genetic marker used for rapid and direct confirmation of MRSA. The genotypic analysis of 49 *S. aureus* isolated from ewes milk, showed that 8 (16.32%) had mecA genes. These results in agreement with (19) who found that not all *Staph. aureus* were carry mecA. Also this finding is in compatible with data recorded by (20) in Turkey who isolate 93 *Staph. aureus*, 16 isolate (17.2%) were MRSA. All 49 isolates of MRSA showed negative results to mecC. These results different from the study by (21) they represents the detection of mecC-positive in MRSA isolation and would confirm that sheep might was a mecC MRSA reservoir, they thought mecC-positive MRSA is difficult to confirm by routine diagnostic methods which employed for mecA-positive MRSA, so diagnosis laboratories should be aware of searching for the mecC gene. Worthwhile the mecA and mecC negative MRSA in the current study may contribute to the presence of other β lactam resistances such as found of blaZ gene slao may depend on the fragment of sequencing. Our suggestion for control of SCM due to MRSA, must be screened lactating ewes by chemical test for detection of SCM and must treat these animals on the basis of antibiotic sensitivity tests to reduce resistance to the antibiotics. Furthermore, other studies on the *Staphylococcus spp.* explore resistant genes to antibiotics in addition to the mecA gene due to its importance in public health.

Our results of the phylogenetic analysis showed that all *Staph aureus* were compatible 100% with other rescuers, strains in japan (22) with ID: P024511.1 found methicillin resistant *S. aureus* isolates were isolated from dogs, environment, dog patients and humans. In Hungary, MRSA...
with ID: CP032468.1 revealed that Hungarian Staph. aureus isolates 60% were recovered previously from wounds, blood, tracheas, ears, lungs, nostrils, skins and throats while in German S. aureus isolates 40% were recovered from body sites (23). Compatibility showed 99% with strains of S. aureus with ID: MW682923.1 isolated in Russia: Kazan by (24) they isolated Staphylococcus aureus from the patients with chronic infections of the upper respiratory tract and the middle ear.

Conclusion

The finding of these results demonstrate the importance of S. aureus in the causes of sheep subclinical mastitis. All isolated Staph. aureus was resistant to methicillin antibiotic but only 8 strains possess meca gene. However, the detecting of meca in some isolates and the absence in other MRSA isolates requires detecting the alternative genetic possibilities related to the resistance profile and fragment of gene used.

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Conflict of interest

Authors declares, no conflict of interest

References

دراسة جزيئية وتحليل التطورات الجينية للمكورات العنقودية الذهبية المقاومة للميثاسيلين المعزولة من التهاب الضرع تحت السريري في النعاج الحليب

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