MOLECULAR DETECTION AND GENETIC CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE MASTITIC MILK IN MISAN PROVINCE, IRAQ

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Abstract

During the period between March and May 2018, a total of 20 isolates of *Staphylococcus aureus* were detected during processing of 80 bovine milk samples in Misan province, South of Iraq. The bacterial isolation rate of *S. aureus* was 20 (25%) out of 80 positive CMT samples. The amplification of the clumping factor (*clfA*) gene resulted in a single amplicon with a size of approximately 1000 bp in all 20 isolates of current study. The sequence analysis based on (*clfA* gene) showed high identity (99%) between local *S. aureus* isolates (No.1 & No. 2) and NCBI-BLAST *S. aureus* strain BPH2986. The Phylogenetic tree analysis revealed that the isolates of present study with BPH2986 (LR130518.1) were grouped together in the same cluster. This study characterized a high number of isolates (obtained from clinical mastitis cases) and associated isolates showed a higher genetic similarity based on (*clfA*) gene. The higher frequency of clinical mastitis cases reported in this study reflect the incorrect management in the local bovine farms of Misan province. Therefore, it is imperative to strengthen farmers’ awareness for using a hygienic procedures in all dairy farms.

**Key words :** Bovine Mastitis, *Staphylococcus aureus*, *clfA* gene, phylogenetic analysis.

Introduction

Mastitis is the inflammation of the mammary gland mainly due to a bacterial infection and it is characterized by a variety of local and systemic symptoms (Vasudevan et al., 2003; Fueyo et al., 2005; Smith et al., 2005; Kozytska et al., 2010). Globally mastitis is the most common infectious disease affecting the dairy cows and remains the most economically important disease of dairy industries around the world (Abd Ellah, 2013). Mastitis is a multi factorial disease and very difficult to control. It results from injury, chemical irritation and infection caused by different bacterial species (Baloch et al., 2011). Mastitis is characterized by physical, chemical and bacteriological change in the milk and pathological changes in the glandular tissue of the udder and affects the quality and quantity of milk (Sharma et al., 2011).

*Staphylococcus aureus* is one of the most important pathogens in humans and animals (Haghkhah, 2003). It is among the most common etiologic agents of bovine mastitis and probably represents the most lethal agent because it causes chronic and deep infection in the mammary glands that is extremely difficult to be cured (Barkema et al., 2009; Miles et al., 1992). It causes big financial economic losses to the dairy industry worldwide, mainly due to reduced milk production and the need to discard contaminated milk (Seegers et al., 2003). The virulence of *S. aureus* is generally considered to be multifactorial and due to the combined action of several virulence determinants (Bien et al., 2011). *Staphylococcus aureus* is an important food borne pathogen involved in a variety of invasive diseases (Morandi et al., 2009).

Clumping factor is an important adhesion protein of *S. aureus* that is governed by *clfA* gene and is thought to be essential for colonization and establishment of infections (Stephan et al., 2001). It participates in the infection process by facilitating bacterial binding via soluble or immobilized fibrinogen as fibrinogen plays a significant
role in platelet thrombus formation (Karahan et al., 2011). Clumping factor has been reported to be present in the majority of isolates and the gene clfA and has been reported to be important in pathogenicity of bovine mastitis (Salasia et al., 2004; Stephanet et al., 2001). The antibodies to clfA enhanced the protection against infection provided by capsular polysaccharides antibodies (Tuchscherr et al., 2008). Therefore, genotyping of isolates is necessary to identify the genetic relatedness of strains and their source of spread and one of the reliable and broad genotyping methodologies is repetitive element sequence based PCR or REP-PCR (Del Vecchio et al., 1995). This study was aimed to detect the genetic characterization of S. aureus isolated from mastitic cows in Misan province on the basis of clfA gene, which has been used firstly for detection of S. aureus in mastitic milk in Iraq.

Materials and Methods

Samples collection

A total of 80 bovine milk samples were collected from 4 local dairy farms (20, 20, 25, 15 samples from A, B, C and D farm, respectively) during the period between March and May 2018, in Misan province, South of Iraq, as it’s shown in table 3. Milk samples were collected after cleaning the udder from the grimes, bole and dirt by water and drying by a piece of clean cloth then used cotton moistened by alcohol 70% and removing the first flowage of milk, then California Mastitis Test (CMT) was carried out according (Markey et al., 2013).

Isolation of S. aureus

Positive CMT milk samples were directly inoculated onto manitol salt agar (MSA) and incubated at 37 °C for 24 hrs (Quinn et al., 1994), then Mannitol fermented colony from primary cultures were purified by subculture onto MSA medium and incubated at 37°C for 24-48 h. Gram stain slides were investigated according to Barrow and Feltham (2003). And biochemical tests that included catalase and coagulase tube were performed according to Macfaddin (2000).

Bacterial genomic DNA extraction

Bacterial genomic DNA was extracted from Staphylococcus aureus isolated by using (Presto™ Mini DNA Bacteria Kit, Geneaid, USA) and according to method described by Sreevatsan. The extraction method was done depend on the manufacturing instructions by using gram positive bacteria DNA Protocol extraction method by using “20 mg/ml” lysozyme buffer. One ml of overnight bacterial growth on BHI broth were placed in 1.5ml micro centrifuge tubes and then transferred in centrifuge at 10000rpm for 1 minute. After that, the supernatant was discarded and the bacterial cells pellets were used in genomic DNA extraction and the extraction was done according to company instruction. Then, the extracted DNA was checked by Nano drop spectrophotometer and store in -20°C at freezer until perform PCR assay.

Polymerase chain reaction (PCR)

PCR assay was performed for detection of virulence factor clumping factor A (clfA gene) in S. aureus isolates according to method described by Monistero et al. (2018) by using specific clfA forward primers (5’-GGG TCTAGTTGTGTTAGG-3’) and clfA reverse primer (5’- TTTTCAGG GTCAATATAAGC-3’) (Stephan et al., 2001) at PCR product size approximately 1000bp (table 1). These primers were provided by Macrogen Company, Korea. Then PCR master mix was prepared by using (Maxime PCR PreMix kit. iNtRON. Korea). The PCR premix tube contains freeze-dried pellet of Taq DNA polymerase, dNTPs, Tris-HCl (pH 9.0), KCl, MgCl, and tracking dye and the PCR master mix reaction was prepared according to kit instructions in (Bioneer, Korea) (table 2). The reaction was performed in a thermocycler (Mygene Bioneer.Korea) by set up the following thermocycler conditions (table 3). The PCR products were examined by electrophoresis in a 1% agarose gel, stained with ethidium bromide and visualized under UV transilluminator.

DNA sequencing method

DNA sequencing method was performed for study the genetic relationship analysis between local S. aureus isolates (clfA gene) partial sequence and NCBI-Genbank submitted S. aureus isolated (clfA gene) by using Phylogenetic tree analysis NCBI-BLAST Homology sequence identity. The PCR product samples were sent to Macrogen Company in Korea to perform the DNA sequencing by AB DNA sequencing system. The phylogenetic analysis was performed based on NCBI- Blast Alignment identification and Maximum Composite Likelihood method by phylogenetic tree UPGMA method (MEGA 7.0 version). Two partial sequences of clfA gene reported were submitted to genbank under accession numbers (MK001021 and MK001022).

Results and Discussion

The results of current study showed that all mastitis cases (80), which have been clinically diagnosed appeared positive to CMT. Based on bacterial isolation and biochemical tests the isolation rate of S. aureus was 20 (25%) out of 80 positive CMT samples. The highest
isolation frequency 30% (6/20) and 28% (7/25), were reported in B and C farms, respectively. While the lowest rates of isolation 20% (4/20) and 20% (3/15) were reported in A and D farms, respectively (table 2). These results seem to be similar to the results of earlier studies of Al-Iedani (2016), who noted that, among 143 milk samples, S. aureus were detected in 36 (25.17%) samples and also similar ratios were reported by Dehkordi et al. (2015), who found the rate of S. aureus isolation in dairy products (24.8%) whereas, these result was higher than that was earlier reported (20.59%) in cows suffering from acute mastitis in Baghdad government-Abu-Ghraib zone (Al-Ani, 2009). The difference between our result and result of others may attributed to some factors such as breed difference, different hygiene and management practices followed in each farm, age and parity of the animal and type of the milking ( Hammadi and Yousif, 2013). Or due to variation in geographical areas and climatic condition, according to the differences in temperature, humidity, environment and nature of society (International Mastitis Conference, 1995).

Clumping factor A (clfA) is necessary in pathogenicity of bovine mastitis, and it is important in colonization because it is a cell surface associated protein of S. aureus and this promotes binding of this pathogen to both soluble and immobilized fibrinogen (Stephan et al., 2001). The amplification of the clumping factor (clfA) gene resulted in a single amplicon with a size of approximately 1000 bp in all 20 isolates of current study (fig. 1), indicating no size polymorphisms of this gene. This result is identical with Momtaz et al. (2011), who recorded amplicons of same sizes with no polymorphism. Likewise, Akineden et al. (2001) and Salasia et al. (2004) reported amplification of the clumping factor gene clfA resulting in a single amplicon with a size of approximately 1000 bp from S. aureus. But contrary to the result of the previous study, polymorphism was reported 84 (91.3%) out of 92 S. aureus isolates showed presence of clfA with two different amplicons at the molecular length of approximately 900 bp and 1000 bp (Karahan et al., 2011), another similar study Memon et al. (2013) also explained that the size of clfA gene amplicon varied from 900 bp to 1000 bp in S. aureus isolates associated with bovine mastitis. Even though sequence variances were reported

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>57°C</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: The PCR program for the (clfA) gene amplification.

![Fig. 1: Agarose gel electrophoresis image for PCR product in virulence factor clumping factor A (clfA gene) S. aureus isolates. Where, Lane (M) DNA marker (1500-100bp), Lane (1-8) showed positive S. aureus isolates for clfA gene at 1000bp PCR product size.](image)

<table>
<thead>
<tr>
<th>Farms</th>
<th>No. of cow per farm</th>
<th>Samples</th>
<th>Positive S. aureus isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>150</td>
<td>20</td>
<td>4(20%)</td>
</tr>
<tr>
<td>Farm B</td>
<td>130</td>
<td>20</td>
<td>6(30%)</td>
</tr>
<tr>
<td>Farm C</td>
<td>150</td>
<td>25</td>
<td>7(25%)</td>
</tr>
<tr>
<td>Farm D</td>
<td>145</td>
<td>15</td>
<td>3(20%)</td>
</tr>
<tr>
<td>Total</td>
<td>575</td>
<td>80(13.9%)</td>
<td>20(25%)</td>
</tr>
</tbody>
</table>

Table 3: Isolation of S. aureus from milk samples collected from four farms located in Miasn province, South of Iraq.

<table>
<thead>
<tr>
<th>Staphylococcus aureus</th>
<th>Genbank accession</th>
<th>Homology sequence identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus isolate No.1</td>
<td>MK001021</td>
<td>LR130518.1 (99%)</td>
</tr>
<tr>
<td>S. aureus isolate ++No.2</td>
<td>MK001022</td>
<td>LR130518.1 (99%)</td>
</tr>
</tbody>
</table>
in previous studies, there is still insufficient knowledge related to the polymorphism in this gene (Karahan et al., 2011).

The sequence analysis based on (clfA gene) showed high identity (100%) between local *S. aureus* isolates (No.1 & No.2) and NCBI-BLAST *S. aureus* strain BPH2986 (LR130518.1) (table 4). The Phylogenetic tree analysis revealed that the isolates of present study with BPH2986 (LR130518.1) were grouped together in the same cluster (figs. 2, 3). The similarity among *S. aureus* isolated from bovine mastitis could reflect a long-term persistence into the bovine mammary gland, this indicates that these strains exist among bovine mastitis isolates from other countries (Rabello et al., 2007; Sung et al., 2008). On other hand, Kate et al. (2016) mentioned that *S. aureus* isolates from bovine raw milk sources in Victoria, Australia showed a higher genetic diversity relative to each other and interestingly, the Sa14-004 bovine isolate clustered closer to the caprine and ovine isolates, suggesting it is more distantly related to the other bovine genomes. The results of molecular detection of *S. aureus* from clinical samples (nasal swab, wound, burn sputum and ear swab) were collected from different Iraqi patients showed a type of mutation of Iraqi isolates with the reference strain *S. aureus* strain MN-082 Gen Bank Acc. based on the alignment of partial clumping factor A (clfA gene) sequences (Mohammed, 2015). Whereas, no mutations have been detected in the isolates of current study when compared with the reference strain *S. aureus* strain BPH2986 (LR130518.1).

**Conclusion**

This study characterized a high number of isolates (obtained from clinical mastitis cases) and associated isolates showed higher genetic similarity based on (clfA gene). The higher frequency of clinical mastitis cases reported this study reflect the incorrect management in the local bovine farms of AL-amara city. Therefore, it is imperative to strengthen farmers’ awareness for using hygiene procedures in all dairy farms.

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References


