Presence of methicillin-resistant Staphylococcus aureus in raw cow’s milk: adhesive capacities and extracellular enzymes characterization

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Presence of methicillin-resistant *Staphylococcus aureus* in raw cow’s milk: adhesive capacities and extracellular enzymes characterization

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**ABSTRACT:** Raw milk contamination with methicillin-resistant *S. aureus* (MRSA) threatens food safety and leads to public health concerns. It is a hazard for the consumer while also depleting therapeutic resources. Our study evaluates biofilm formation and virulence factors among 21 MRSA in raw cow’s milk. Methicillin resistance was confirmed by cefoxitin screening using the automated VITEK2 system, with a minimum inhibitory concentration greater than 8 mg/l. Phenotypic characterization of biofilm-producing strains was performed on Congo red agar (CRA), with a semi-quantitative adhesion test on 96-well tissue culture plates (TCP). The ability to produce different enzymes was evaluated, such as caseinase, lipase, and phospholipase (lecithinase). The surface hydrophobicity of the bacteria was determined, and the autoaggregation test was used to predict the interactions between bacterial cells. Among the tested strains, 61.9% were biofilm producers in the CRA, developing a positive (black colonies with a rough surface) and variable phenotype (colonies with black centers and red outlines, or red centers and black outlines). Furthermore, 19.05% and 80.95% of isolates were high and low biofilm formation on TCP. The enzymatic activity showed that lecithinase, caseinase, and lipase activities were detected in 100%, 80.95%, and 80.95% of tested strains respectively. Highly hydrophilic (85.71%) and weakly hydrophobic (14.29%) were detected in MRSA isolates. 71.43% of the MRSA strains exhibited a moderate autoaggregation and 28.57% of them showed a low autoaggregation. No significant difference was found between the CRA method and TCP (p>0.05). A significant association was found between adhesion capacity and bacterial autoaggregation in *S. aureus* strains (p<0.05). On the other hand, no statistical association between the hydrophobicity of microbial strains and adhesion capacity (p<0.05) was found. The same result was for the hydrophobicity of microbial strains and autoaggregation (p<0.05). This investigation could be beneficial for developing new control measures, prevention, and effective treatment against infections caused by antibiotic-resistant staphylococci.

**Keywords:** *Staphylococcus aureus*; Biofilm; Enzymatic activities; Hydrophobicity; Autoaggregation.
INTRODUCTION

Staphylococcus aureus is a pathogen responsible for many human and animal diseases (Papadopolouset al., 2021; Chen et al., 2020). Raw milk contamination at the farm level threatens food safety and public health (Ren et al., 2020). In many parts of the world, the methicillin-resistant S. aureus (MRSA) is the most widely known antibiotic-resistant pathogen. It has an intrinsic capacity to form biofilms on different surfaces (Rodriguez-Lázaro et al., 2018). Biofilms are organized communities of microorganisms that can attach and grow on surfaces (Hoveidaet al., 2019). They are formed by bacterial adhesion to the surface, followed by aggregation, maturation, and separation, which are essential for staphylococcal propagation (Yonget al., 2019). Biofilm formation is an important virulence factor, promoting the adherence and colonization of S. aureus on the mammary gland epithelium. Milk is an ideal nutrient medium for the bacteria; it facilitates their access to the galactophorous ducts, causing infection of the teat canal epithelium (Zaatout et al., 2020).

The formation of S. aureus biofilms is affected by several factors, such as cell surface hydrophobicity (Hoveidaet al., 2019). Microorganisms encircled by this matrix are more resistant to antimicrobial agents and are protected against phagocytosis (Rakshaet al., 2020). Adhesion capacity precedes the penetration of microorganisms into the host tissue, which is promoted by the production of toxins and extracellular enzymes (Wu et al., 2019). This bacterium, furthermore, produces a myriad of cellular and extracellular proteins involved in virulence (Tam and Torres, 2019). S. aureus secretes the lipase enzyme lecinthinase (Jooet al., 2016), allowing it to invade and destroy host tissue. Moreover, this bacterial genus is known for producing extracellular enzymes with protease (caseinase) activity (Marqueset al., 2013).

The characterization of potential virulence factors of S. aureus isolated from raw cow’s milk has become necessary to understand this foodborne disease in the Sidi-Bel-Abbes region. We conducted this study to evaluate biofilm formation among MRSA isolates and to establish a phenotypic characterization of their ability to secrete exoenzymes. Also, the occurrence of autoaggregation and cell surface hydrophobicity of the isolates were investigated.

MATERIAL AND METHODS

Sample collection and identification

The twenty-one strains of MRSA recovered from the milk of 200 dairy cows were used. The isolates were obtained from 25 dairy farms in the Sidi-Bel-Abbes region of Algeria. In the present study, lactating off-ground breeding Prim’Holstein cows were examined, and 30 mL of milk was collected from each quarter. The cows were at least primiparous with clinically healthy udders. Bacterial identification was performed based on Gram staining; colony morphology, using mannitol salt agar (MSA); catalase test, and tube coagulase test, using conventional methods by Markey et al. (2013). Species identification was performed using API-20-Staph Galleries (Bio-Mérieux, France) according to the manufacturer’s recommendation, and the results were interpreted using the numerical profile by the Apiweb software version 4.1 of Bio-Mérieux (France). Methicillin resistance was detected by the Cefoxitin Disc Diffusion Test (30 µg) with an inhibition zone of less than 22 mm (Canninget al., 2020) and confirmed by screening for cefoxitin by the automated VITEK2 system (Bio-Mérieux, France), with a minimum inhibitory concentration greater than 8 mg/l, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2008).

Characterization of biofilm production

Qualitative characterization of biofilm-producing strains was performed on congo agar (CRA) and incubated for 24 hours at 37°C in aerobic conditions. The CRA medium consists of 36 g of sucrose (Sigma Chemical Company, St. Louis, MO) in 1 L of brain heart infusionagar (BHIA) (Biorad, USA) with 0.8 g of congo reddye (Torlaket al., 2017). Biofilm-producing strains give black colonies a rough surface after 48 hours, whereas red colonies with a smooth surface for non-producing strains (Meloet al., 2013). Strains of variable phenotype yielded colonies with black centers and red outlines, or red centers and black outlines were considered positive biofilm producers (Touatieetal., 2007). A semi-quantitative adherence test on 96-well tissue culture plates (TCP) (Nunc, Roskilde, Denmark) was used to determine biofilm production by MRSA strains grown in BHI (Biorad, France) according to the method defined by Haddad et al. (2018). 95% ethanol was used to fix the adherent bacteria and stained with 100 mL of 1% crystallized violet (Merck, France) for 5 minutes in each well. The microplates were air-dried, and the optical density of each well was calculated at 570 nm (OD570) using an automated multi-scan reader (GIO. DE VITA E C, Italy). Biofilm formation has been interpreted as strong formation (OD570 > 1), weak formation (0.1<
OD570 < 1) and no formation (OD570 < 0.1) (Mack et al., 2001).

**Characterization of enzymatic activity**

The capacity to produce different enzymes was evaluated by inoculation of the cultures on TSA-1 (Biorad) supplemented with 1% (weight/volume) of skim milk for caseinase, 1% (weight/volume) of Tween 80 for lipase, 5% (weight/volume) of egg yolk for phospholipase (lecithinase) according to the method described by Merghni et al. (2014).

**Hydrophobicity and autoaggregation activity**

The surface hydrophobicity of the bacteria was determined by Borges et al. (2008). The bacterial cells were harvested by centrifugation at 5000 xG for 10 min after cultivation in BHI broth for 24 h, after being washed three times in 0.85% NaCl. The washed cells were resuspended in phosphate-buffered saline (PBS) to an absorbance of about 0.5 at 500 nm (DO500), and the initial optical density was measured at 500 nm. Three milliliters of bacterial suspension were mixed with 0.3 mL hexadecane per vortex for 1 min. After 30 to 60 min of settling, the aqueous phase was removed, and its OD500 was measured (final optical density). Cell surface hydrophobicity was calculated according to the formula:

\[
\text{Hydrophobicity (\%)} = \left[1 - \left(\frac{\text{DO (final)}}{\text{DO (initial)}}\right)\right] \times 100
\]

The hydrophobicity of a bacterial cell is classified into three categories: Hydrophobicity > 70%: bacteria are highly hydrophobic; 30% ≤ Hydrophobicity < 70%: bacteria are weakly hydrophobic; Hydrophobicity < 30%: bacteria are highly hydrophilic.

The interactions between bacterial cells were determined using the autoaggregation test described by Xu et al. (2009). Bacterial cells were reaped for 10 min at room temperature by centrifugation at 5000 rpm, washed with PBS, and resuspended in PBS to an absorbance of approximately 0.4 at 600 nm (DO600). Every 3 mL bacterial suspension was vortexed for 10 sec and incubated for 2 h at 37°C. The absorbance of the supernatant was measured at 600 nm. Autoaggregation was calculated according to the formula:

\[
\text{Autoaggregation (\%)} = \left[1 - \left(\frac{\text{A2h/A0h}}{1}\right)\right] \times 100
\]

Where A0h is the DO600 of the bacterial suspension at 0 h, and A2h is the DO600 of the bacterial suspension at 37°C incubation after 2 h. According to Rahman et al. (2008), the strains are classified following autoaggregation percentage: Autoaggregation ≥70%: Strong autoaggregation; Autoaggregation between 20 and 70%: Moderate autoaggregation; Autoaggregation < 20%: Weak autoaggregation.

**Statistical analysis**

Mean values and standard deviation were calculated from data obtained from three independent experiments (n=3). The statistical evaluation of biofilm formation between CRA and TCP methods was performed by the Chi-Square test (χ² test) with a significance level of P < 0.05. The Non-Parametric Test Friedman’s 2-way ANOVA by ranks (k samples) was performed with a significance level of P < 0.05 to illustrate whether there was a significant difference between adhesion, autoaggregation, and bacterial hydrophobicity. Statistical studies were performed using SPSS 25.0 software (IBM Corp).

**RESULTS**

**Characterization of biofilm production**

This was the first survey in western Algeria to study the adhesion potential of 21 MRSA strains isolated from raw cow’s milk. The biofilm production capacity in CRA of all tested isolates is shown in Table 1. In our study, 13 (61.9%) of the 21 strains were biofilm producers on CRA.

Semi-quantitative adhesion tests on a TCP (OD570) showed that all 21 strains were biofilm producers, with four strains (19.05%) being highly positive (Table 1). The remaining strains were weak producers of biofilm (0.1 < OD570 < 1). These results suggest a difference in adhesion capacity between the tested strains. Our results showed that there were no statistically significant differences (Chi-Square Test) in the ability to form biofilm by the CRA method and the ability to adhere to the TCP method (p>0.05).

**Characterization of enzymatic activity**

In our study, we also determined the characterization of hydrolytic enzyme production, which showed that 100% of the strains were lecithinase-positive and 80.95% were lipase-producing. In our work, 80.95% of the tested strains were caseinase-producing.

**Hydrophobicity and autoaggregation activity**

Based on the obtained results, it was found that the majority of the MRSA tested isolates 18 (85.71%) were found to be highly hydrophilic. In comparison,
Table 1. Biofilm production, adhesion ability, hydrophobicity, and autoaggregation of 21 MRSA strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Biofilm Phenotype (CRA)</th>
<th>OD570 ± SD</th>
<th>Adherence State</th>
<th>Hydrophobicity %</th>
<th>Autoaggregation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive phenotype</td>
<td>1.12 (0.34)</td>
<td>Highly positive</td>
<td>38.86</td>
<td>20.72</td>
</tr>
<tr>
<td>2</td>
<td>Positive phenotype</td>
<td>0.28 (0.04)</td>
<td>Low positive</td>
<td>30.66</td>
<td>24.98</td>
</tr>
<tr>
<td>3</td>
<td>Positive phenotype</td>
<td>0.54 (0.02)</td>
<td>Low positive</td>
<td>27.03</td>
<td>23.69</td>
</tr>
<tr>
<td>4</td>
<td>Negative phenotype</td>
<td>0.28 (0.03)</td>
<td>Low positive</td>
<td>29.13</td>
<td>24.02</td>
</tr>
<tr>
<td>5</td>
<td>Variable phenotype*</td>
<td>1.79 (0.64)</td>
<td>Highly positive</td>
<td>40.59</td>
<td>26.93</td>
</tr>
<tr>
<td>6</td>
<td>Variable phenotype</td>
<td>0.61 (0.03)</td>
<td>Low positive</td>
<td>15.17</td>
<td>20.68</td>
</tr>
<tr>
<td>7</td>
<td>Negative phenotype</td>
<td>0.16 (0.01)</td>
<td>Low positive</td>
<td>11.97</td>
<td>24.00</td>
</tr>
<tr>
<td>8</td>
<td>Positive phenotype</td>
<td>0.20 (0.02)</td>
<td>Low positive</td>
<td>20.30</td>
<td>18.91</td>
</tr>
<tr>
<td>9</td>
<td>Variable phenotype</td>
<td>0.77 (0.09)</td>
<td>Low positive</td>
<td>10.68</td>
<td>12.05</td>
</tr>
<tr>
<td>10</td>
<td>Variable phenotype</td>
<td>3.07 (0.19)</td>
<td>Highly positive</td>
<td>18.22</td>
<td>15.14</td>
</tr>
<tr>
<td>11</td>
<td>Negative phenotype</td>
<td>0.85 (0.10)</td>
<td>Low positive</td>
<td>18.32</td>
<td>23.66</td>
</tr>
<tr>
<td>12</td>
<td>Negative phenotype</td>
<td>0.52 (0.02)</td>
<td>Low positive</td>
<td>25.74</td>
<td>25.35</td>
</tr>
<tr>
<td>13</td>
<td>Variable phenotype</td>
<td>1.78 (0.60)</td>
<td>Highly positive</td>
<td>11.77</td>
<td>18.23</td>
</tr>
<tr>
<td>14</td>
<td>Variable phenotype</td>
<td>0.42 (0.05)</td>
<td>Low positive</td>
<td>12.51</td>
<td>22.14</td>
</tr>
<tr>
<td>15</td>
<td>Negative phenotype</td>
<td>0.69 (0.29)</td>
<td>Low positive</td>
<td>10.87</td>
<td>16.88</td>
</tr>
<tr>
<td>16</td>
<td>Negative phenotype</td>
<td>0.22 (0.00)</td>
<td>Low positive</td>
<td>11.62</td>
<td>20.71</td>
</tr>
<tr>
<td>17</td>
<td>Negative phenotype</td>
<td>0.83 (0.04)</td>
<td>Low positive</td>
<td>17.54</td>
<td>24.45</td>
</tr>
<tr>
<td>18</td>
<td>Negative phenotype</td>
<td>0.95 (0.01)</td>
<td>Low positive</td>
<td>22.79</td>
<td>22.65</td>
</tr>
<tr>
<td>19</td>
<td>Positive phenotype</td>
<td>0.49 (0.02)</td>
<td>Low positive</td>
<td>18.98</td>
<td>21.05</td>
</tr>
<tr>
<td>20</td>
<td>Positive phenotype</td>
<td>0.58 (0.04)</td>
<td>Low positive</td>
<td>20.56</td>
<td>21.04</td>
</tr>
<tr>
<td>21</td>
<td>Variable phenotype</td>
<td>0.23 (0.04)</td>
<td>Low positive</td>
<td>11.07</td>
<td>16.50</td>
</tr>
</tbody>
</table>

*Strains with variable phenotype were considered to be positive biofilm producers.

Table 2. Relationship between the adhesion capacity, autoaggregation, and hydrophobicity of MRSA strains

<table>
<thead>
<tr>
<th>Number</th>
<th>Friedman’s test</th>
<th>Middle Rank</th>
<th>Paired comparisons (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>χ²</td>
<td>Df</td>
<td>P</td>
</tr>
<tr>
<td>30.400</td>
<td>2</td>
<td>&lt;0.001</td>
<td>2.67</td>
</tr>
</tbody>
</table>

A_{dh}= Adherence; A_{Ag}= Autoaggregation; H_b= Hydrophobicity

Our finding corroborate with those of Acheket al. (2020), who reported that 18 of the 28 S. aureus (64.3%) associated with ovine milk were biofilm producers. According to Darwish and Asfour (2013), 27 of 40 S. aureus strains (67.5%) isolated from bovine milk were biofilm producers. Study conducted by Marques et al. (2017) in Brazil found that 20 tested strains of S. aureus (100%) were biofilm producers, with 11 (55%) classified as strong (55% = 11/20), 6 (30%) moderate and 3 (15%) weak producers. On the other hand, a lower prevalence rate of biofilm producers among S. aureus was observed, with a value of 29.41% (Dhanawadeet al., 2010). It is important to note that there is no statistically significant difference in biofilm formation by the CRA and the TCP methods (p>0.05) in our results. Similarly, previous studies have shown a high correlation between the two methods (Vasil et al., 2017). Biofilms tend to be related to many diseases, supported by a shift in antimicrobial susceptibility (Komodromos et al., 2022). However, other reports have shown a lack of correlation between MRSA isolates and biofilm formation (Cha et al., 2011).
During infection, *S. aureus* hydrolyzes host lipid substrates through the secretion of lipases and phospholipases for nutrient acquisition and colonization (Chen and Alonzo, 2019). Our study has shown 100% and 80. 95% for lecinthinase, lipase, and caseinase production, respectively, by MRSA isolates. These results differ from those obtained by El-Jakeet et al. (2008), who found that 81. 1% of *S. aureus* among bovine isolates were lecinthinase-positive, while only 47. 2% of isolates were lipase-positive. A lower percentage of isolates, 54. 72% lipase-positive, was reported by Parthet et al. (2016). Numerous enzymes secreted by *S. aureus* degrade host tissues, several of which are proteases (Kot et al., 2016;Marques et al., 2013; Gayatri et al., 2017).

The adhesion of bacterial cells is generally related to their cell surface characteristics (Sharma et al., 2017). Factors such as proteins and teichoic acids on the bacterial cell wall are involved in adhesion, autoaggregation, and hydrophobicity (Li et al., 2015). We found that 18 (85. 71%) MRSA were highly hydrophilic, and 15 (71. 43%) showed moderate autoaggregation. The significant association between adhesion capacity and bacterial autoaggregation of *S. aureus* strains is noted in this study (p<0. 05). Similarly, Colladoet al. (2007) found that autoaggregation correlated with the adhesion capacity in lactic acid bacteria. Autoaggregation is one of the first steps in biofilm formation, providing the bacteria with the benefits of advanced evasion of host defenses and antimicrobial treatment (Trunk et al., 2018). The cell surface characteristics, including hydrophobicity, could affect bacterial adhesion and autoaggregation, as reported by DelRe et al. (2000). The MRSA strains we studied did not show a significant association between hydrophobic capacity and adhesion capacity (p<0. 05), as well as hydrophobic capacity and autoaggregation (p<0. 05), in agreement with the results of Auger et al. (2009). In contrast, some authors consider bacterial hydrophobicity as crucial for adhesion (Waśko et al., 2014). An association between autoaggregation activity and bacterial surface hydrophobicity has been reported by Kanjan and Sakpetch (2020) in a coagulase-negative staphylococcosisolate, *Staphylococcus simulans* PMRS35, isolated from fermented foods.

**CONCLUSION**

The present study demonstrated that MRSA isolates from raw cow’s milk in the western region of Algeria formed biofilm to varying degrees, as well as a variety of hydrolytic enzymes, which play an important role in *S. aureus* animal infection. This indicates an alarming situation, posing a risk to consumers, which requires the development of new control measures, prevention, and effective treatment against infections caused by antibiotic-resistant staphylococci. This study has shown the necessity for future research to provide recommendations for the rapid detection of MRSA and its virulence factors to assist in control measures on our dairy farms.

**ACKNOWLEDGMENTS**

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**REFERENCES**


