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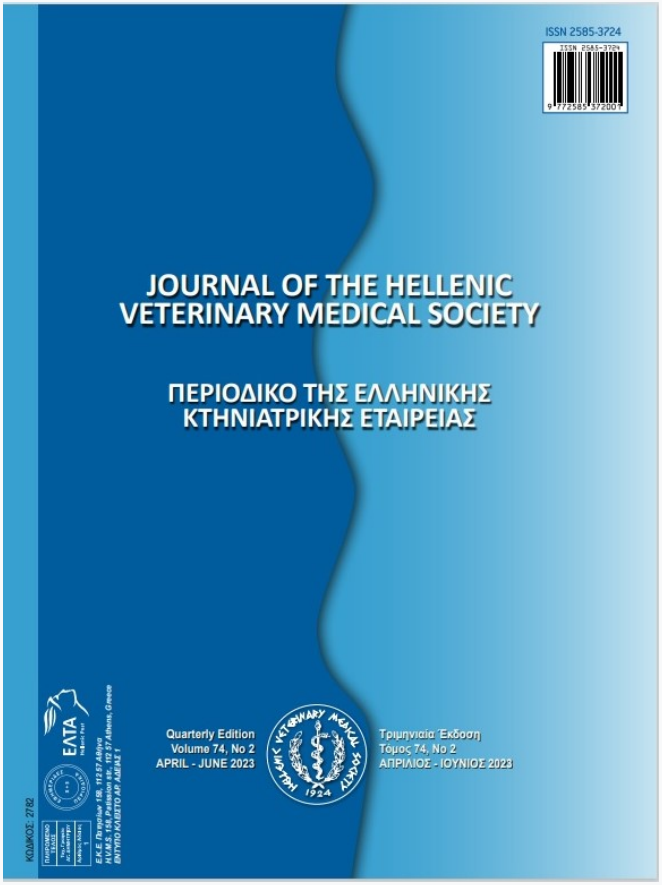

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Research article
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Presence of methicillin-resistant *Staphylococcus aureus* in raw cow's milk: adhesive capacities and extracellular enzymes characterization

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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 auto aggregation 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 strainsrespectively. Highly hydrophilic (85. 71%) and weakly hydrophobic (14. 29%) were detected in MRSA isolates. 71. 43% of the MRSA strains exhibited a moderate auto aggregation and 28. 57% of them showed a low auto aggregation. No significant difference was found between the CRA method and TCP ($p>0.05$). A significant association was found between adhesion capacity and bacterial auto aggregation 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 auto aggregation ($p<0.05$). 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; Auto aggregation.

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INTRODUCTION

Staphylococcus aureus is a pathogen responsible for many human and animal diseases (Papadopoulos *et 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. Ithas an intrinsic capacity to form biofilms on different surfaces (Rodríguez-Lázaro *et al.,* 2018). Biofilms are organized communities of microorganisms that can attach and grow on surfaces (Hoveida *et al.,* 2019). They are formed by bacterial adhesion to the surface, followed by aggregation, maturation, and separation, which are essential for staphylococcal propagation (Yong *et 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 (Hoveida *et al.,* 2019). Microorganisms encircled by this matrix are more resistant to antimicrobial agents and are protected against phagocytosis (Raksha *et 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 lecithinase (Joo *et al.,* 2016), allowing it to invade and destroy host tissue. Moreover, this bacterial genus is known for producing of extracellular enzymes with protease (caseinase) activity (Marques *et 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 auto aggregation 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 (Canning *et 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 red 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 red dye (Torlak *et 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 (Melo *et 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 (Touati*etal.,* 2007). A semi-quantitative adhesion 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 auto aggregation 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:

Hydrophobicity (%) = [1- (DO (final) / DO (initial)] × 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 auto aggregation 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. Auto aggregation was calculated according to the formula:

Auto aggregation % = [1- (A2h/A0h)] x 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 auto aggregation percentage: Auto aggregation ≥70%: Strong auto aggregation; Auto aggregation between 20 and 70%: Moderate auto aggregation and Auto aggregation< 20%: Weak auto aggregation.

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 (χ2test) 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, auto aggregation, 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 Table1. 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 testedstrains were caseinase-producing.

Hydrophobicity and auto aggregation activity

Based on the obtained results, it was found that the majority of the MRSA testedisolates 18 (85. 71%) were found to be highly hydrophilic. In comparison,

Table 1. Biofilm production, adhesion ability, hydrophobicity, and autoagregation of 21 MRSA strains

Strains	Biofilm Phenotype (CRA)	OD570 ± SD	Adherence State	Hydrophobicity %	Autoagregation %
1	Positive phenotype	1, 12 (0, 34)	Highly positive	38, 86	20, 72
2	Positive phenotype	0, 28 (0, 04)	Low positive	30, 66	24, 98
3	Positive phenotype	0, 54 (0, 02)	Low positive	27, 03	23, 69
4	Negative phenotype	0, 28 (0, 03)	Low positive	29, 13	24, 02
5	Variable phenotype[a]	1, 79 (0, 64)	Highly positive	40, 59	26, 93
6	Variable phenotype	0, 61 (0, 03)	Low positive	15, 17	20, 68
7	Negative phenotype	0, 16 (0, 01)	Low positive	11, 97	24, 00
8	Positive phenotype	0, 20 (0, 02)	Low positive	20, 30	18, 91
9	Variable phenotype	0, 77 (0, 09)	Low positive	10, 68	12, 05
10	Variable phenotype	3, 07 (0, 19)	Highly positive	18, 22	15, 14
11	Negative phenotype	0, 85 (0, 10)	Low positive	18, 32	23, 66
12	Negative phenotype	0, 52 (0, 02)	Low positive	25, 74	25, 35
13	Variable phenotype	1, 78 (0, 60)	Highly positive	11, 77	18, 23
14	Variable phenotype	0, 42 (0, 05)	Low positive	12, 51	22, 14
15	Negative phenotype	0, 69 (0, 29)	Low positive	10, 87	16, 88
16	Negative phenotype	0, 22 (0, 00)	Low positive	11, 62	20, 71
17	Negative phenotype	0, 83 (0, 04)	Low positive	17, 54	24, 45
18	Negative phenotype	0, 95 (0, 01)	Low positive	22, 79	22, 65
19	Positive phenotype	0, 49 (0, 02)	Low positive	18, 98	21, 05
20	Positive phenotype	0, 58 (0, 04)	Low positive	20, 56	21, 04
21	Variable phenotype	0, 23 (0, 04)	Low positive	11, 07	16, 50

[a] Strains with variable phenotype were considered to be positive biofilm producers.

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

Number	Friedman's test			Middle Rank			Paired comparisons (P)		
21	$\chi 2$	Df	P	A_{dh}	A_{Ag}	H_b	H_b- A_{Ag}	H_b-A_{dh}	A_{Ag} -A_{dh}
	30. 400	2	<0. 001	2. 67	2. 1	1. 24	0. 016	<0. 001	0. 192

A_{dh}= Adherence; A_{Ag}= Auto aggregation; H_b= Hydrophobicity

three isolates (14. 29%) were weakly hydrophobic. The auto aggregation properties of the strains are shown in Table1. MRSA strains showed auto aggregation values ranging from 15. 14 to 26. 93%. 15 isolates (71. 43%) showed moderate auto aggregation, while six (28. 57%) low auto aggregation. Non-parametric statistical analysis showed a significant association between adhesion capacity and bacterial auto aggregation in MRSA strains (p>0. 05).

In the present study, nosignificant association between the hydrophobicity of microbial strains and adhesion capacity (p<0. 05) was found (Table 2). On the other hand, no significant association between the hydrophobicity of microbial strains and auto aggregation (p<0. 05) was found too.

DISCUSSION

In Africa, the use of antibiotics in animal production is difficult to control (Belkadi *et al.,* 2022). The development of biofilms in MRSA isolates allows the bacteria to withstand harsh environmental conditions and become resistant to antibiotics (Shivaee *et al.,* 2019). This bacterium threatens human health as it can be responsible for zoonosis and foodborne infections (Belmamoun *et al.,* 2017; Ahmed *et al.,* 2020).

Our finding corroborate with those of Achek *et 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% (Dhanawade *et 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 lecithinase, lipase, and caseinase production, respectively, by MRSA isolates. These results differ from those obtained by El-Jakee *et al.* (2008), who found that 81. 1% of *S. aureus* among bovine isolates were lecithinase-positive, while only 47. 2% of isolates were lipase-positive. A lower percentage of isolates, 54. 72% lipase-positive, was reported by Parth *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 were involved in adhesion, auto aggregation, and hydrophobicity (Li *et al.,* 2015). We found that 18 (85. 71%) MRSA were highly hydrophilic, and 15 (71. 43%) showed moderate auto aggregation. The significant association between adhesion capacity and bacterial auto aggregation of *S. aureus* strains is noted in this study ($p>0.05$). Similarly, Collado *et al.* (2007) found that auto aggregation correlated with the adhesion capacity in lactic acid bacteria. Auto aggregation 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 auto aggregation, as reported by Del Re *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 auto aggregation ($p<0.05$), 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 auto aggregation activity and bacterial surface hydrophobicity has been reported by Kanjan and Sakpetch (2020) in a coagulase-negative staphylococci isolate, *Staphylococcus simulans* PMRS35, isolated from fermented foods.

CONCLUSION

The present study demonstrated that MRSA isolates from raw cow's milkin 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.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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