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The Ciliate Protist *Tetrahymena pyriformis* as a Cellular Adhesion Model for the Pathogenic Bacterium *Staphylococcus aureus*

Bouchra El Khalfi[1], Mohammed Benlahfid[1], Sofia Jarmouni[1], Nezha Senhaji[2], Aurelio Serrano Delgado[3], Abdelaziz Soukri[1]

[1]Laboratory of Physiopathology, Genetics Molecular and Biotechnology (PGMB), Faculty of Sciences Ain Chock, Research Center: Health & Biotechnology, University Hassan II of Casablanca, Casablanca, Morocco
[2]Laboratory of Genetic and Molecular Pathology (LGPM), Medical School, Hassan II University, Casablanca, Morocco
[3]Institute of Biochemistry Vegetal and Photosynthesis (IBVF-CSIC University of Seville), Seville, Spain
Email: ab.soukri@gmail.com

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Abstract

Staphylococcus aureus is one of the main pathogenic agents responsible for nosocomial and community-acquired bacterial infections. The pathogenicity of this Gram-positive bacterium is ensured by its different adhesion factors. Collagen and the extracellular glycoprotein adhesin are among the *Staphylococcus* most important virulence factors. It has been shown that most of the *S. aureus* strains carry the *ica* operon, responsible for biofilm production. However, the coexpression of the *icaA* and the *icaD* genes is necessary for complete biofilm synthesis. The aim of our study was to study a collection of 15 clinical strains of *S. aureus* from different sources for the presence of *cna* and *icaD* genes coding intercellular adhesion proteins. We also intended to estimate the strains' ability to form biofilms by the red Cong method and to test the adhesion ability of *S. aureus* to the ciliated protist *Tetrahymena pyriformis*, which we used as a novel cellular adhesion model. Finally, we checked the adhesion's inhibition capacity of some plants extracts. The molecular detection of adhesion genes revealed that 80% of strains are cna positive, and 73% are *icaD* positive. Qualitative biofilm production of *S. aureus* revealed that 66.6% of strains were slime producers. The adhesion test revealed that 20% of strains are strongly adhering to *T. pyriformis* and that the *Clematis cirrhosa* extract has an anti-adhering effect of *S. aureus* to the ciliate *T. pyriformis*.

Keywords

Staphylococcus aureus, Adhering Genes (*cna* and *icaD*), *Tetrahymena pyriformis*, Biofilm Production, Plant Extract, Anti-Adhesion Effect

1. Introduction

Staphylococcus (from the Greek: σταφυλή, staphylē, "grape" and κόκκος, kókkos, "granule") is a genus of Gram-positive bacteria in shells shape, without flagellum, which has a cellular envelope made of a unique plasma membrane, surrounded by a relatively thick cell wall [1] [2]. *Staphylococcus* strains are able to grow in aerobic or anaerobic conditions. They are ubiquitous species: having the ability to live in soil, water or diverse animal tissues [3] [4].

Among the *Staphylococci*, there are three main human pathogenic species: *S. aureus, S. epidermidis and S. saprophyticus. Staphylococcus aureus* is usually called golden *Staphylococcus* because of the yellow colored colonies that it forms on the agar. As described in 1881 by Alexander Ogston, it is a Gram-positive spherical bacterium, optional anaerobic, immobile and making regular clusters like bunch of grapes of 0.5 to 1.5 μm. It has the most important pathogenicity potential among the *Staphylococci*, and is the only strain able to produce coagulase, an exoenzyme able to coagulate blood plasma, which allows *Staphylococcus* fast and easy identification. It is also one of the main causes of nosocomial and community-acquired infections. Most often, these infections involve biofilms, which are multicellular communities omnipresent in natural, industrial and medical areas and can affect human health [3] [4].

S. aureus infection pathogenicity is related to its ability to colonize host tissues, to proliferate and to evade the immune defense system of the host thanks to specific virulence factors (adhesion factors, exoenzymes and toxin production) [5] [6].

Bacterial adhesion to host cells or surfaces is often an essential first stage in pathogenic mechanisms. *S. aureus* has a large number of surface proteins called adhesins, belonging to the MSCRAMMs class (Microbial Surface Components Recognizing Adhesive Matrix Molecules) [7] [8] that enable specific adhesion to components of the host tissue, namely, the fibrinogen binding protein (Clumping factor A or *ClfA*), the fibronectin binding proteins *FnBPA* and *FnBPB* (fibronectin-binding protein A and B) [9] [10], the collagen binding protein (collagen adhesin or *Cna*) and the elastin binding protein (*EbpS*) [8]. Other surface proteins also described in *S. aureus*, such as *Eap* (extracellular adherence protein) [11], *Ebh* (extracellular matrix binding homologue protein) [12] and *Emp* (extracellular matrix binding protein) [13] may have a role in colonization.

Biofilms formation is the outcome of a set of physical, chemical and biological processes. According to the model suggested by Mack *et al.* [14], it consists of two phases: initial attachment and accumulation. The first phase is the initial attachment or adhesion of cells to a solid support, this support can either be the host tissue (skin, epithelium…), materials used in medical field (catheters, prosthesis···) or a food industry support (cutting surface, floors, walls…). The initial adhesion is the result of non covalent physicochemical interactions between support and bacteria such as Van der Waals forces, electrostatic forces, Lewis acid-base properties and hydrophobic/hydrophilic properties [15] [16]. Adhe-

sion is also affected by components of the bacterial cell wall such as teichoic acids and surface proteins (adhesins and autolysins identified in *S. aureus* and *S. epidermidis*) [17] [18].

The second phase is the intercellular aggregation, which leads to micro colonies formation. This step includes cell division and exopolysaccharides production and leads to a mature biofilm establishment. Among the responsible factors for intercellular aggregation, there are three main surface compounds such as: polysaccharide intercellular adhesion (*PIA*) described in *S. epidermidis, S. aureus* and *S. caprae* [14] [19] [20], Accumulation Associated Protein (*AAP*) described in *S. epidermidis* [21] and the Biofilm Associated Protein (*BAP*) originally described in bovine *S. aureus* strains [22]. Biofilm maturation is sometimes followed by a detachment phase that allows bacteria to colonize other sites. This phenomenon has been slightly studied in *Staphylococci.* In *S. epidermidis*, this phase has been studied using electric currents or enzymes that would reverse the establishment of biofilms on catheters, causing thereby the detachment [23]. In *S. aureus*, the biofilm viscoelasticity allows resistance to detachment caused by mechanical stress or surrounding flux [24]. In addition, this viscoelasticity leads to rolling phenomenon in micro-colonies that allows them to migrate to other sites. Stoodley *et al.* showed that cells spontaneous removal is divided into two processes: erosion and sloughing [25]. Erosion is a continual detachment of single cells and small cell aggregates while the sloughing is the rapid and massive loss of biofilm. Erosion occurs during the whole biofilm maturation period whereas sloughing occurs after a nutritional deficiency [26] [27]. The detachment step seems to be genetically programmed, and would facilitates the propagation of the infection and/or the colonization of other sites [28] [29] [30] [31].

In the current study, we screened the presence of *cna* and *icaD* genes coding for adhesion proteins in 15 clinical *S. aureus* strains and assessed their ability to produce biofilms. We also tested, for the first time, the adhesion ability of *S. aureus* to ciliated protist *Tetrahymena pyriformis*, a well-known model organism in biomedical research with a cellular architecture similar to human cell [32], and finally evaluated the anti-adhesion effect of some plant extracts.

2. Material and Methods

1) Microorganisms and Culture Condition

Staphylococcus aureus

Fifteen *Staphylococcus aureus* bacterial strains of different origins have been studied, including a resistant strain to methicillin (SARM). These strains were provided by the molecular bacteriology laboratory of the Pasteur Institute of Morocco (IPM) and the bacteriology department of the Ibn Rochd hospital of Casablanca (Morocco).

Identification of the 15 strains of *S. aureus* was performed using primers specific for the AF gene, which is specific to the identification of *Staphylococcus aureus*.

The reference strain *Staphylococcus aureus* ATCC 25923, which was provided by the Laboratory of Analysis, Treatment and Valorization of Environmental and Products Pollutants, Faculty of Pharmacy, Monastir (Tunisia) was used as a control.

Culture of *Staphylococcus aureus* was performed on Nutrient Broth (NB) medium, and/or brain heart infusion broth (BHI) and incubated at 37˚C.

Tetrahymena pyriformis

The ciliate protist *Tetrahymena pyriformis* (strain GL, ATCC 30005) was grown in PPY medium composed of: meat Peptic digest (1.5%), yeast extract (0.25%) and was then incubated at 28˚C. *Pseudomonas syringae* and *Eschericha coli BL21* strains were used as controls.

2) Plant Extracts

Plant extracts were prepared from the aerial part (stem + leaf) of *Pisenlit (Taraxacum officinale)*, *Clematis cirrhosa, Mesembryanthemum crystallinum* and *Rubia pergrina*, and stored at 4˚C in the National Institute of Medicinal and Aromatic Plants, Taounate, Morocco.

3) Bacterial DNA Extraction

Colonies of *S. aureus* were scraped using an inoculation loop and mixed with 200 µl of distilled water in a conical centrifuge tube under sterile conditions. Tubes were placed in a water bath for 10 min at 100˚C, then immediately placed on ice for 5 min (heat shock), and centrifuged at 12,000 rpm for 10 min at 4˚C. The supernatant representing the DNA was collected in sterile Eppendorf tubes of 1.5 ml which were stored at −20˚C.

4) Polymerase Chain Reaction

Molecular detection of *AF, icaD* and *can* genes was performed with PCR using the primers shown in Table 1. For all the PCR experiments, reaction mixtures contained 10 µM of each forward and reverse primer, 25 mM of $MgCl_2$, 5× flexi buffer Promega, 10 µM of dNTPs and 5 U/µl of Taq DNA polymerase.

The PCR program used for strains identification was: 1 cycle of 3 min at 96˚C, 30 cycles (30 sec at 95˚C, 30 sec at 52˚C and 1 min at 72˚C) and a final elongation step of 10 min at 7˚C. For *cna* and *icaD* genes detection, we used the following PCR program: 1 cycle of 5 minutes at 94˚C, 30 cycles (30 sec at 94˚C, 30 sec at 57˚C (for the *cna* gene, 55˚C for the *icaD* gene) and 1 min at 72˚C) and a final elongation step of 10 min at 72˚C.

Table 1. Primers sequences and PCR conditions.

	Primer	5'-3' Sequence	PCR product length (bp)	Annealing temperature	References
1	*AF*	AATCTTTGTCGGTACACGATATTCTTCACG CGTAATGAGATTTCAGTAGATAATACAACA	108	52˚C	Murdoch *et al.* 2004 (Num réf)
2	*icaD*	ATGGTCAAGCCCAGACAGAG AGTATTTTCAATGTTTAAAGCAA	198	55˚C	Rohde *et al.* 2001 (Num réf)
3	*cna*	AAAGCGTTGCCTAGTGGAGA AGTGCCTTCCCAAACCTTTT	192	57˚C	Arciola *et al.* 2005 (Num réf)

5) Detection of Biofilm-Forming Strains

Biofilm-forming strains detection was performed on Congo Red Agar medium (CRA). 0.4 g of CRA (Panreac C.I 22120) was added to 500 ml of aqueous solution, which was autoclaved for 15 min at 120˚C. 26.5 g of BHI, 25 g of sucrose and 5 g of agar were then added to the solution that was autoclaved and poured into Petri dishes.

6) Adhesion Test of *Staphylococcus aureus* to *Tetrahymena pyriformis* Cells

Using the following protocol, we aimed to study the adhesion capacity of *Staphylococcus aureus* to the ciliated protist *T. pyriformis* cells. Seventeen cover slips were prepared: The first one served as a control in which the protist cells were incubated alone, in the second one *T. pyriformis* cells were incubated with *Pseudomonas syringae* and for the 15 remaining cover slips, the protist cells were incubated with the 15 strains of *Staphylococcus aureus*. Then, each cover slip was placed in a sterile Petri box and 100 µl of an exponential phase culture of *T. pyriformis* were added. Afterward, using a toothpick, 30 colonies of each bacterial culture were took from the gelose medium, spread over the entire cover slip and incubated for 2 h at 28˚C. Subsequently, the cover slips were fixed with 1 ml of methanol for 20 min, stained with 1 ml of Giemsa solution for 20 min and washed 2 - 3 times with 1 ml of PBS. After drying, cover slips were placed on slides. Finally, a drop of immersion oil was added and preparations were observed on light microscope. The experiment was carried out in triplicate.

7) Anti-Adhesion Effect of Plant Extracts

Study of the antibacterial effect of plant extracts

The antibacterial effect of *Pisenlit*, *Clematis cirrhosa*, *Mesembryenthemum hallinum* and *Rubia peregrina* plant extracts on *S. aureus* was determined by the well diffusion technique on solid medium. The antimicrobial activity is determined in terms of the inhibition zone diameter generated around the wells.

Three boxes were used, the first one contained only the culture medium (negative control), the second one contained the bacterial culture without plant extracts (positive control) and the third box contained bacterial suspension incubated with plant extracts. The experiment was carried out in triplicate for each plant extract.

Effects of plant extracts on bacterial adhesion

To study the anti-adhesion effect, 30 colonies of bacteria were incubated with *T. pyriformis* cells and plant extracts, as described above (Section 6. Adhesion test of *Staphylococcus aureus* to *Tetrahymena pyriformis* cells). 50 µl of one of the analyzed plant extracts (*Pisenlit, Clematis cirrhosa, Mesembryentemum hallinum*, and *Rubia pergrina*) were added to each coverslip before 2 h of incubation. The same steps of adhesion test were followed and the result was observed using a light microscope. The experiment was carried out in triplicate for each plant extract.

3. Results and Discussion

Molecular identification of *S. aureus* strains

In this study, all the fifteen clinical bacterial strains collected were identified by PCR using the *AF* gene. The results were revealed by electrophoresis on 1% agarose gel. The Figure 1 is a demonstrative profile representing the result of a set of selected clinical strains.

Thereby as shown in Figure 1, all strains presented the expected DNA fragment of 108 bp, which is in line with the microbiological identification (results not shown), indicating that all clinical strains belong to the *Staphylococcus aureus* species.

Molecular detection of *cna* and *icaD* genes

The *cna* gene

PCR amplification of the *cna* gene encoding the collagen-binding adhesin protein was performed for all *Staphylococcus aureus* clinical strains using genomic DNA as a template, and the results were revealed on 1% agarose gels. Figure 2 shows an example of some selected strains.

Based on the results of PCR amplifications, out of the fifteen clinical strains available, twelve *Staphylococcus aureus* strains (80%) carried the *cna* gene as indicated by the amplified DNA fragment of 192 bp (Figure 2), while only three strains did not possess this gene.

According to literature, the staphylococcal collagen-binding adhesion has been described as one of the most important virulence factor proteins in the infection

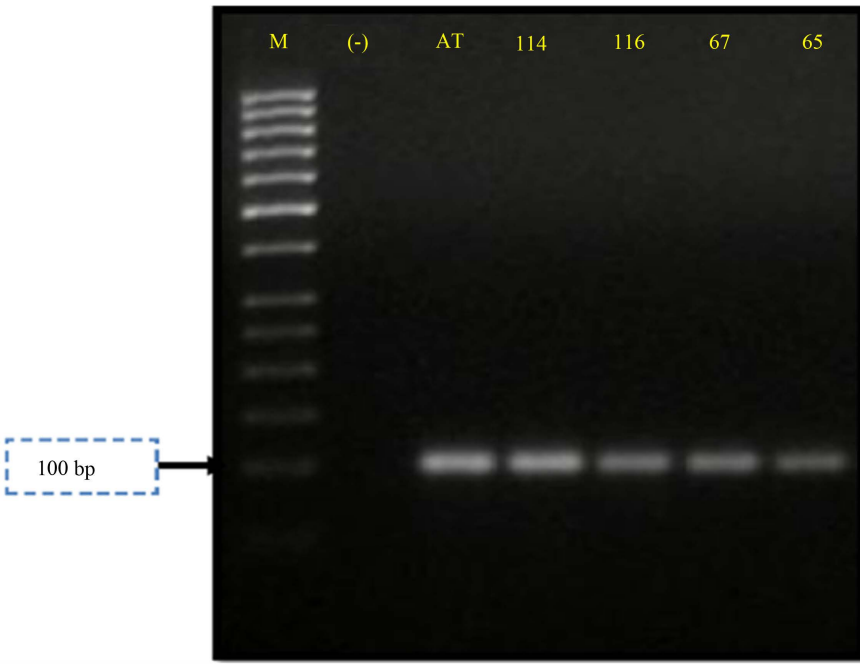

Figure 1. *S. aureus* identification by *AF* gene detection through agarose gel electrophoresis of PCR products. M: DNA marker, (−): Negative control *E. coli*, AT: the reference strain ATCC 25923 (positive control). 114, 116, 67, and 65: selected clinical strains of *Staphylococcus aureus.*

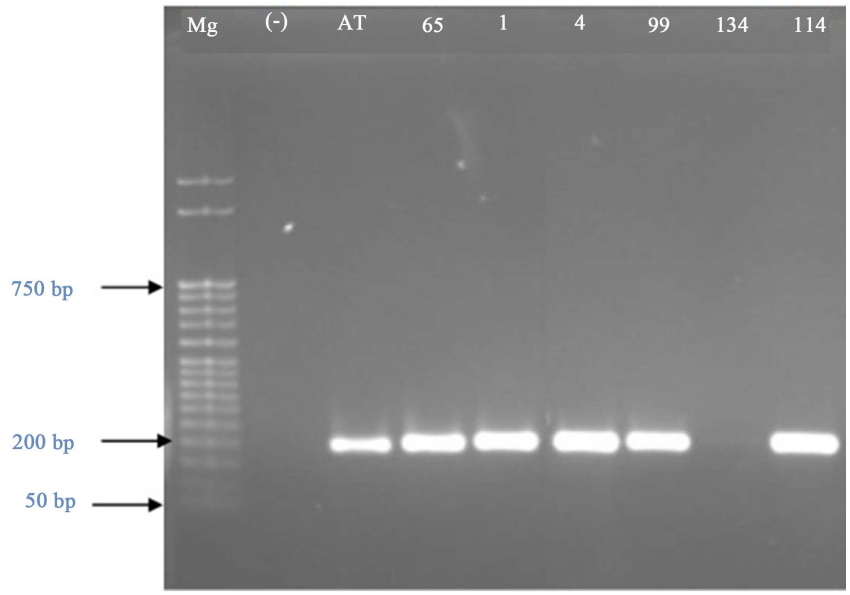

Figure 2. *cna* gene detection through agarose gel electrophoresis of PCR products. Mq: 50 bp molecular weight marker, (−): negative control *E. coli*; AT: the reference strain ATCC 25923 (positive control). 65, 1, 4, 99, 134, 114: *Staphylococcus aureus* clinical strains.

pathogenesis of this bacterium [33]. [34] showed that *cna* gene is carried by 56.5% of the tested *S. aureus* strains while Nizami Duran *et al.* [35] found ***cna*** gene presence in 78.4% of the staphylococcal strains. However, Peacock *et al.* [36] underlined that the prevalence of *cna*-positive strains was 52% while Aricola *et al.* [37] showed that *cna* was carried by 46% of *S. aureus* strains. Furthermore, Tristan *et al.* [38] and Rohdet *et al.* [39] described the presence of *cna* gene in 36% and 22% of the strains respectively.

The *icaD* gene

PCR amplification of the *icaD* gene, which is a member of the *ica* operon encoding the proteins that synthesize the polysaccharide intercellular adhesin (*PIA*), was obtained for most tested *Staphylococcus aureus* clinical strains (**Figure 3**).

Eleven among fifteen *Staphylococcus aureus* strains (73%) presented the *icaD* gene, as revealed by the amplified band on agarose gel which corresponds to a DNA fragment of 198 bp (**Figure 3**). The remaining four strains did not show the highlighted gene.

Phenotypic determination of biofilm production

Slime production by *Staphylococcus aureus* clinical strains was assessed using Congo red agar technique; the results were interpreted according to what has been reported in the literature [37] [40]. Strains forming biofilms presented crystalline dry colonies, in black, dark black or slightly black color. While those not forming biofilm developed pink, red or bordeaux colonies and sometimes can present red colonies with a black dot in the middle known as "bulls eye".

We found that ten of the *Staphylococcus aureus* strains analyzed in this study (66.6%; including reference strain) were biofilm producing strains, giving dry

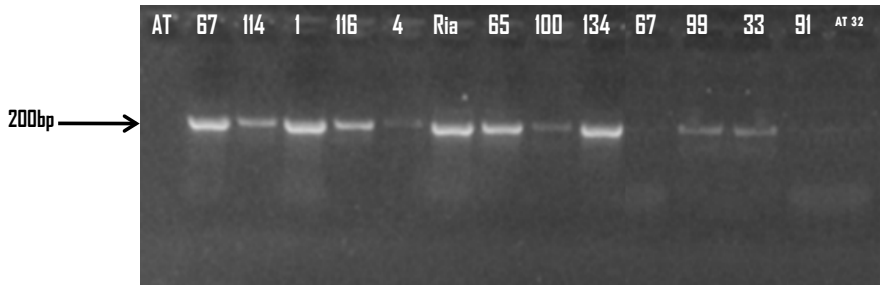

Figure 3. *icaD* gene detection through agarose gel electrophoresis of PCR products. M: 50 bp molecular weight marker; (−): negative control *E. coli*.

crystalline black, slightly black or dark black colonies (**Figure 4**), this difference in coloration intensity can be attributed to the different metabolic pathways incurred by *S. aureus* for biofilm formation [41].

These strains demonstrated an intense biofilm production, indicating a high enzymatic activity of polysaccharide intercellular adhesin (*PIA*), while the remaining five clinical strains (33.3%) were classified as non-biofilm forming strains, and developed pink, red or bordeaux colored colonies (**Figure 4**).

Our results are close to those obtained by Arciola *et al.* [42] who reported 61% of *icaA* and *icaD* genes holders among *S. aureus* strains. While Tarek Zmantar *et al.* [43] found that 78.26% of the tested strains were *icaA* and *icaD* positive.

We also found that nine *icaD* positive strains (including the reference strain) were characterized as slime forming, whereas two strains were positive for this gene but were not producing biofilm. These results are inconsistent with those described by Arciola *et al.* [42] who found that *icaA* and *icaD* genes were only detected in slime forming strains. These differences may be caused by environmental conditions [44] [45]. In the case of many biofilm-forming bacteria, differentiation from planktonic state to sessile state is associated to environmental stress factors [29]. However, biofilm formation by *Staphylococcus* is subject to complex regulation influenced by a number of environmental factors including osmolarity, glucose, anaerobiosis and temperature [46] [47].

On the other hand, Gundogan *et al.* [48] found that 58 out of 110 (52.7%) *S. aureus* strains were slime producing; furthermore, Vasudevan *et al.* [49] underlined that 32 out of 35 (91.42%) *S. aureus* strains were slime positive after 24 - 48 h of incubation, indicating that *S. aureus* biofilm production depends on the incubation time.

Staphylococcus aureus is able to adhere and form biofilms and therefore cause severe infections [6]. This pathogen has the ability to produce a number of exoenzymes, some of them are involved in virulence [50]. PIA production is responsible for the staphylococcal biofilm development [43]. Biofilm formation is considered as a two-step process that requires the adhesion of bacteria to a surface or substrate followed by a cell-cell adhesion, forming the multiple layers of the biofilm [18].

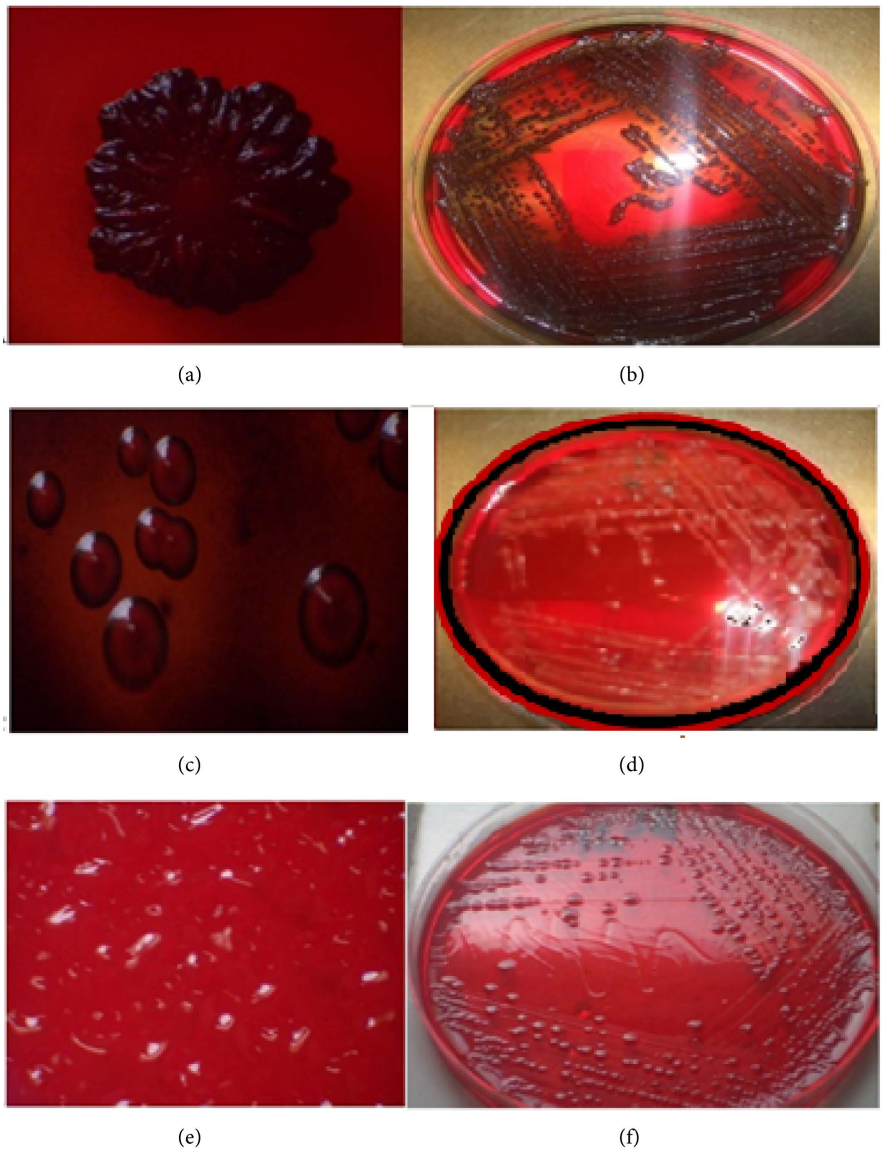

Figure 4. Appearance of *Staphylococcus aureus* colonies on Congo red agar (*CRA*). (a) Crystalline dry and slightly black colony; (b) *S. aureus* biofilm-forming strain; (c) "bulls eye" bearing colonies; (d) *S. aureus* non biofilm-forming strain; (e) Negative control 1 (*Pseudomonas syringae*); (f) Negative control 2 (*E. coli BL* 21).

It was shown by several authors that most of *S. aureus* strains have the entire *ica* operon [20] [51]. Thereby, the single *icaA* expression induces only low enzyme activity, whereas the co-expression of *icaA* and *icaD* leads to a significant increase of activity and is related to the phenotypic expression of capsular polysaccharide PIA [52].

S. aureus Adhesion Test to *Tetrahymena pyriformis* cells

Adhesion is an essential step in the development of the infectious process. It is recognized that, in order to adhere to human tissue surfaces, a bacterium must first adhere to appropriate host cells or to their extracellular matrix, in order to withstand the various mechanisms that may eliminate it [53].

Different human cell lines were used to study the phenomenon of bacterial adhesion but most of them are very expensive and are likely to be contaminated during subcultures. For this reason, we chose to use the ciliated protist *Tetrahymena pyriformis* as a more suitable host to which *S. aureus* may adhere.

Comparative analysis of the obtained results showed that among the fifteen bacterial strains studied, three of them {Sa67, Sa99 and Sa114} (20%) showed a strong adhesion to protist cells, six {Sa65, Sa116, Sa122, SARM, Ria and the reference strain ATCC25923} (40%) had a moderate adhesion capacity, and the remaining six strains (40%) presented poor adhesion to the protist cells (**Figure 5**).

The capacity of *S. aureus* to adhere to host cells is considered the first step towards colonization and then cell infection. It is generally accepted that the ability of the bacteria to adhere to the host cells surface is an important factor in the initial interaction between *S. aureus* and its host [54] [55] [56].

Anti-adhesion effect of some plant extracts

The antibacterial effect of a number of plant extracts on *S. aureus* was first studied in order to search for the optimal concentration of each extract for which bacterial adhesion is inhibited without stressing the protist. For this purpose, the well diffusion technique was used. The obtained results are shown in **Table 2**.

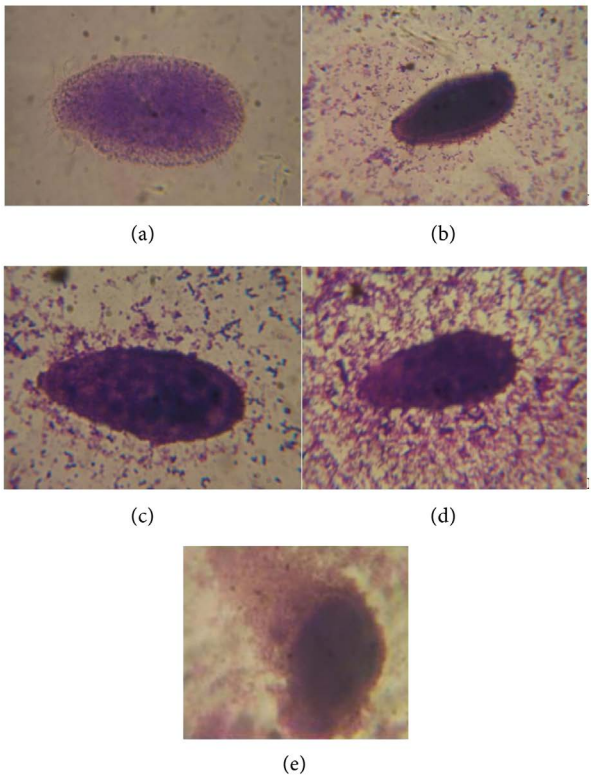

Figure 5. Adhesion test of *Staphylococcus aureus* to *Tetrahymena pyriformis* cells observed under light microscope (magnification, ×100). (a) Control; (b) *S. aureus* strains with low adhesion capacity; (c) Strains with moderate adhesion capacity; (d) and (e) Strains with strong adhesion capacity.

The well diffusion technique revealed that all the tested plant extracts (*Pisenlit, C. cirrhosa, M. crystallinum* and *R. peregrina*) exhibit an antibacterial effect. Interestingly, the *R. peregrina* extract produced a growth inhibition zone of 1 cm and showed the strongest effect, followed by *C. cirrhosa* and *M. crystallinum* with an inhibition zone of 0.8 cm. Thereby, *R. peregrina* and *C. cirrhosa* extracts were selected for further tests.

Our results are consistent with a study on the antibacterial effect of *R. peregrina*, which showed that the ethyl acetate and chloroform fractions of the extract are effective against *S. aureus* and *Escherichia coli*, respectively [57]. The anti-adhesion effects of *R. peregrina* and *C. cirrhosa* extracts were assessed and the results are shown in **Figure 6**.

Our results clearly show that both plant extracts exhibit an anti-adhesion effect. However, it seems that *C. cirrhosa* features a better effect on *S. aureus* adhesion-inhibition without stressing *T. pyriformis cells*.

The results obtained with the *S. aureus* strains on the relationships between the presence of genes encoding adhesion proteins, biofilm formation phenotypes and adhesion tests with *Tetrahymena pyriformis* are summarized in **Table 3**.

Table 2. Antibacterial effect of plant extracts on the *S. aureus* strain "114" in solid medium. The antibacterial effect was determined by measuring the diameter of the growth inhibition zone. 50 µl of plant extract were poured on the wells.

Ampicilline (100 µg/ml)	*Pisenlit*	*C. cirrhosa*	*M. crystallinum*	*R. peregrina*
0.4 cm	0.6 cm	0.8 cm	0.8 cm	1 cm

Table 3. Summary of *S. aureus* strains features showing the relationships between presence of *cna* and *icaD* genes, biofilm formation and adhesion tests with *Tetrahymena pyriformis*.

Strains	*AF*	*cna*	*icaD*	Biofilm phenotype	Slime production	*Adhesion to Tetrahymena pyriformis
ATCC 25923	+	+	+	Black	Producer	++
Sa 1	+	+	+	Almost Black	Producer	+
Sa 4	+	+	+	Dark Black	Producer	+
Sa 33	+	+	−	Red	Non Producer	+
Sa 65	+	+	+	Almost Black	Producer	++
Sa 100	+	+	+	bordeaux	Non Producer	+
Sa 91	+	−	−	bordeaux	Non Producer	+
Sa 114	+	+	+	Almost Black	Producer	+++
SARM	+	+	−	Red	Non Producer	++
Sa 67	+	+	+	Almost Black	Producer	+++
Sa 122	+	−	+	Black	Producer	++
Sa 99	+	+	+	Black	Producer	+++
Sa 116	+	+	+	Bordeaux	Non Producer	++
Sa 134	+	−	−	Dark black	Producer	+
Ria	+	+	+	Almost Black	Producer	++

*+++: Strong adhesion capacity; ++: Moderate adhesion capacity; +: Low adhesion capacity.

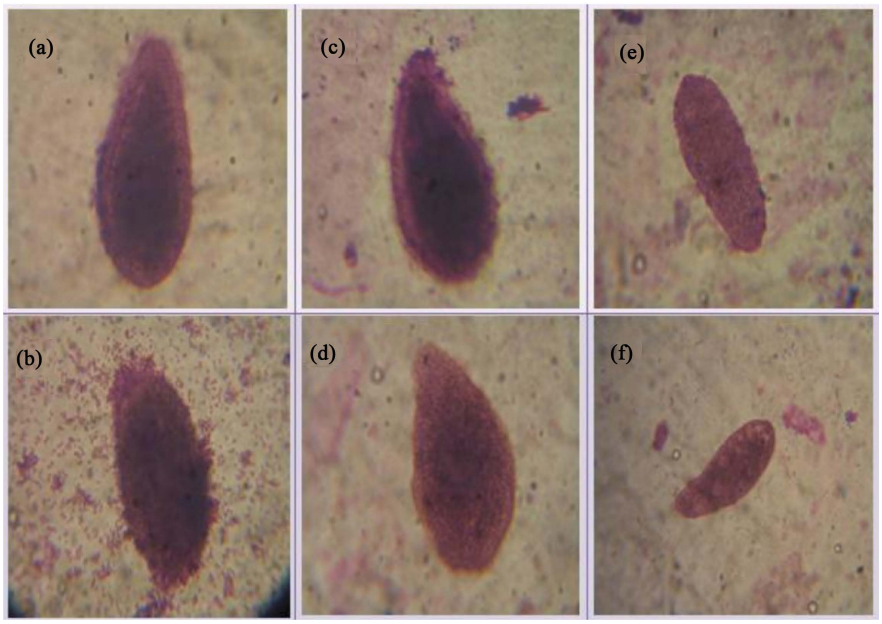

Figure 6. Determination of the inhibitory effect of *C. cirrhosa* and *R. peregrina* extracts on adhesion of *S. aureus* to *T. pyriformis* cells. (a) *T. pyriformis* control; (b) *T. pyriformis* plus *S. aureus* 114 ; (c) *T. pyriformis* plus *C. cirrhosa* extract; (d) *T. pyriformis* plus *S. aureus* 114 plus *C. cirrhosa* extract; (e) *T. pyriformis* plus *R. peregrina* extract; (f) *T. pyriformis* plus *S. aureus* 114 plus *R. peregrina* extract (magnification, ×100).

4. Conclusions

In conclusion, our study confirms variations in the presence and expression of genes encoding important adhesion proteins among clinical *S. aureus* strains. Slime production is also subjected to strain selection and can be affected by the environmental conditions.

In addition, we suggest that our study on *S. aureus* adhesion to *Tetrahymena pyriformis* cells may be considered as a first step in understanding the establishment of different adhesion mechanisms in the pathogenesis of medical device-associated staphylococcal infections. Furthermore, the anti-adhesion effect of plant extracts could be relevant in the development of new preventive and therapeutic approaches against staphylococcal infections.

References

[1] Navarre, W.W. and Schneewind, O. (1999) Surface Proteins of Gram-Positive Bacteria and Mechanisms of Their Targeting to the Cell Wall Envelope. *Microbiology and Molecular Biology Reviews*, **63**, 174-229.

[2] Smith, E.J., Visai, L., Kerrigan, S.W., Speziale, P. and Foster, T.J. (2011) The Sbi Protein Is a Multifunctional Immune Evasion Factor of *Staphylococcus aureus*. *Infection and Immunity*, **79**, 3801-3809. https://doi.org/10.1128/IAI.05075-11

[3] Le Loir, Y., Baron, F. and Gautier, M. (2003) *Staphylococcus aureus* and Food Poisoning. *Genetics and Molecular Research,* **2**, 63-76.

[4] Stefani, S., Chung, D.R., Lindsay, J.A., Friedrich, A.W., Kearns, A.M., Westh, H. and MacKenzie, F.M. (2012) Meticillin-Resistant *Staphylococcus aureus* (MRSA): Glob-

al Epidemiology and Harmonisation of Typing Methods. *International Journal of Antimicrobial Agents*, **39**, 273-282. https://doi.org/10.1016/j.ijantimicag.2011.09.030

[5] Alegre, M.-L., Chen, L., David, M.Z., Bartman, C., Boyle-Vavra, S., Kumar, N., Chong, A.S. and Daum, R.S. (2016) Impact of *Staphylococcus aureus* USA300 Colonization and Skin Infections on Systemic Immune Responses in Humans. *The Journal of Immunology*, **197**, 1118-1126. https://doi.org/10.4049/jimmunol.1600549

[6] Doster, R.S., Kirk, L.A., Tetz, L.M., Rogers, L.M., Aronoff, D.M. and Gaddy, J.A. (2016) *Staphylococcus aure*us Infection of Human Gestational Membranes Induces Bacterial Biofilm Formation and Host Production of Cytokines. *The Journal of Infectious Diseases*, jiw300. https://doi.org/10.1093/infdis/jiw300

[7] Chavakis, T., Preissner, K.T. and Herrmann, M. (2007) The Anti-Inflammatory Activities of *Staphylococcus aureus*. *Trends in Immunology*, **28**, 408-418. https://doi.org/10.1016/j.it.2007.07.002

[8] Foster, T.J. and Höök, M. (1998) Surface Protein Adhesins of *Staphylococcus aureus*. *Trends in Microbiology*, **6**, 484-488. https://doi.org/10.1016/S0966-842X(98)01400-0

[9] Greene, C., McDevitt, D., Francois, P., Vaudaux, P.E., Lew, D.P. and Poster, T.J. (1995) Adhesion Properties of Mutants of *Staphylococcus aureus* Defective in Fibronectin-Binding Proteins and Studies on the Expression of Fnb Genes. *Molecular Microbiology*, **17**, 1143-1152. https://doi.org/10.1111/j.1365-2958.1995.mmi_17061143.x

[10] Vaudaux, P.E., François, P., Proctor, R.A., McDevitt, D., Foster, T.J., Albrecht, R.M., Lew, D.P., Wabers, H. and Cooper, S.L. (1995) Use of Adhesion-Defective Mutants of *Staphylococcus aureus* to Define the Role of Specific Plasma Proteins in Promoting Bacterial Adhesion to Canine Arteriovenous Shunts. *Infection and Immunity*, **63**, 585-590.

[11] Harraghy, N., Hussain, M., Haggar, A., Chavakis, T., Sinha, B., Herrmann, M. and Flock, J.-I. (2003) The Adhesive and Immunomodulating Properties of the Multifunctional *Staphylococcus aureus* Protein Eap. *Microbiology*, **149**, 2701-2707. https://doi.org/10.1099/mic.0.26465-0

[12] Clarke, S.R., Harris, L.G., Richards, R.G. and Foster, S.J. (2002) Analysis of Ebh, a 1.1-Megadalton Cell Wall-Associated Fibronectin-Binding Protein of *Staphylococcus aureus*. *Infection and Immunity*, **70**, 6680-6687. https://doi.org/10.1128/IAI.70.12.6680-6687.2002

[13] Hussain, M., Becker, K., von Eiff, C., Schrenzel, J., Peters, G. and Herrmann, M. (2001) Identification and Characterization of a Novel 38.5-Kilodalton Cell Surface Protein of *Staphylococcus aureus* with Extended-Spectrum Binding Activity for Extracellular Matrix and Plasma Proteins. *Journal of Bacteriology*, **183**, 6778-6786. https://doi.org/10.1128/JB.183.23.6778-6786.2001

[14] Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H. and Laufs, R. (1996) The Intercellular Adhesin Involved in Biofilm Accumulation of *Staphylococcus epidermidis* Is a Linear Beta-1,6-Linked Glucosaminoglycan: Purification and Structural Analysis. *Journal of Bacteriology*, **178**, 175-183. https://doi.org/10.1128/jb.178.1.175-183.1996

[15] Bellon-Fontaine, M.-N., Rault, J. and van Oss, C.J. (1996) Microbial Adhesion to Solvents: A Novel Method to Determine the Electron-Donor/Electron-Acceptor or Lewis Acid-Base Properties of Microbial Cells. *Colloids and Surfaces B: Biointerfaces*, **7**, 47-53. https://doi.org/10.1016/0927-7765(96)01272-6

[16] Briandet, R., Lacroix-Gueu, P., Renault, M., Lecart, S., Meylheuc, T., Bidnenko, E., Steenkeste, K., Bellon-Fontaine, M.-N. and Fontaine-Aupart, M.-P. (2008) Fluorescence Correlation Spectroscopy to Study Diffusion and Reaction of Bacteriophages inside Biofilms. *Applied and Environmental Microbiology*, **74**, 2135-2143. https://doi.org/10.1128/AEM.02304-07

[17] Gross, M. (2011) Revived Interest in Bacteriophages. *Current Biology*, **21**, R267-R270. https://doi.org/10.1016/j.cub.2011.04.008

[18] Heilmann, C., Gerke, C., Perdreau-Remington, F. and Götz, F. (1996) Characterization of *Tn*917 Insertion Mutants of *Staphylococcus epidermidis* Affected in Biofilm Formation. *Infection and Immunity*, **64**, 277-282.

[19] Allignet, J., Aubert, S., Dyke, K.G.H. and Solh, N.E. (2001) *Staphylococcus caprae* Strains Carry Determinants Known to Be Involved in Pathogenicity: A Gene Encoding an Autolysin-Binding Fibronectin and the *Ica operon* Involved in Biofilm Formation. *Infection and Immunity*, **69**, 712-718. https://doi.org/10.1128/IAI.69.2.712-718.2001

[20] Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W. and Götz, F. (1999) The Intercellular Adhesion (ica) Locus Is Present in *Staphylococcus aureus* and Is Required for Biofilm Formation. *Infection and Immunity*, **67**, 5427-5433.

[21] Hussain, M., Herrmann, M., von Eiff, C., Perdreau-Remington, F. and Peters, G. (1997) A 140-Kilodalton Extracellular Protein Is Essential for the Accumulation of *Staphylococcus epidermidis* Strains on Surfaces. *Infection and Immunity*, **65**, 519-524.

[22] Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, Í. and Penadés, J.R. (2001) Bap, a *Staphylococcus aureus* Surface Protein Involved in Biofilm Formation. *Journal of Bacteriology*, **183**, 2888-2896. https://doi.org/10.1128/JB.183.9.2888-2896.2001

[23] van der Borden, A.J., van der Werf, H., van der Mei, H.C. and Busscher, H.J. (2004) Electric Current-Induced Detachment of *Staphylococcus epidermidis* Biofilms from Surgical Stainless Steel. *Applied and Environmental Microbiology*, **70**, 6871-6874. https://doi.org/10.1128/AEM.70.11.6871-6874.2004

[24] Stoodley, P., Cargo, R., Rupp, C.J., Wilson, S. and Klapper, I. (2002) Biofilm Material Properties as Related to Shear-Induced Deformation and Detachment Phenomena. *Journal of Industrial Microbiology & Biotechnology*, **29**, 361-367. https://doi.org/10.1038/sj.jim.7000282

[25] Stoodley, P., Wilson, S., Hall-Stoodley, L., Boyle, J.D., Lappin-Scott, H.M. and Costerton, J.W. (2001) Growth and Detachment of Cell Clusters from Mature Mixed-Species Biofilms. *Applied and Environmental Microbiology*, **67**, 5608-5613. https://doi.org/10.1128/AEM.67.12.5608-5613.2001

[26] Donlan, R.M. (2002) Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases*, **8**, 881-890. https://doi.org/10.3201/eid0809.020063

[27] Stoodley, P., Sauer, K., Davies, D.G. and Costerton, J.W. (2002) Biofilms as Complex Differentiated Communities. *Annual Review of Microbiology*, **56**, 187-209. https://doi.org/10.1146/annurev.micro.56.012302.160705

[28] Allison, K.R., Brynildsen, M.P. and Collins, J.J. (2011) Metabolite-Enabled Eradication of Bacterial Persisters by Aminoglycosides. *Nature*, **473**, 216-220. https://doi.org/10.1038/nature10069

[29] Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999) Bacterial Biofilms: A Common Cause of Persistent Infections. *Science*, **284**, 1318-1322. https://doi.org/10.1126/science.284.5418.1318

[30] Lee, J.C., Park, J.S., Shepherd, S.E., Carey, V. and Fattom, A. (1997) Protective Efficacy of Antibodies to the *Staphylococcus aureus* Type 5 Capsular Polysaccharide in a Modified Model of Endocarditis in Rats. *Infection and Immunity*, **65**, 4146-4151.

[31] Wrangstadh, M., Conway, P.L. and Kjelleberg, S. (1986) The Production and Release of an Extracellular Polysaccharide during Starvation of a Marine *Pseudomonas sp*. and the Effect Thereof on Adhesion. *Archives of Microbiology*, **145**, 220-227. https://doi.org/10.1007/BF00443649

[32] Zhang, Y.-Y., Yang, J., Yin, X.-X., Yang, S.-P. and Zhu, Y.-G. (2012) Arsenate Toxicity and Stress Responses in the Freshwater Ciliate *Tetrahymena pyriformis*. *European Journal of Protistology*, **48**, 227-236. https://doi.org/10.1016/j.ejop.2012.01.005

[33] Holderbaum, D., Spech, T., Ehrhart, L.A., Keys, T. and Hall, G.S. (1987) Collagen Binding in Clinical Isolates of *Staphylococcus aureus*. *Journal of Clinical Microbiology*, **25**, 2258-2261.

[34] Zmantar, T., Chaieb, K., Makni, H., Miladi, H., Abdallah, F.B., Mahdouani, K. and Bakhrouf, A. (2008) Detection by PCR of Adhesins Genes and Slime Production in Clinical *Staphylococcus aureus*. *Journal of Basic Microbiology*, **48**, 308-314. https://doi.org/10.1002/jobm.200700289

[35] Duran, N., Dogramaci, Y., Ozer, B., Demir, C. and Kalaci, A. (2010) Detection of Adhesin Genes and Slime Production among *Staphylococci* in Orthopaedic Surgical Wounds. *African Journal of Microbiology Research*, **4**, 708-715.

[36] Peacock, S.J., de Silva, I. and Lowy, F.D. (2001) What Determines Nasal Carriage of *Staphylococcus aureus*? *Trends in Microbiology*, **9**, 605-610. https://doi.org/10.1016/S0966-842X(01)02254-5

[37] Arciola, C.R., Campoccia, D., Gamberini, S., Baldassarri, L. and Montanaro, L. (2005) Prevalence of *cna fnbA* and *fnbB* Adhesin Genes among *Staphylococcus aureus* Isolates from Orthopedic Infections Associated to Different Types of Implant. *FEMS Microbiology Letters*, **246**, 81-86. https://doi.org/10.1016/j.femsle.2005.03.035

[38] Tristan, A., Ying, L., Bes, M., Etienne, J., Vandenesch, F. and Lina, G. (2003) Use of Multiplex PCR to Identify *Staphylococcus aureus* Adhesins Involved in Human Hematogenous Infections. *Journal of Clinical Microbiology*, **41**, 4465-4467. https://doi.org/10.1128/JCM.41.9.4465-4467.2003

[39] Rohde, H., Knobloch, J.K.M., Horstkotte, M.A. and Mack, D. (2001) Correlation of *Staphylococcus aureus icaADBC* Genotype and Biofilm Expression Phenotype. *Journal of Clinical Microbiology*, **39**, 4595-4596. https://doi.org/10.1128/JCM.39.12.4595-4596.2001

[40] Freeman, D.J., Falkiner, F.R. and Keane, C.T. (1989) New Method for Detecting Slime Production by Coagulase Negative *Staphylococci*. *Journal of Clinical Pathology*, **42**, 872-874. https://doi.org/10.1136/jcp.42.8.872

[41] Zapotoczna, M., O'Neill, E. and O'Gara, J.P. (2016) Untangling the Diverse and Redundant Mechanisms of *Staphylococcus aureus* Biofilm Formation. *PLOS Pathogens*, **12**, e1005671. https://doi.org/10.1371/journal.ppat.1005671

[42] Arciola, C.R., Baldassarri, L. and Montanaro, L. (2001) Presence of *icaA* and *icaD* Genes and Slime Production in a Collection of *Staphylococcal* Strains from Catheter-Associated Infections. *Journal of Clinical Microbiology*, **39**, 2151-2156. https://doi.org/10.1128/JCM.39.6.2151-2156.2001

[43] Zmantar, T., Kouidhi, B., Miladi, H., Mahdouani, K. and Bakhrouf, A. (2010) A Microtiter Plate Assay for *Staphylococcus aureus* Biofilm Quantification at Various

pH Levels and Hydrogen Peroxide Supplementation. *New Microbiologica*, **33**, 137.

[44] Chaieb, K., Hajlaoui, H., Zmantar, T., Kahla-Nakbi, A.B., Rouabhia, M., Mahdouani, K. and Bakhrouf, A. (2007) The Chemical Composition and Biological Activity of Clove Essential Oil, *Eugenia caryophyllata* (*Syzigium aromaticum L. Myrtaceae*): A Short Review. *Phytotherapy Research*, **21**, 501-506. https://doi.org/10.1002/ptr.2124

[45] Rashid, M.H., Rumbaugh, K., Passador, L., Davies, D.G., Hamood, A.N., Iglewski, B.H. and Kornberg, A. (2000) Polyphosphate Kinase Is Essential for Biofilm Development, Quorum Sensing, and Virulence of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences*, **97**, 9636-9641. https://doi.org/10.1073/pnas.170283397

[46] Schlag, S., Nerz, C., Birkenstock, T.A., Altenberend, F. and Götz, F. (2007) Inhibition of *Staphylococcal* Biofilm Formation by Nitrite. *Journal of Bacteriology*, **189**, 7911-7919. https://doi.org/10.1128/JB.00598-07

[47] Shanks, R.M.Q., Meehl, M.A., Brothers, K.M., Martinez, R.M., Donegan, N.P., Graber, M.L., Cheung, A.L. and O'Toole, G.A. (2008) Genetic Evidence for an Alternative Citrate-Dependent Biofilm Formation Pathway in *Staphylococcus aureus* That Is Dependent on Fibronectin Binding Proteins and the GraRS Two-Component Regulatory System. *Infection and Immunity*, **76**, 2469-2477. https://doi.org/10.1128/IAI.01370-07

[48] Gündoğan, N., Citak, S. and Turan, E. (2006) Slime Production, DNase Activity and Antibiotic Resistance of *Staphylococcus aureus* Isolated from Raw Milk, Pasteurised Milk and Ice Cream Samples. *Food Control*, **17**, 389-392. https://doi.org/10.1016/j.foodcont.2005.01.006

[49] Vasudevan, P., Nair, M.K.M., Annamalai, T. and Venkitanarayanan, K.S. (2003) Phenotypic and Genotypic Characterization of Bovine Mastitis Isolates of *Staphylococcus aureus* for Biofilm Formation. *Veterinary Microbiology*, **92**, 179-185. https://doi.org/10.1016/S0378-1135(02)00360-7

[50] Dinges, M.M., Orwin, P.M. and Schlievert, P.M. (2000) Exotoxins of *Staphylococcus aureus*. *Clinical Microbiology Reviews*, **13**, 16-34. https://doi.org/10.1128/CMR.13.1.16-34.2000

[51] Martín-López, J.V., Pérez-Roth, E., Claverie-Martín, F., Gil, O.D., Batista, N., Morales, M. and Méndez-Álvarez, S. (2002) Detection of *Staphylococcus aureus* Clinical Isolates Harboring the *ica* Gene Cluster Needed for Biofilm Establishment. *Journal of Clinical Microbiology*, **40**, 1569-1570. https://doi.org/10.1128/JCM.40.4.1569-1570.2002

[52] Gerke, C., Kraft, A., Süßmuth, R., Schweitzer, O. and Götz, F. (1998) Characterization of the N-Acetylglucosaminyltransferase Activity Involved in the Biosynthesis of the *Staphylococcus epidermidis* Polysaccharide Intercellular Adhesin. *The Journal of Biological Chemistry*, **273**, 18586-18593. https://doi.org/10.1074/jbc.273.29.18586

[53] Jiang, X., Qin, Y.X., Lin, G.F., Huang, L., Huang, B., Huang, W.S. and Yan, Q.P. (2015) FlgN Plays Important Roles in the Adhesion of *Aeromonas hydrophila* to Host Mucus. *Genetics and Molecular Research*, **14**, 6376-6386. https://doi.org/10.4238/2015.June.11.13

[54] Agerer, F., Lux, S., Michel, A., Rohde, M., Ohlsen, K. and Hauck, C.R. (2005) Cellular Invasion by *Staphylococcus aureus* Reveals a Functional Link between Focal Adhesion Kinase and Cortactin in Integrin-Mediated Internalisation. *Journal of Cell Science*, **118**, 2189-2200. https://doi.org/10.1242/jcs.02328

[55] Hauck, C.R., Agerer, F., Muenzner, P. and Schmitter, T. (2006) Cellular Adhesion Molecules as Targets for Bacterial Infection. *European Journal of Cell Biology*, **85**, 235-242. https://doi.org/10.1016/j.ejcb.2005.08.002

[56] Hauck, C.R. and Ohlsen, K. (2006) Sticky Connections: Extracellular Matrix Protein Recognition and Integrin-Mediated Cellular Invasion by *Staphylococcus aureus*. *Current Opinion in Microbiology*, **9**, 5-11. https://doi.org/10.1016/j.mib.2005.12.002

[57] Özgen, U., Houghton, P.J., Ogundipe, Y. and Coşkun, M. (2003) Antioxidant and Antimicrobial Activities of *Onosma argentatum* and *Rubia peregrine*. *Fitoterapia*, **74**, 682-685. https://doi.org/10.1016/S0367-326X(03)00161-8