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Study of the Adhesion of Clinical Strains of *Staphylococcus aureus* on an Abiotic Surface Using the Biofilm Ring Test®

J. M. Liesse Iyamba[1,2], N. B. Takaisi-Kikuni[2], S. Dulanto[1], J. P. Dehaye[1]

[1]Laboratoire de Chimie biologique et médicale et de Microbiologie pharmaceutique, Faculté de Pharmacie, Université libre de Bruxelles, Brussels, Belgium; [2]Laboratoire de Microbiologie Expérimentale et Pharmaceutique, Faculté des Sciences Pharmaceutiques, Université de Kinshasa, Kinshasa, Democratic Republic of Congo.
Email: jliessei@ulb.ac.be

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ABSTRACT

Four methicillin-sensitive (MSSA) and 4 methicillin-resistant (MRSA) strains of *Staphylococcus aureus* were collected and isolated at the Laboratory of Bacteriology of the Provincial General Reference Hospital of Kinshasa in the Democratic Republic of Congo. The microbial adhesion to solvents (MATS) test showed that the MRSA strains had a less hydrophobic membrane than the MSSA strains. Using the Biofilm Ring Test® (BFRT®) to investigate on the adhesion of these bacterial strains to smooth surfaces, we observed that the MSSA strains adhered more rapidly than the MRSA strains. The biomass of the produced biofilm measured by the Crystal violet staining method (CVSM) was more important with MSSA than with MRSA strains. Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) inhibited the adhesion and the formation of a biofilm by MRSA strains; this inhibition was reversed by calcium, magnesium and manganese. The MRSA strains adhered less to silicon tubing and the adhesion was inhibited by EGTA in 2 of the 4 MRSA strains and none of the MSSA strains. In conclusion, the MSSA and MRSA strains adhered on an abiotic surface and formed a biofilm at distinct rates and with different sensitivities to ions. The results also confirm the utility as well as the limits of the BFRT® to study the adhesion of bacteria on a surface.

Keywords: Biofilm Ring Test®; Crystal Violet; Cell Surface; Hydrophobicity; Adhesion; Catheter Tube

1. Introduction

Staphylococcus aureus is a human pathogen that causes both chronic and nosocomial infections; many of which are mediated by their ability to adhere to medical devices and to form biofilms. This bacteria is responsible of a large variety of diseases, including endocarditis, osteomyelitis, and foreign body infections [1]. A biofilm is defined as aggregated, microbial cells surrounded by a polymeric self-produced matrix, which may contain host components [2]. According to the Center for Disease Control and Prevention, 65% of human bacterial infections are associated to a biofilm [3]. Furthermore, biofilms infections are a major problem in the clinic and cause many deaths and high health costs, e.g. 12% to 25% of patient mortality are attributable to catheter-related bloodstream infections [4]. Biofilm-associated bacteria are generally resistant to antibiotics [5], and to host immune responses [6].

Two main stages are involved in *S. aureus* biofilm formation [7]. In a first step, the cells adhere on an abiotic or a biotic surface. The adherence of *S. aureus* to foreign bodies depends on the cell surface characteristics of the micro-organisms and the nature of foreign body material and involves physicochemical forces such as polarity, London-van der Waals forces and hydrophobic interactions [8]. Divalent cationic ions (e.g. magnesium, calcium) may enhance the attachment of bacteria to surface by reducing electrostatic repulsion and stabilizing interaction between the negatively charged surface of bacteria and anionic substrates [9]. Cell surface hydrophobicity and initial adherence of micro-organisms to a surface have been attributed to different bacterial surface-associated adhesins. The attachment of *S. aureus* on a biotic surface is likely to be mediated by cell-wall associated proteins such as the microbial surface components recognizing adhesive matrix molecules (MSCRAMM). The second stage of biofilm development includes cells multiplication and formation of a mature structure consisting of many cell layers. This stage is associated with the production of extracellular factors which may include exo-polysaccharides, proteins and extracellular DNA (eDNA) [10]. Excretion of polysaccharide intercellular adhesion (PIA) polymers in *Staphylococcus* species and the presence of divalent cations interact to form stronger

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bonding between cells [11]. The detachment of cells from established biofilms allows the spreading and the colonization of new sites by planctonic bacteria [12].

Colorimetric microtiter plate systems are widely used to determine bacterial adhesion [13]. Among these techniques, the Crystal Violet staining method (CVSM) has been modified to increase its accuracy and to allow the quantification of the biomass of the biofilm in the entire well [14]. Despite the fact that this method is suitable for estimating the adherent cells stained after washing steps, it requires cultures of at least 24 to 48 h. The Biofilm Ring Test® (BFRT®), a newly described method, has been recently proposed as an alternative to the CVSM for studying bacterial adhesion [15]. This technique based on the immobilization of magnetic beads by adherent cells had not only been implemented to study the initial steps of biofilm formation [16,17], but also to explore the composition of *Staphylococcus aureus* and *Leuconostoc mesenteroides* biofilm matrix [15,18]. The aim of this study was to compare the adhesion of clinical methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) strains and to determine the effect of divalent cations on the inhibition of bacterial adhesion by ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA).

2. Materials and Methods

2.1. Origin of the Strains and Growth Conditions

The 8 strains used in this study were collected for diagnostic purposes in the Laboratory of Bacteriology of the Provincial General Reference Hospital of Kinshasa (HGPRK) in the Democratic Republic of Congo. Among the 4 MSSA strains, 2 strains (the 5668/B and 5741/B strains) were isolated from blood samples and the 2 other strains (the 1532/SW and 1620/SW) were from skin wounds. Among the 4 MRSA strains, 2 strains (the 007/FV and the 028/FV) were collected from a vaginal smear test, 1 strain (the 011/LP) from prostatic fluid and 1 strain (027/U) from urine. The bacteria were grown and isolated on Brain Heart Agar with 5% (v/v) sheep blood and on Mannitol Salt Agar (Difco, BD Franklin Lakes, NJ, USA). The identification of *S. aureus* was performed with the latex agglutination test (Pastorex Staph-Plus, Biorad, Marnes-la-Coquette, France). All MRSA strains were positive for *mecA* gene (data not shown).

2.2. Characterization of Cell Surface Properties of the Strains

The microbial adhesion to solvents (MATS) test was performed to estimate, simultaneously, the hydrophobicity and the electron-acceptor and electron-donor characteristics of the bacterial membranes [19]. Briefly, the bacteria strains were cultivated on TSA medium and incubated for 24 h at 37˚C. TSB medium was inoculated with 2 or 3 colonies and incubated for 24 h at 37˚C. The bacteria were then harvested by centrifugation and washed twice with 150 mM potassium phosphate. The bacteria were suspended in the same buffer and adjusted to a concentration of ~10^8 CFU/mL. An aliquot of this suspension (2.4 mL) was mixed with 0.4 mL of one of 2 pairs of organic solvents and vigorously shaken by vortexing for 2 min. Each pair of polar and non-polar solvents had similar Lifshitz-van der Waals surface tension properties. The two pairs of solvents tested were 1) chloroform (an acidic solvent) and hexadecane and 2) ethyl acetate (a strong basic solvent) and hexane, its *n*-alkane apolar control [19]. The mixture was allowed to stand for 15 min to ensure complete separation of the two phases. The OD of the aqueous phase was measured at 400 nm. The adhesion to each solvent was calculated by the equation: % adhesion = $(1 - A/A_0) \times 100$, where A_0 was the absorbance of the bacterial suspension (aqueous phase) before mixing and A was the absorbance after mixing. The experiment was repeated at least 3 times for each strain.

2.3. Evaluation of Bacterial Adherence in a Smooth Surface Using the BFRT®

The Biofilm Ring Test® (BFRT®) is based on the immobilization of the magnetic beads by adherent cells. Consequently, the more beads are entrapped by cells, the fewer they are detectable after magnetization and, subsequently, the more cells are attached to the surface [15]. Bacterial adhesion was assessed using an appropriate kit commercially available (Biofilm Control, Saint Beauzire, France). The toner solution (TON005) containing negatively charged magnetic beads of 1 - 3 μm was mixed with a calibrated Initial Bacterial Suspension (IBS) containing ~10^7 bacteria/mL. Two hundred μL of this mixture were placed in the wells of a 96-wells polystyrene plate formed by the assembly of 12 individual 8-wells strips (Strip Well MSW002B). After incubation time at 37˚C, wells of the strip were covered with a few drops of contrast liquid (inert opaque oil used for the reading step) and scanned with the plate reader to get I_o image. Then, the plate was placed for 1 min on the magnet (Blok test) and scanned again to get I_1 image. The adhesion capability of each strain was expressed as the Biofilm Index (BFI) calculated by the software and based on the comparison of the two images. When BFI ≥ 7, a spot corresponding to the microbeads attracted by the magnet is visible and indicates the absence of adhesion or biofilm. When BFI ≤ 2, no spot can be observed after magnetiza-

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tion indicating that microbeads are blocked by adherent cells. Biofilm in formation results in microbeads partially blocked $2 \leq BFI \leq 7$. The experiment was repeated at least 3 times for each strain.

2.4. Evaluation of the Formation of a Biofilm with the CVSM

Polystyrene sterile strips were inoculated with 200 µL of IBS and incubated for various times at 35˚C in a humid atmosphere. A control well was inoculated with sterile medium. Each strain was evaluated in triplicate. Medium was removed from the wells which were washed 3 times with 200 µL sterile distilled water. The strips were air-dried for 45 min and the adherent cells were stained with 200 µL of 0.1% Crystal violet solution. After 45 min, the dye was eliminated and the wells were washed 5 times with 300 µL of sterile distilled water to remove excess stain. The dye incorporated by the cells forming a biofilm was dissolved with 200 µL of 33% (v/v) glacial acetic acid and the absorbance of each well was read at 540 nm in the microplate reader. The results were expressed as variation of $OD_{540\ nm}$ ($OD_{540\ nm}$ sample - $OD_{540\ nm}$ control). The experiment was repeated at least 3 times, for each strain and incubation time.

2.5. Study of the Adhesion of the Bacteria on Catheter Tubing

The bacteria were grown overnight and the $OD_{600\ nm}$ adjusted to 1.00 ± 0.05. They were then diluted 250-fold. A silicon tubing (2 cm long, 3 mm inner diameter) was incubated with the bacteria at 37˚C for 18 h. At the end of the incubation, the tubes were rinsed twice with water before adding 1 mL phosphate-buffer solution (pH 7.2). The cells were detached from the tubing by incubation in an ultrasound bath at 25˚C for 5 min. After serial dilutions of the bacterial suspension with PBS, the bacteria were plated on Petri dishes containing 15 mL TSA medium and the Petri dishes were incubated at 35˚C for 48 h before counting the colonies. Dishes with less than 50 colonies or with more than 500 colonies were not counted. Results are expressed as colonies forming units per mL (CFU/mL).

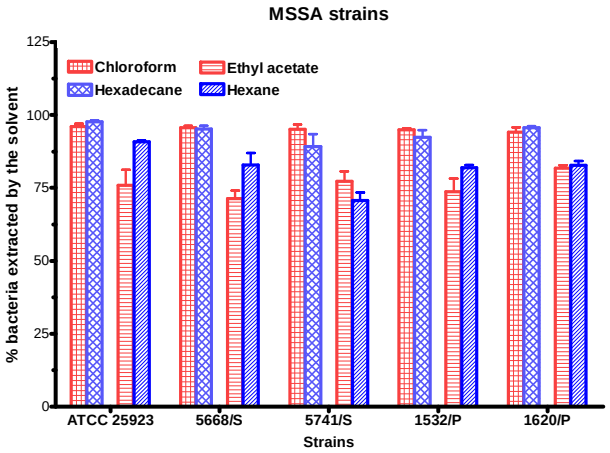
2.6. Statistical Analysis

Results were analyzed with the Mann-Whitney non-parametric test. $^{***}P < 0.005$; $^{**}P < 0.01$; $^{*}P < 0.05$.

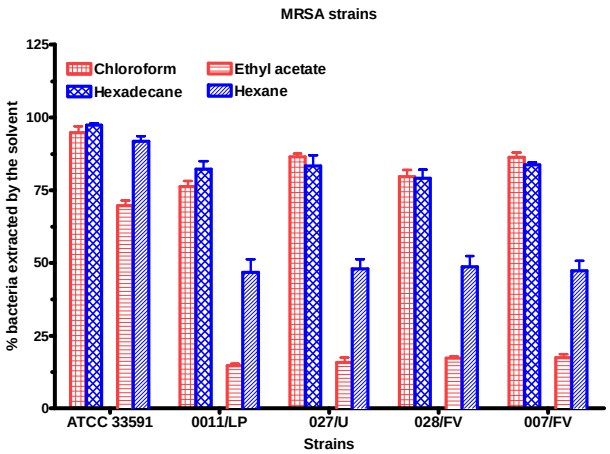
3. Results

3.1. Measure of the Adhesion of the Strains to Solvents

The hydrophobicity and the electron-donor and electron-acceptor properties of the cell walls of the various strains were tested according to Bellon-Fontaine *et al.* [19]. The 4 MSSA strains migrated nearly totally (from 90% to 97%) from the aqueous to the hexadecane phase establishing that their membrane was very hydrophobic. The 4 MRSA strains migrated slightly less (from 80% to 83%). The difference between MSSA (93.1% ± 1.4%) and MRSA strains (82.1% ± 1.2%) was significant ($P < 0.001$, $n = 12$) suggesting that MRSA strains were slightly less hydrophobic than MSSA strains (**Figures 1(a)** and **(b)**). The bacteria were also extracted with chloroform. The extraction of MSSA strains (95% ± 0.6%) was better than the extraction of MRSA strains (82.1% ± 1.5 %) ($P < 0.001$, $n = 12$) showing that the cell walls of MSSA strains were more basic (electron-donor capacity) than cell walls from



(a)



(b)

Figure 1. Study of the interaction of *S. aureus* with organic solvents. The affinity of 4 MSSA strains (a) or 4 MRSA strains (b) for chloroform, hexadecane, ethyl acetate or hexane was measured. Results are expressed as percentages of bacteria transferred from the aqueous phase to the organic solvent. Values are the means ± s.e.m. of 3 experiments.

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MRSA strains. The comparison of the extraction of the strains by ethyl acetate and hexane gave an estimate of the electron-accepting property of the strains. The 2 solvents extracted comparable percentages of the 4 MSSA strains showing that the cell walls of these bacteria had no electron-accepting propensity; ethyl acetate extracted the 4 MRSA strains 3-times less than hexane and it could be concluded that their walls were rather acidic.

3.2. Comparison between the BFRT® and the CVSM to Evaluate the Adhesion of the Bacteria

In a next experiment, the adhesion of the 8 strains on an abiotic surface (the bottom of the wells of a multi-well plate) was estimated using the BFRT® and the CVSM. As shown in **Figure 2(a)**, the 4 MSSA strains immobilized the magnetic beads of the BFRT®. Two MSSA strains (1620/SW and 1538/SW) fully blocked the beads within 2 h (drop of the BFRT® from 17.0 ± 0.1 to 2.4 ± 0.3, n = 18). The drop of the BFI was slightly slower for the 5668 and the 5741 strains (from 17.6 ± 0.2 to 9.3 ± 1.0 after 2 h). The BFI further dropped for the next 2 hours and reached 1.4 ± 0.1 after 4 h. The 4 MRSA stains immobilized the beads at a slower rate (**Figure 2b)**. After 2 hours, 3 strains (011/LP, 027/U and 028/FV) decreased the BFI from 15.5 ± 0.9 to 9.1 ± 0.6, n =27). These strains fully blocked the beads after 3 h (BFI: 2.1 ± 0.1, n = 27). One strain (007/FV) was much less effective and significantly blocked the beads only after 4 h (7.3 ± 1.6, n = 7). Similar experiments were performed in the wells of a multi-well plate and the biomass adhering at the bottom of the wells was assayed after staining with Crystal violet. As shown in **Figures 3(a)** and **(b)**, the formation of a biomass became significant after 3 h and differed among the strains. Some MSSA strains produced a very significant biomass after 4 h (5668/B > 1620/SW > 1532/SW > 5741/B). Among the MRSA strains, the 007/FV strain did not produce any significant biofilm after 4 h. From these results it could be concluded that the results obtained with the BFRT® and the CVSM were consistent but that the BFRT® was more efficient for the study of short-term effects.

3.3. Effect of Ions on the Formation of a Biofilm

Divalent cations contribute to the interaction among bacteria or their interaction with components of the extracellular matrix [20]. The purpose of these experiments was to test the effect of EGTA, a chelator of divalent cations, on the adhesion of bacteria and the interaction between EGTA and divalent cations. Preliminary experiments were performed to test whether these ionic conditions affected by themselves the mobility of the

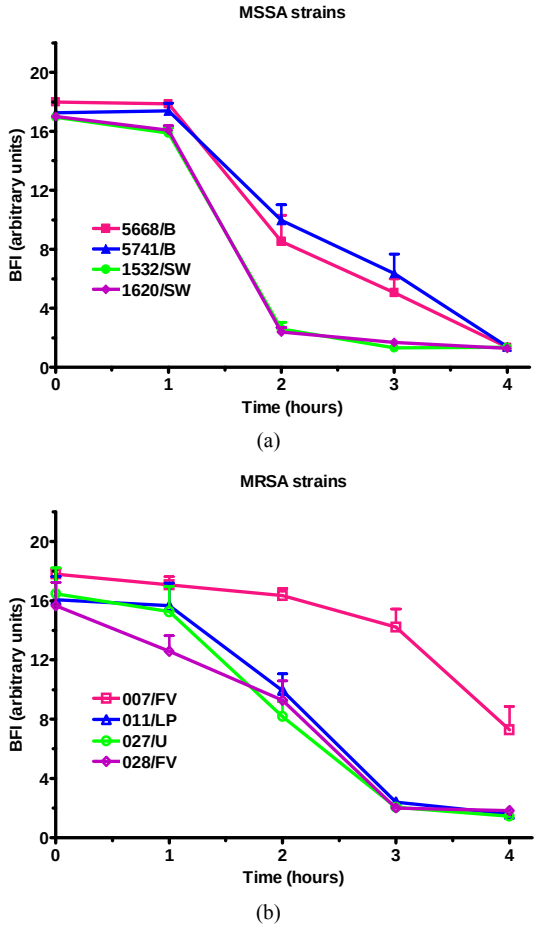

Figure 2. Time-course of the immobilization of magnetic beads by clinical strains of *S. aureus*. MSSA strains (a) and MRSA strains (b) of S. aureus were incubated at 35˚C in the presence of magnetic beads. The immobilization of the beads was measured after 1, 2, 3 or 4 h using the Biofilm Ring Test®. Results are expressed as the Biofilm Index (BFI). Data are the means ± s.e.m. of 3 experiments.

beads. As shown in **Figure 4(a)**, EGTA (from 100 µM to 1 mM) had no effect on the BFI. One mM calcium, magnesium and manganese decreased the BFI below 2. Only 1 mM EGTA was tested on the adhesion of the bacteria using the BFRT®. At this concentration, EGTA has no effect on the viability and the doubling time of the tested strains (data not shown). The bacteria were exposed to the chelator for 6 h. As shown in **Figure 4(b)**, the metal-chelator had no effect on the drop of the BFI provoked by the 4 MSSA strains. EGTA increased the BFI measured in the presence of 3 MRSA strains (the 011/LP, 027/U and 028/FV strains). The 007/FV was not affected by EGTA (**Figure 4(c)**). Considering the interaction of the ions with the BFRT®, the reversibility of the inhibition by EGTA was tested using the CVSM. The 4 MRSA strains were cultured for 24 h in control conditions or in the presence of 1 mM EGTA, in the absence

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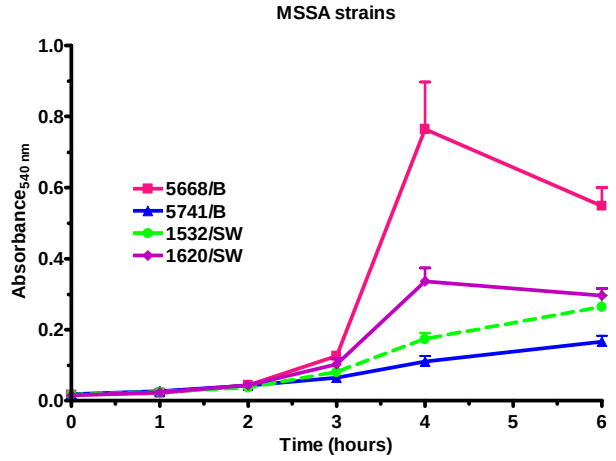
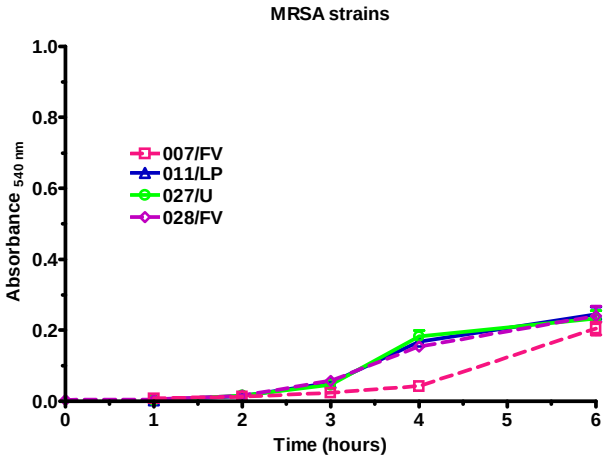


(a)



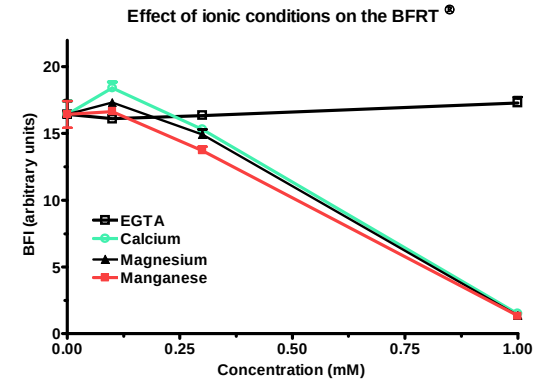
(b)

Figure 3. Study of the formation of a biofilm by clinical strains of *S. aureus* using the CVSM. MSSA strains (a) and MRSA strains (b) of *S. aureus* were incubated at 35˚C. The formation of a biofilm was measured after 1, 2, 3, 4 or 6 h using the CVSM. Results are expressed as the absorbance measured at 540 nm. Data are the means ± s.e.m. of 3 experiments.

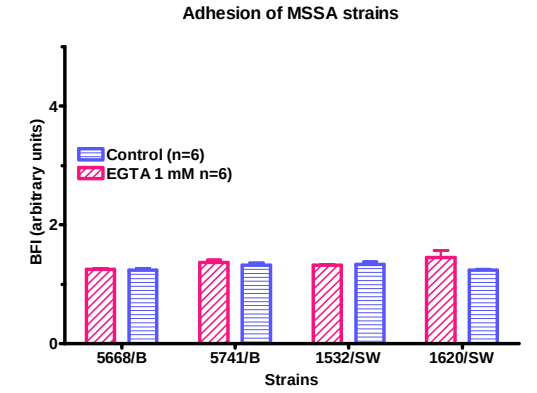
or in the presence of 1 mM Ca^{2+}, Mg^{2+} or Mn^{2+}. As shown in **Figure 5**, EGTA significantly decreased the biomass measured after 24 h with the 4 MRSA strains. Adding a divalent cation to the medium (calcium, magnesium or manganese) blocked the inhibition exerted by EGTA.

3.4. Study of the Adhesion of the Microbial Strains on Catheter Tube

Fragments of a catheter tube (2 cm long, 3 mm inner diameter) were incubated for 24 h in the presence of cellular suspensions from each strain (10^8 CFU/mL). After the incubation, the catheter tubes were washed twice and the adhering bacteria were detached from the tubing by



(a)



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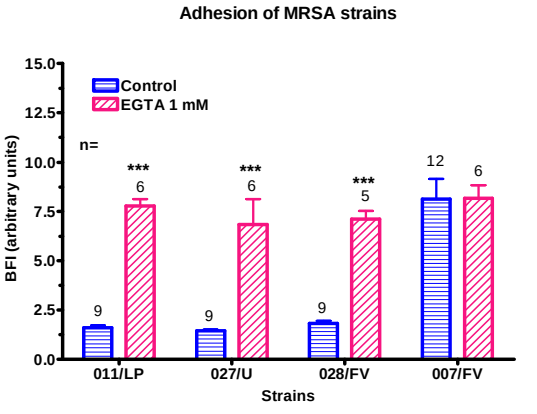

(c)

Figure 4. Effect of EGTA on the adhesion of bacteria measured with the BFRT®. (a) Magnetic beads were incubated for 4 h in the presence of increasing concentrations of EGTA, calcium, magnesium or manganese and in the absence of bacteria. At the end of the incubation, the BFI was measured. Results are the means ± s.e.m. of 3 experiments; (b) and (c) MSSA strains (middle panel) and MRSA strains (lower panel) of *S. aureus* were incubated at 35˚C in the presence of magnetic beads in the absence or in the presence of 1 mM EGTA. The immobilization of the beads was measured after 6 h using the Biofilm Ring Test®. Results are expressed as the Biofilm Index (BFI). Data are the means ± s.e.m of 6 (MSSA strains) or of n (MSSA strains) experiments. ***: P < 0.005 when compared to control.

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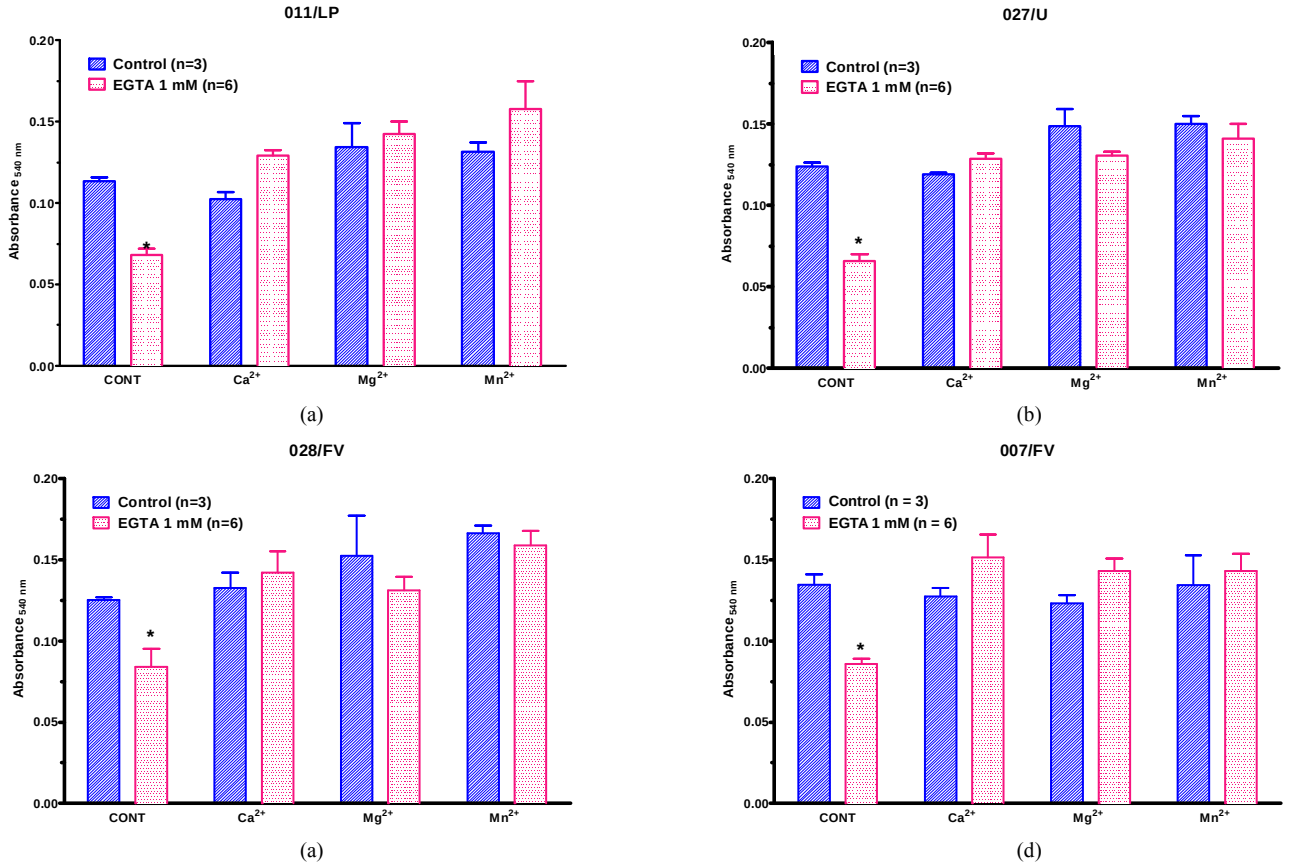

Figure 5. Reversibility of the inhibition by EGTA of the formation of a biofilm by MRSA strains. The 4 MRSA strains of *S. aureus* were incubated at 35˚C in the control conditions or in the presence of 1 mM EGTA, in the absence or in the presence of 1 mM calcium, magnesium or manganese. The formation of a biofilm was measured after 24 h using the CVSM. Results are expressed as the absorbance measured at 540 nm. Data are the means ± s.e.m. of n experiments. *: P < 0.05.

sonication for 5 minutes in an ultrasound bath.

The MSSA strains adhered more on the tubing ($1.095 \times 10^7 \pm 5.37 \times 10^6$) than the MRSA strains ($2.4 \times 10^5 \pm 6.75 \times 10^4$). Adding EGTA to the culture medium increased the adhesion of the MSSA strains ($1.176 \times 10^8 \pm 1.59 \times 10^7$) and decreased the adhesion of MRSA strains ($7.67 \times 10^4 \pm 2.24 \times 10^4$). The ANOVA analysis of the results followed by the Bonferroni post test confirmed that the adhesion of the MSSA strains was significantly more important than the adhesion of MRSA strains. EGTA had no significant effect on either group. Considering the large variations of the measurements, the results for each strain were analyzed. As shown in **Figures 6(a)-(d)**, the 4 MSSA strains were comparable and there was no effect of EGTA on either strain. Two MRSA strains (007/U and 028/FV) were not affected by EGTA. The chelator inhibited the adhesion of the 2 other MRSA strains (011/LP and 027/FV).

4. Discussion

In this work, we studied the interaction of clinical strains of *S. aureus* with an abiotic surface. We show that the BFRT® was a more rapid method than the CVSM to detect the adhesion of the bacteria to the surface. MSSA strains adhered more rapidly and the interaction of MRSA strains with an abiotic surface was inhibited by EGTA. Calcium, magnesium and manganese reversed the inhibition exerted by EGTA.

Different methods have been used to evaluate the adhesion of bacterial strains. Bacterial adhesion and the early stage of biofilm formation can be studied using reactors developed for the cultivation of biofilms like flow cells [21,22], annular biofilm reactors [23], fowler cells [24], modified Robbins devices [25], CDC biofilm reactors [26,27]. Disadvantages of such flow cell devices include limited sample area, and if operated on once-through flow basis, the potential exists for gradients in biofilm amount and fluid phase concentrations to develop in longitudinal direction. The examination and characterization of biofilms in medical devices, and human infections can also be performed using microscopy techniques (transmission electron, scanning electron, confocal

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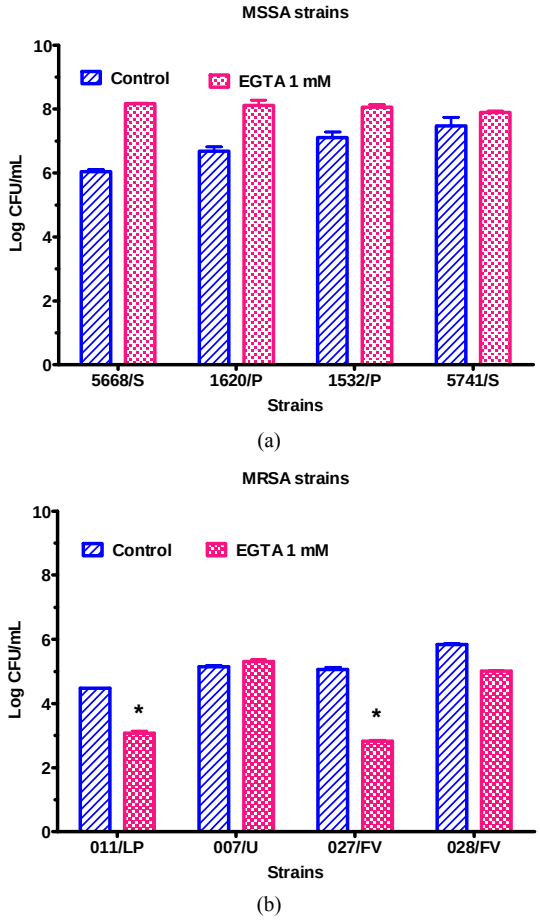

Figure 6. Adhesion of the clinical strains of *S. aureus* to a catheter. The 4 MSSA strains (a) and the 4 MRSA strains (b) were incubated for 18 h in the presence of a piece of catheter. After washing, the bacteria were detached by sonication and the number of colony forming units was estimated. Results are the means ± s.e.m. of 4 measurements. *: P < 0.05.

laser-scanning, epifluorescence, atomic force microscopy) [28-31]. These methods are mostly qualitative and descriptive, but they do not directly measure the adhesion of bacterial populations [32]. The enumeration of bacteria on plate counts after the removal of biofilms or biofilm-associated bacteria by mechanical forces such as vortexing or sonication is also widely used [33,34]. This method gives indication only on the number of bacteria inside the biofilm, and not on the number of bacteria adhering on a surface. This slow and fastidious method is also highly dependent on cell recovery which is difficult to estimate and is not fitted to large scale screening experiments. Microtiter plate methods have been also developed. Culture of the bacteria in the wells of the microplate followed by the estimation of the biomass remaining in the well after washing using the CVSM [35] is a very convenient and widespread method to evaluate biofilm adhesion. It has been modified to increase its accuracy and to improve its reproducibility [13,14]. As shown in this study, this method has a low sensitivity and requires rather long incubation times to get significant increase of the absorbance. The washing steps, used to remove non-adherent cells, are also critical. The lack of standardization of these washing steps makes difficult the comparison of results obtained by different laboratories [15]. The BFRT® is a newly described method for studying biofilm formation. This technique is based on the immobilization of magnetic beads by adherent cells [15, 17]. In this paper, we presented evidence that this method could detect adhesion of *S. aureus* to the bottom of the wells within a few hours, before any significant biofilm could be detected with the CVSM. These observations confirmed our previous results on *S. aureus* [18] or *P. aeruginosa* [17]. The BFRT® could also demonstrate that MSSA strains interacted more rapidly with the surface than MRSA strains. The adhesion of the MRSA strains was partly reverted by the presence in the culture medium of EGTA, while the adhesion of the MSSA strains was not affected by the metal-chelator. These results were confirmed with the CVSM which illustrated that, at later times, the MSSA strains formed a more important biofilm than MRSA strains. Our results were in agreement with those of O'Neill *et al.* [36]. These authors reported that MRSA strains were less likely to form a biofilm than MSSA strains and that their sensitivity to salts differed. They concluded that the mechanism involved in the formation of the biofilm was different among MSSA and MRSA strains. Another explanation for this difference between MSSA and MRSA strains could be their distinct membrane properties. The MATS test demonstrated that the two populations diverged: the membranes of the MSSA strains were more hydrophobic and had a higher propensity to donate electrons. These properties should affect the adherence of the bacteria [8]. Cations (calcium, magnesium, manganese) interfered with the behavior of the beads in the BFRT® and decreased the BFI. Similar artefactual interactions have also been described with pronase [37] or with culture media with high ionic strength [38]. Such interaction has also been observed with antimicrobial cationic peptides derived from cathelicidin and which, at neutral pH, have many positive charges (C. Nagant, personal communication). These interactions might thus be a consequence of some electrostatic interactions between the negatively charged beads and the cations or between some components of the medium. These results also illustrate the fact that the mobility of the beads can be affected not only by adhering bacteria but also by modifications of rheological properties of the medium. The interaction of the cations with EGTA was thus tested with the CVSM. The

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ions had no effect on the formation of the biofilm by the MRSA strains but the 3 cations reversed the inhibition by EGTA. This lack of specificity suggested that the effect of the ions was indirect. Considering the dissociation constant of EGTA for the 3 cations [39], the concentration of free EGTA should be very low (in the micromolar range) in solutions containing 1 mm EGTA and 1 mM calcium, magnesium or manganese. The most likely explanation is thus that, in the absence of any added divalent cation, EGTA probably binds another endogenous cation (iron [40] or zinc [41]) contributing to the formation of the biofilm. It has been recently reported that zinc contributes to the rod-like structure of SasG, a protein involved in the formation of a biofilm by *S. aureus* [42].

The results obtained in the wells of a microplate were, at least, partly confirmed by measuring the adhesion of the bacteria on catheter tubing. In agreement with the results of the BFRT and the CVSM, the MRSA strains adhered less than the MSSA strains. The 2 MRSA strains with less adherence on the tubing in the presence of EGTA were also the 2 strains mostly affected by EGTA in the BFRT®. The conclusions of the studies comparing the adhesion of MSSA and MRSA strains were not consistent. Amaral *et al.* [43] reported that the adhesion of clinical MRSA strains on bronchial epithelial cells was higher than the adhesion of clinical MSSA strains. More recently, Pozzi *et al.* [44] showed that the expression of a gene responsible with resistance to methicillin was associated with increased adhesion on a catheter [44]. At the opposite, Aathitan *et al.* [45] reported that MRSA and MSSA strains similarly interacted with liver epithelial cells [45], whereas Karauzum *et al.* [46] observed that MRSA strains were less adherent on human airway epithelial cells than MSSA strains [46]. There is thus a large panel of opinions on the ability of the two populations of strains to adhere on a surface.

In conclusion, the formation of a biofilm by MSSA and MRSA strains proceeds at a different rate and is differently affected by cations. This should help in the formulation of washing solutions to clean material contaminated by MRSA-carriers. The BFRT® could contrib.-ute to the search for these new cleaning solutions: it is a very easy and efficient technique to study the initial steps of the formation of a biofilm by *S. aureus*, at a time when the growth of the bacterial population only marginally affects the results. However, the results obtained with the BFRT are also consistent with the investigation on catheters. Further studies should also look for a correlation between the BFRT® and the adhesion of *S. aureus* on biotic surfaces like wounds. It should also be kept in mind that the results of an assay might be indirectly affected by modifications of the properties of the medium provoked by the tested drugs.

5. Acknowledgements

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