International Journal of Molecular and Clinical Microbiology 8(1) (2018) 942-949

International Journal of
Molecular and Clinical Microbiology

Visualization of acidic and alkaline pH effect on biofilm formation of *Staphylococcus aureus* isolates by Atomic force microscope

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ARTICLE INFO

Article history:
Received 3 February 2018
Accepted 24 May 2018
Available online 1 June 2018

Keywords:
pH, Biofilms, Staphylococcus aureus, Atomic force microscope

ABSTRACT

Staphylococcus aureus is the most common pathogen causing nosocomial infections that their treatment by antibiotics is difficult. Biofilm potential of *S. aureus* is considered to be one of the main reasons for its survival and is influenced by many environmental factors. The purpose of this study was to evaluate the effect of pH on biofilm formation of *S. aureus* and its visualization by atomic force microscope (AFM). 100 *S. aureus* strains were isolated from clinical specimens of patients who were referred to Milad Hospital, Tehran and diagnosed by biochemical tests. A microtiter plate method was used to determine the strength of biofilm formation under acidic and alkaline pH. The effect of pH on biofilm formation was visualized by using AFM. In pH 7 and 9 the biofilm formation of *S. aureus* strains was at highest level, 78.1% and 71.9% but in pH3 and 12 was at lowest rate 35% and 35.4%. There is a significant association between pH and biofilm formation. AFM microscopy analysis of effect of pH 3 and 12 in *S. aureus* showed reduction biofilm structures. In pH 7 and pH 9 more biofilm and less planktonic cells were observed. The increase or decrease in pH value was involved in decrease of biofilm formation. The AFM was a useful tool for visualization of *S. aureus* biofilm.

1. Introduction

Bacterial adhesion is a very important concept in the area of bacterial disease and control (Nicolau Korres et al., 2013). Many bacteria are surrounded within an extracellular matrix as biofilm and can live in many environments (Branda et al., 2005; Kolter & Greenberg, 2006). Biofilm is known to facilitate the colonization and the persistence of a large variety of microbial species (Wang et al., 2011). Most of the bacterial populations are in the form of biofilm at different stages of growth (Dalton & March, 1998). Researchers have discovered that bacterial biofilms have strong resilience in the host immune system and resistance to antibiotics (Costerton, 2005; Rodríguez-Martínez & Pascual, 2006). Also bacterial biofilms play an important role in a range of chronic infections (Costerton et al., 1999; Hall-Stoodley et al., 2004). *Staphylococcus aureus* can attach to a surface, accumulate biomass and form a biofilm (Kiedrowski & Horswill, 2011). *S. aureus* is the cause of many diseases associated with morbidity and mortality such as nosocomial infections (Jabra-Rizk et al., 2006). The biofilms of *S. aureus* are involved in cystic fibrosis (CF), chronic otitis media and

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osteomyelitis (Lindsay & Von Holy, 2006). Many physicochemical factors affect the premier attachment of bacteria to abiotic materials. Some factors such as surface charge, hydrophobicity and surface chemistry have been well studied, previously (Maheshwari et al., 2000; Hassan & Frank, 2004; Hou et al., 2007). Studies showed that the surface properties such as roughness, energetics pH, surface tension and proteins affect bacterial biofilm formation (Chung et al., 2007; Mosier & Cady, 2011; Zmantar et al., 2011). Today there is increasing interest in evaluation and controlling factors that affect the formation and development of biofilm structures. Detailed study of bacterial adhesion for multicellular biofilm formation is necessary to use strategies to control biofilm development. Atomic force microscopy (AFM) has been proved to be suitable tools in order to follow the initial stages of biofilm formation. AFM is used to obtain information about biofilms structure by exploring the aggregation and forces involved during cell attachment (Schilardi et al., 2010). The aim of this study was evaluation of biofilm formation of *S. aureus* at different acidic and alkalin pH and visualization of biofilm formation under these conditions by AFM.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

This descriptive study was conducted in 2016 in a total of 100 *S. aureus* isolates that were collected from September 2013 and June of 2014, from clinical specimens of patients who admitted to the Milad Hospital, Tehran. Isolates were diagnosed by biochemical tests such as culturing on Manitol Salt Agar and DNase, Catalase and Coagulase tests. The collective Strains were storaged in Brain Heart infusion (BHI) media (Merck,German) containing 15% glycerol and were frozen in -80ºC until used.

2.2. In vitro biofilm assay

Biofilm formation of all *S. aureus* isolates were detected by Microtitere plate method in Trypticase soy broth (TSB) on Round Bottom 96-well Microplates microtiter plate (SPL Life sciences, Korea) as described previously (Christensen et al., 1985). An overnight culture grown in TSB (Merck, German) at 37°C, adjusted to 0.5 Mac-Farland and was diluted to 1:100 in TSB with 2% (w/v) glucose. 200 µl of these suspensions was transferred in a U - bottomed well. The plates were incubated aerobically for 24 h at 37°C. Furthermore, the culture was removed and plates were washed three times with 200 µl of phosphate-buffered saline (PBS, pH 7.4; Sigma, USA). Adherent biofilm was fixed with 95% ethanol and was stained with 0.1% crystal violet (Merck, German) for 15 min. Then, the dye was removed and the wells were washed three times with 300 µl of sterile distilled water, then cleared and the microtiter plate was air dried. The crystal violet from stained biofilm was resuspended in 250 µL of 95% ethanol (22). The optical density (OD) of each well was measured at 570 nm using an Elisa reader (Biotek, cytation3, USA). The cut-off optical density (OD) for a tissue culture-plate is defined as three standard deviations above the mean OD of the negative control. Each strain was tested in triplicate. Wells with sterile TSB alone was served as controls. *S. aureus* ATCC 25923 was used as the positive control. The interpretation of biofilm production was done according to the criteria, previously (Stepanović et al., 2007) (Table1) .

Table1. Interpretation of biofilm production

Average OD value	Biofilm production
OD ≤ ODc	Non-adherent
ODc<OD ≤ 2 × ODc	Weakly adherent
2×ODc <OD ≤ 4 × ODc	Moderately adherent
4 × ODc < OD	Strongly adherent

Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control

2.3. Quantitative biofilm production assay of S. aureus isolates by pH levels

To analyze the effect of pH on biofilm formation, the pH of the TSB medium was adjusted (3, 5, 9 and 12) (Zmantar et al., 2010). Each assay was performed and repeated at least three times. The Control pH was adjusted to 7.

2.4. AFM microscopic analysis for biofilm formation at various pH

An AFM (Model: JPK nanowizard II, Germany) was used for analyzing the topography of the biofilms. The AFM analysis was performed using one *S. aureus* isolate

attached to polystyrene microliter grown for 24 h in TSBG at different pH values (3, 5, 7, 9 and 12).

2.5. Statistical analysis

Statistical analysis was performed by SPSS-21 (SPSS Inc., Chicago, IL, USA). ANOVA test was employed to evaluate any significant differences between the values obtained in TSBG with different pH. Tukey test was used for comparison of data means. A p value of < 0.05 was considered significant.

3. Results

The frequency of *S. aureus* from urine, blood, wound, and other specimens of patients were 25.14%, 7.85%, 14.28% and 25.71%, respectively.

3.1. The biofilm formation of S. aureus isolates

Biofilm productions assessed by TCP method revealed 71.9% strong biofilm producers, 28.1% moderate producers, there was no weak or non-biofilm producers. This qualitative assay was at pH7 as a control pH (Figure1).

3.2. The biofilm formation of S. aureus isolates at different pH by Microtiter plate method

The results showed that among 100 *S. aureus* isolates by TSBG microtiter plate method based on means OD, at pH 3, 35.55% strains had strong and 63.55% had moderate biofilm formation capacity. Only 1 isolate had no biofilm formation. At pH5, 48% isolates had strong, 50% had moderate biofilm formation capacity and two isolates had no biofilm formation. The biofilm formation among isolates at pH9 was 78.1% strong and 21.9% moderate and at pH12, 35.4% and 64.6% of isolates had strong and moderate biofilm formation, respectively (Figure1). The results showed that at pH3 and pH 12 the biofilm formation of bacteria are weak but at pH9 and the pH 7, strains have highest rate of biofilm formation (figure1 and 2).

The results of data mean comparison demonstrated that at pH3, 5, 7, 9 and 12 OD' means were, 1.0045, 1.41705, 1.94933, 2.20578 and 2.20578, respectively (figure2). Standard error for two means was 0.1519. Data analysis by ANOVA test showed that there is a significant relationship between pH variation and biofilm formation (P 0.0001). By comparison of biofilm formation of strains at different pH with control pH, there is significant differences between pH3, 5 and 12 except pH9 with control pH (p<o.o5)*(Table2).

3.3. Visualization of biofilm formation of S. aureus isolate at different pH by AFM microscope

AFM micrographs of a strain of *S. aureus* that grown for 24 h in TSBG with different pH and according to the results of OD means showed that at pH3 and pH5 bacterial cells grew in as looser colonies and the amount of biofilm was sensibly less (Figure 3A, B) . At pH7 much aggregates or biofilm and less loose colonies were visualized (Figure 3C). At pH 9 very much more aggregates and full structure biofilm without any loser colonies was observed (Figure 3D). At pH12 no aggregates and biofilm was observed but there was much more singular colonies (Figure 3E). The results of AFM images were according to the results of *S. aureus* biofilm formation by microtiter paltes at different pH.

Table 2. OD means and differences between different pH with control pH

pH	MeanOD$_{570}$	Standard Error	Difference
3	1.0045	0.09516	-0.9448*
5	1.4170	0.10954	-0.5323*
9	2.2058	0.119105	0.2565
12	0.9994	0.089062	-·0.9499*
Control	1.9493	0.12579	

Standard error (mean): 0.1074
Standard error (two means): 0.1519
*P value <0.05

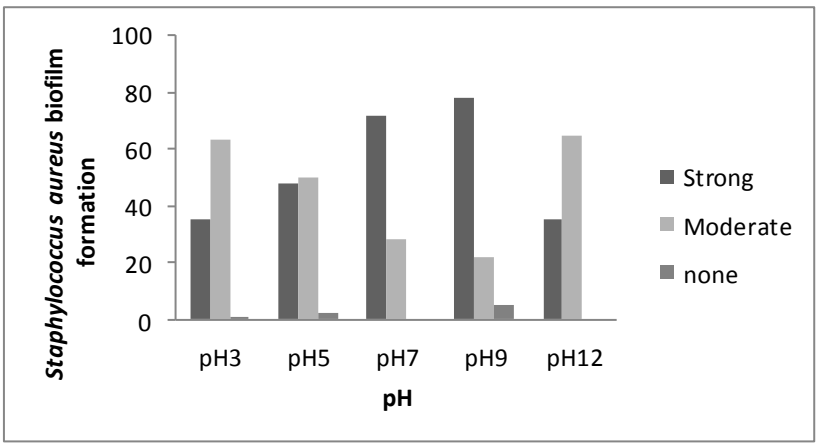

Figure1. The frequency of *S. aureus* isolates based on biofilm formation at different pH

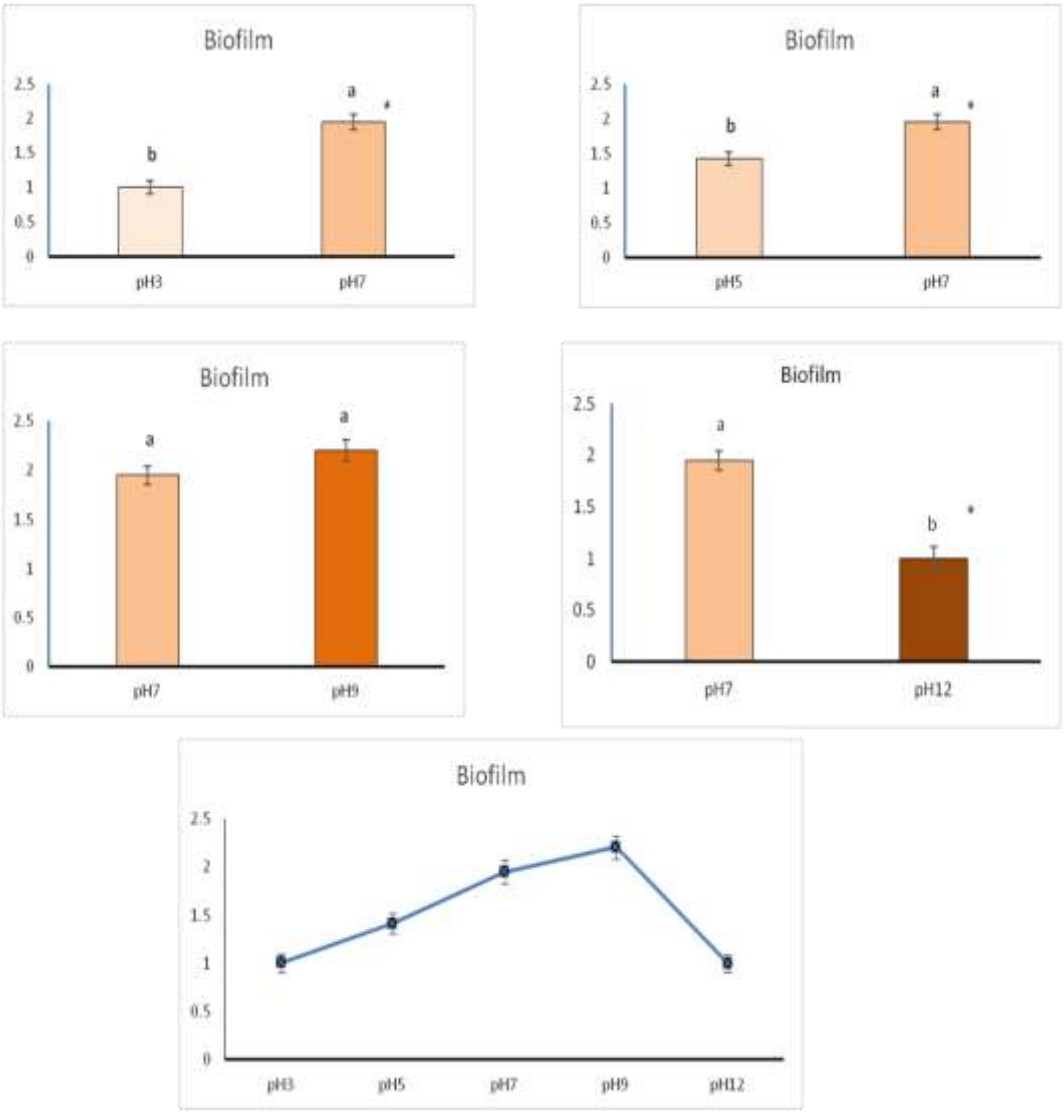

Figure 2. Comparison of biofilm formation of *S. aureus* isolates at differ pH with control pH

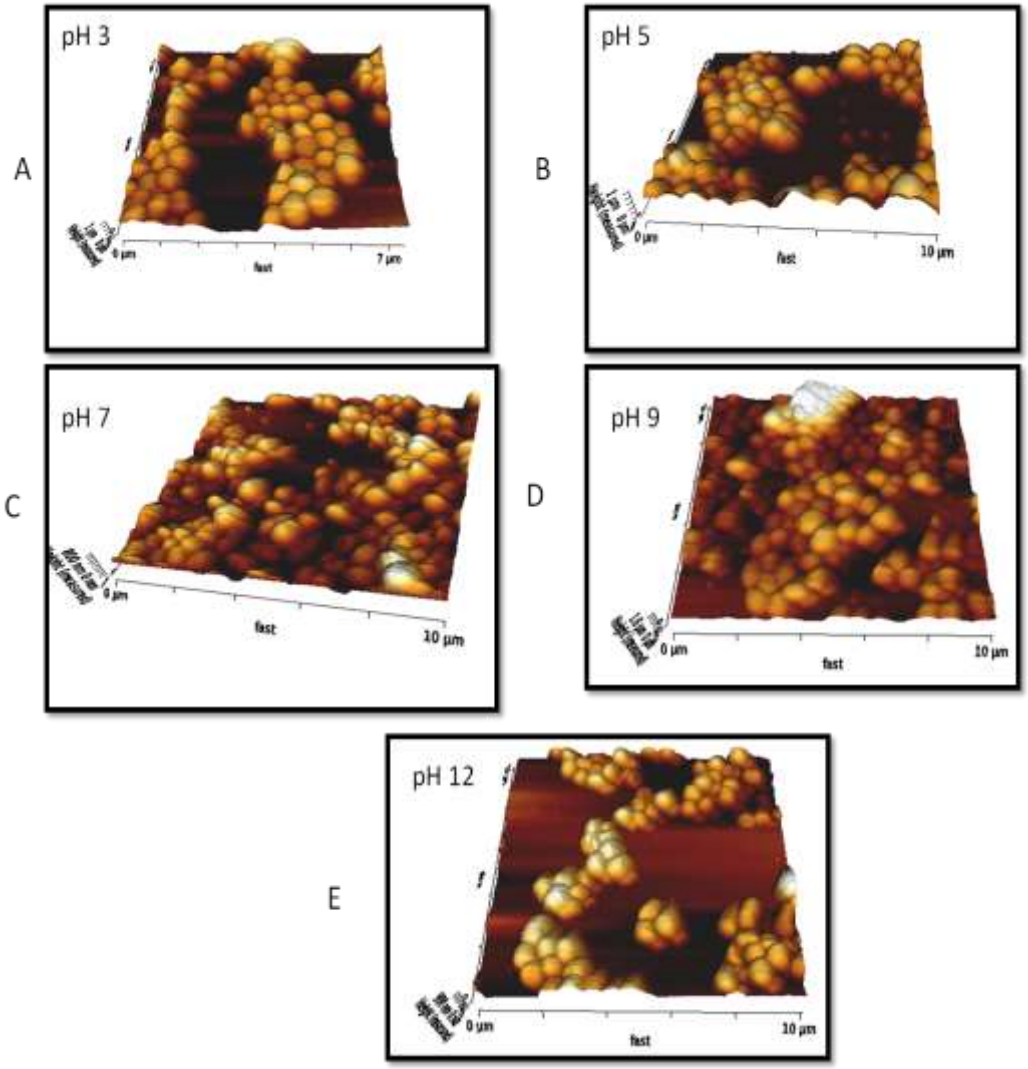

Figure 3. The AFM images of *S. aureus* isolate that was grown for 24 h in TSBG with different pH on polystyrene microtiter plates.

4. Discussion

S. aureus forms biofilm and may causing severe infections (Bennett et al., 2014). Biofilm formation is a complex process that the first stage of its formation is affected by many factors including the chemical properties and environmental factors (Simões et al., 2007). Several studies have shown that with respect to pH, growth in acidic or alkaline conditions can change the biofilm formation of bacteria (Doyle, 2000). In this study we evaluated the quantitative biofilm production of *S. aureus* isolates at various pH. At pH 3, 35.55% strains had strong and 63.55% had moderate biofilm formation and at pH12, 35.4% and 64.6% of isolates had strong and moderate biofilm formation, respectively. At pH5, 48% isolates had strong, 50% had moderate biofilm formation capacity and at pH9, 78.1% and 21.9% had strong and moderate biofilm formation. The results showed that at pH3 and pH 12 the biofilm formation of bacteria are weak but at pH9 and the pH 7, strains have highest rate of biofilm formation. Statistical analysis revealed a significant difference between the OD570 obtained at pH 7 as a control pH and pH 3 and 12 (P<0.05). However, the difference between the OD570 at pH 7 and that pH 9 was not significant. In a study by Chaieb et al., 2007, it was demonstrated that biofilm formation of staphylococci bacteria is inhibited at pH 3

(Chaieb et al., 2007). Hamadi et al in 2005 found that the cells adhere to glass surfaces strongly at pH range 4 to 6 but weakly at highly acidic (pH 2, pH 3) and alkaline pH levels(Hamadi et al., 2005). Zmantar in 2010 showed that at pH 5 , pH 9 and pH12 there is an increase in *S. aureus* strains biofilm formation (Zmantar et al., 2010). There are different studies on factors affecting biofilm formation such as oxygen level, pH, temperature, osmolarity among different bacterial species (O'Toole et al., 2000; Di Bonaventura et al., 2007; Vivas et al., 2008). Tang et al., in 2012 showed that many factors such as environmental factors and cultivation conditions influence the biofilm development in many *S. aureus* clinical isolates (Tang et al., 2012). In a study by Nostra et.al In 2014, the biofilm of *S. aureus* and *Staphylococcus epidermidis* was reduced after exposition to different acids like acetic, lactic, and hydrochloric acids (Nostro et al., 2014). Atomic force microscopy (AFM) is an advanced technology that have many advantages for observation of biological samples like bacterial biofilms (Touhami et al., 2004; Andre et al., 2010; Qin et al., 2009). In the present study, the topography of *S. aureus* biofilm formation under acidic and alkaline pH was examined by AFM microscope.

The results showed that at pH3 and 5, bacterial cells grew as single cells, at pH 7 bacteria were found to attach in a random, loosely aggregated manner without fully biofilm formation. At pH9 more aggregates and full structure biofilm without any losser colonies was observed but at pH12 there was much more singular colonies too, like at pH3. Tollersrud et al., in 2001 examined the surfaces of *S. aureus* strains by atomic force microscopy (Tollersrud et al., 2001). They showed that it is AFM is suitable for getting some information about surface characteristics of *S. aureus* Chatterjee in 2014 by study the biofilm formation of gram-positive and gram-negative bacteria by AFM showed that it is one of the important equipment for the evaluation of bacterial biofilm (Chatterjee et al. 2014). Aru et al in 2014, used the AFM and microtiter plate assay for evaluation of *Streptococcus mutans* biofilm isolated from dental plaque(Arul & Palanivelu, 2014). In a study by Bazari et al in 2017, the biofilm surfaces of 3 *S. aureus* isolates were observed by AFM. They showed that AFM is a useful tool for observation of bacterial biofilm formation (Bazari, 2017).

Conclusion

In conclusion Acidic and alkaline environment maybe have important implications to prevent bacterial colonization and control biofilm formation. The increase or decrease in pH value (12 or ≤5) was involved in the decrease of biofilm formation. The AFM was a useful tool for visualization of *S. aureus* biofilm formation. Also, the results observed by AFM microscopic topography of bacterial biofilm formation at varius pH on polystyrene microtiter plate was in agreement with quantitative biofilm assay by microtiter plate. So an understanding of bacterial community basis as targets that may provide a strategy for controlling of biofilm associated infections, is necessary. For this purpose, both physical and chemical properties of bacterial cell envelope and the expression of genes that influence bacterial adhesion and biofilm are effective factors that recommended further studies.

Refereces

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