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Assessment of biofilm formation among *Acinetobacter baumannii* strains isolated from burned patients

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ABSTRACT

Acinetobacter baumannii strains have emerged as problematic hospital pathogens, which can survive in healthcare settings and medical devices. Due to biofilm formation ability of A. baumannii and antibiotic resistance, making treatment difficult. This study was performed in order to investigate the potential of biofilm formation of A. baumannii strains. A total of 120 samples suspected Acinetobacter spp. were collected from burned patients and were assessed by using conventional phenotypic and biochemical tests. Biofilm formation of A. baumannii isolates was evaluated by using crystal violet staining and scanning electron microscopy. Results showed that 100 isolates were A. baumannii that among them, 17% of clinical isolates were weak biofilm producers while 44% and 39% of them were moderate and strong biofilm producers, respectively. It can be concluded that most of A. baumannii isolates can form biofilm and may contribute to its persistence in the hospital environment, increasing the probability of causing nosocomial infections among burned patients.

Key words: *Acinetobacter baumannii*, multi drug resistance, biofilm formation, Iran

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INTRODUCTION

Burn wound surfaces are more susceptible and provide a favorable niche for colonization and proliferation of microorganisms [1]. Infection is one of the most sever and serious complications among burned patients [2]. *Acinetobacter baumannii*, important opportunistic pathogens responsible for nosocomial infections, in burned patients is a crucial concern and a global threat that may lead to delays in wound healing [3]. The rates of morbidity and mortality due to *Acinetobacter* infections are increasing in hospitals. On the other hand, outbreaks caused by multidrug resistant *A. baumannii* (MDRAB) are difficult to control [3, 4].

Biofilm formation ability is one the most virulence factor among *A. baumannii* that is effective in the intensify of speciation [5]. Actually, biofilms can be defined as communities of microorganisms in which cells stick to each other and attach to a surface in an extracellular polymeric matrix [6]. Biofilm microbiota are up to 1,000-fold more resistant to antibiotics than their planktonic phases [7].Elimination of microbial biofilms and effective killing of microorganism's biofilm are critical in the management of *A. baumannii* infections [8].Center for Disease Control and Prevention (CDC) reported that biofilms have been involved in over 65% of hospital infections [9]. Forasmuch as there is increasing evidence that biofilm infections often resist to the highest levels of antibiotics, clinicians who deal with chronic biofilm associated infections frequently faced with problems to cure their patients [10].The current

study was performed to elucidate the potential of biofilm formation of *A. baumannii* strains isolated from burned patients.

MATERIALS AND METHODS

Isolation and identification of A.baumannii strains

A total of 120 clinical isolates which were recovered from specimens of patients with burn wounds suspected with *A.baumannii* infection were collected from Motahari hospital in Tehran, Iran, from Oct 2012 to Jun 2013. The isolates were identified as *A. baumannii* according to conventional phenotypic and biochemical tests including growth on MacConkey agar; catalase and oxidase tests; indole, urease and hemolysin production tests; motility test; citrate utilization test; lactose fermentation test; and discoloration of blood agar containing D-glucose test. The analytical profile index (API® 20E) assays (BioMerieux, Marcy l'Etoile, France) was used to confirm the biochemical characterization and identification of these isolates at genus and species levels.

Quantitation of biofilm formation ability of A. baumannii strains

Quantitative analysis of the biofilm formation ability of *A. baumannii* was performed according to a previous study [11]. Briefly, the *A. baumannii* strains were grown aerobically overnight in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) at 37°C. Bacterial cultures were then diluted with BHI broth to a final concentration of 1.0×10^8 colony forming units (CFU)/mL, which was verified by spectrophotometry (optical density [OD]600: 0.08-0.12) [12]. 200 µL of these bacterial suspensions were individually inoculated into flat-bottomed sterile polystyrene microplates (TPP; Trasadingen, Switzerland)and the microplates were incubated for 24 h at 37°C to allow for biofilm formation. After incubation, the microplates contents shaked out and were then washed with phosphate buffered saline (PBS) (10 mM Na2HPO4, 2 mM NaH2PO4, 2.7 mMKCl, 137 mMNaCl, pH 7.4) to remove non-adherent bacteriaand media and air dried for 15 min. For fixed adherent bacterial cells prior to staining, 100 µL of 95% ethanol was added to each well and the plates were incubated at room temperature for 10 min. The microplate contents were then emptied out from each well and added 200 µL of 0.1% (wt/vol) crystal violet solution to each well of micropletes at room temperature for 15 min. After this time, removed excess stain by repeated washing (3–4 washes) with PBS. The crystal violet could be eluted from stained biofilms by adding 200 µL 33% (v/v)acetic acid to each well. The optical density at 570 nm (OD_{570}) of each well was measured in a microplate reader (Thermo Fisher Scientific, US). The adhesion of isolates is classified into four groups. Strains were classified as follows: a) OD ≤ODc = non-adherent; b) ODc< OD ≤ 2ODc = weakly adherent; c) 3ODc < OD ≤ 4ODc = moderately adherent; d) 4OD <ODc = strongly adherent; e) ODc = OD of control. *Pseudomonas aeruginosa* PA01 was used as positive control for biofilm formation tests.

Microscopic analysis of biofilmsformation ability of A. *baumannii* strains

The biofilm formation ability of *A. baumannii* strains was visualized by scanning electron microscope (SEM).Biofilm was formed on the MBEC™ high-throughput (HTP) plates (Innovotech, Alberta, Canada) as previously described [13].*A. baumannii* suspensions (200 µL) were inoculated into each well (containing BHI broth supplemented with 0.1% glucose) and then incubated overnight at 37°C. Biofilms that formed were then washed twice with PBS to remove any unattached and floating cells and were fixed with 4% glutaraldehyde in 0.1 M cacodylic acid at 4°C for 24 h and post fixed with 2% osmium tetroxide at room temperature for 60 min. After incubation, plates were washed once with $dH_2O$for 15 min, followed by gradual dehydration with ethanol, and 1.5 h of critical point drying (Bal-Tec CPD 030, the Netherlands). The fixed biofilms were then coated with a layer of gold–palladium (7 nm thick) and examined with SEM (LEO, 1455 VP, Germany).

Statistical Analysis

A student's t-test *P* value of <0.05 was used as a cut-off when testing for significant difference between absorbance readings.

RESULTS

Of the 120 isolates suspected to *A. baumannii*, 100 isolates (83.3%) of them identified as *A. baumannii* by conventional biochemical assesments. An API ® 20E assay identified 100 isolates obtained from burned patients samples as *A. baumannii*.

The biofilm formation abilities of all the 100 isolates were determined. Quantitative analysis of biofilms formed by *A. baumannii* showed that 17 (17%), 44 (44%), and 39 (39%) produced weak, moderate, and strong biofilm, respectively (Fig. 1). In this study, there was no isolates without biofilm formation ability.

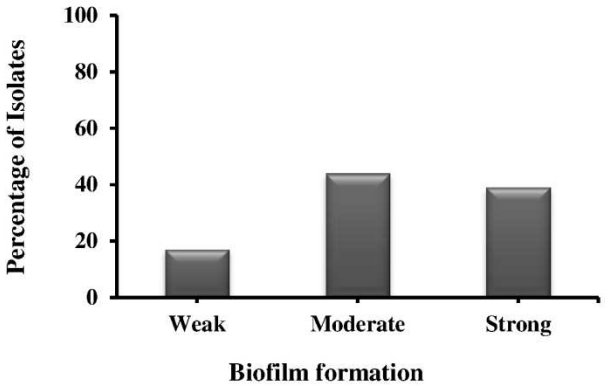

Figure 1.Biofilm formation ability in *A. baumannii*strains

SEM analysis of samples showed that few *A. baumannii* cells clustered together in weak-biofilm-forming strains and large groups of conglomerate *A. baumannii* cells in the moderate- and strong-biofilm-forming strains(Fig. 2). In all cases, the cells' morphology remained unaltered.

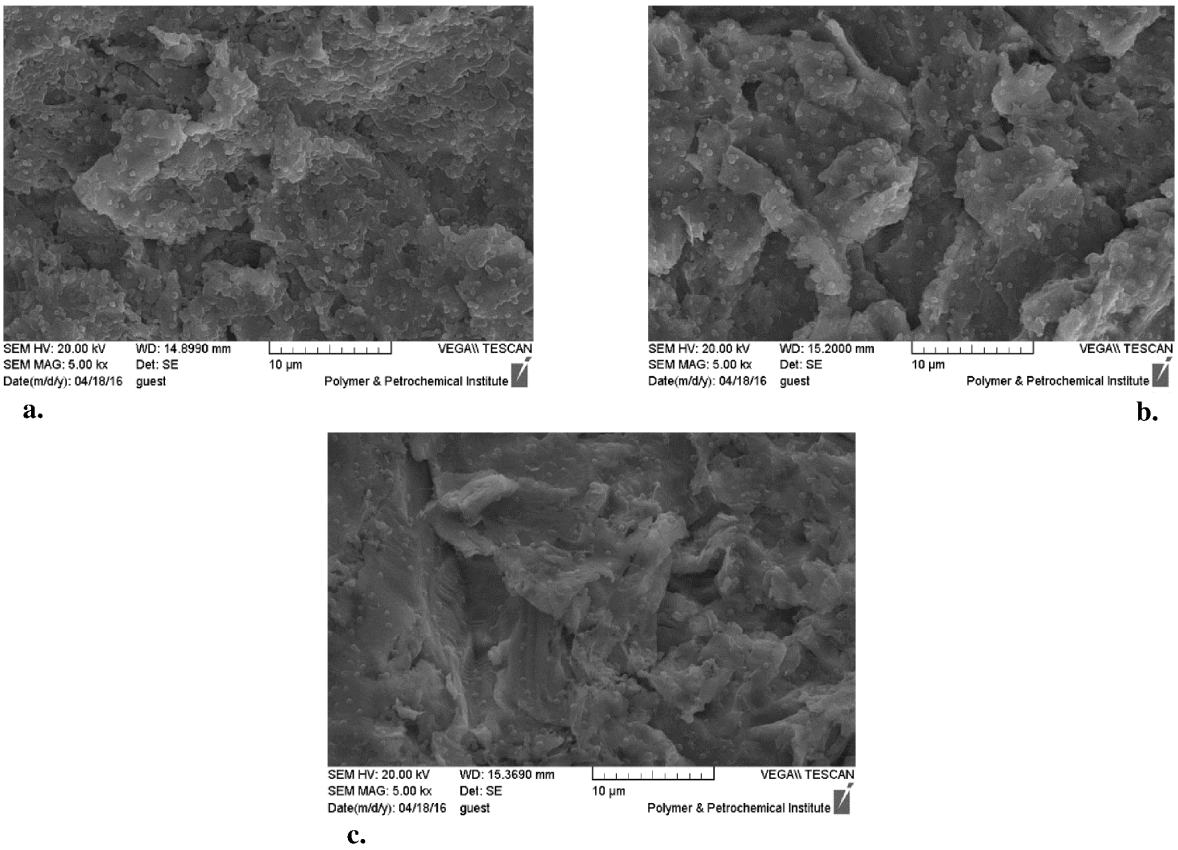

Figure 2.SEM images of *A. baumannii*cells: a) strong, b) moderate, and c) weak biofilm formation

DISCUSSION

A. baumannii is a Gram-negative coccobacilli that can lead to severe infections in immunosuppressed patients admitted into hospital environments, especially in intensive care units (ICU), burn and surgery [14].The emergence of *A. baumannii* strains in the hospital environment has been associated with the presence of multiple genetic elements, virulence factors and the ability to form biofilms [15]. *Acinetobacter* spp. remain as normal skin flora, can remain viable in the hospital setting and on medical devices and hospital equipment and indwelling medical devices, such as urinary catheters, central venous catheters (CVCs), endotracheal tubes, etc. for a long time due to its multi drug resistant status, resistance to desiccation, and tendency to adhere to inanimate surfaces [14,16]. *A. baumannii* capacity for biofilm formation is a reason for persist in environments, as well as its virulence. Antibiotic resistance

related to biofilm formation is the major cause of treatment failure of infected patients with all *Acinetobacter* species, particularly those with *A. baumannii* [17].

Several factors such as pH, temperature, concentration of extracellular free iron, and salt concentration of the medium affect the production of biofilm [18].Some studies have shown that the ability of *A. baumannii* strains for the formation of biofilms on biotic and abiotic surfaces is dependent on a number of gene products have been reported to play a role in adhesiveness and biofilm development such as*csuE* gene expression that is the member of csuA/BABCDE chaperone-usher complex. So, inactivation of *csuE* gene leads to inhibition of biofilm formation [19, 20].

However, there is very limited information about the biofilm formation ability of A. *baumannii*[5-7]. Sechi et al. [21] found that 16 (80%) of 20 isolates of *A. baumannii* formed biofilm, perhaps because of a dominant *A. baumannii* clone. Espinal et al. [22]reported that non-biofilm forming strains were particularly more resistant than biofilm forming strains. Kazemi pour et al. [23] evaluated the variety of conditions for *A. baumannii* biofilm formation and stated that shaking conditions were suitable for biofilm formation. Rodríguez-Bañoet al. [24] showed that 63% of 92 clonally unrelated *A. baumannii* clinical isolates formed biofilm.

Based on the present study, we show that the *A. baumannii* isolates can produce the moderate, strong, and weak biofilm, respectively. Because of the ability to form biofilms (moderate and strong), they have a large dispersion in hospitals circumstances. In our SEM analysis, *A. baumannii* cells connected to each other with extracellular appendages.

CONCLUSION

Adhesiveness and biofilm forming ability of *A. baumannii* play a key role in the pathogen interactions and in medical device associated infections. Consideration of the necessary actions including hand hygiene, personnel protective equipment, training of health care personnels, isolation of patients, and etc. can be useful to control the outbreak of MDRAB in burn unit. To conclude, this study shows that outbreak investigation the biofilm formation ability of *A. baumannii* is the key factors which help in deciding the infection control strategies for control of outbreak. Furthermore, additional evaluations are needed on the correlation between *A. baumannii* ability to adhere and form biofilm with regulator networks and molecular mechanisms.

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