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Comparison of two methods for quantification of *Acinetobacter baumannii* biofilm formation

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Abstract

Introduction: Medical devices are made from a variety of materials such as polypropylene, polycarbonate, poly styrene, glass and etc. by attaching to this surfaces, *Acinetobacter baumannii* can form biofilms and then cause several device associated infections. Biofilms are communities of bacteria attached to the surfaces. In this study, biofilm formation ability in clinical isolates of *Acinetobacter baumannii* was assessed by two methods on different surfaces.

Materials and methods: Biofilm formation by 75 clinical isolates of *A. baumannii* was evaluated on polycarbonate surface (microtiter plate) and polypropylene surface (falcon) by crystal violet and 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT tetrazolium sodium salt) assay methods. Falcon or tube method was carried out under static and agitation conditions.

Results: Results showed the most isolates can form biofilm but higher numbers of isolates form biofilm on polypropylene surface under agitation. XTT method confirmed strong biofilm formation ability of 10 isolates.

Discussion and conclusion: Each of the two assays showed an excellent applicability for the quantification of biofilms. The Crystal violet assay is cheap, easy and is usually used for the quantification of biofilms formed by microorganisms but XTT is more reliable and repeatable. Most of *A. baumannii* isolates have potential to form biofilm on the medical devices which may result in device-associated infections.

Key words: *Acinetobacter baumannii*, Biofilm, Crystal violet, XTT

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Introduction

Biofilms are complex communities of bacteria embedded in a polysaccharide matrix. Biofilm formation occurs in response to a variety of signals, including high cell density, nutrient deprivation and physical environmental stresses (1). Planktonic bacteria are either leaving, joining or surrounding the biofilm. The growth conditions vary between biofilm and planktonic environments. For this reason, proteins expressed by biofilm bacteria may differ from those expressed by their planktonic form (2). Biofilms can cause significant problems in many areas, both in medical settings (e.g. persistent and recurrent infections, device-related infections) and in non-medical settings (e.g. biofouling in drinking water distribution systems) (3).

Members of the genus *Acinetobacter* are non-motile, ubiquitous Gram-negative bacteria that can be isolated from a wide range of sources such as soil, water, food products and medical environments. *Acinetobacter* spp. are aerobic, non-fermentative, able to colonize patients of intensive care units (4). They often have the ability to form biofilms or large communities of bacteria living on a solid surface within a self-produced matrix. *Acinetobacter* cause infections because of their many factors associated with biofilm formation including pili, biofilm associated protein (Bap), quorum sensing and exopolymeric DNA (eDNA) (5). *Acinetobacter* biofilms cause diseases such as burn infection, periodontitis, bloodstream infection and urinary tract infection because of their ability to indwell medical devices including catheters, prosthetic heart valves and joint replacements (6, 7, 8). *A. baumannii* has emerged as an important nosocomial pathogen hospital outbreaks of which have been reported from various geographic areas. This species has potential to form biofilm that explain significant survival properties on hospital and medical devices (9).

Medical devices are made from variety of materials such as polypropylene, polycarbonate, poly styrene, glass and etc. In this study, two methods for quantification of biofilm formation based upon CV and XTT assay methods were compared. In addition biofilm formation of *A. baumannii* isolates were assessed on two different surfaces, polycarbonate and polypropylene, to demonstrate the ability of this bacterium to form biofilm in medical devices

Material and methods

Bacterial strains and culture conditions

A total of 75 *A. baumannii* isolates were collected from different clinical sources, including burn wounds (from burn hospital, 2008) and urinary catheters (from an army hospital, 2011) and evaluated. Frozen stocks were prepared in skim milk (Merck, Germany) containing 15% glycerol (Merck, Germany) and were stored at -70 ° C.

All isolates were transferred from the stock cultures into tryptic soy agar (TSA) (Merck, Germany) and were aerobically incubated at 37°C for 24 hours.

Pseudomonas aeroginosa PAO1 was used as positive control for biofilm formation tests.

Biofilm formation

Polypropylene and polycarbonate surface

For this survey, 15 mL polypropylene falcon tubes were used. Cultures were inoculated in Luria bertani (LB) broth and adjusted to 0.5 McFarland standards. 0.1 mL of each bacterial suspension was transferred to falcons containing 10 mL LB. Tubes under two different conditions: (a) agitation under 200 r.p.m, and (b) stationary were incubated at 37 ° C for 24 hours. Negative control contained only LB. Following the medium was removed, tubes were washed with distilled water and air

dried and falcons were stained for 5 minutes with 0.2 mL of 2% crystal violet (Merck, Germany).The excess of the stain was rinsed off by placing the tubes under running tap water. Later the falcons were air dried; the dye bound to the adherent cells was resolubilized with 160 µL of 33% (v/v) glacial acetic acid and 100 µLof each falcon were transferred to a well of a 96-well microtiter plate., The OD_{650} of each well measured (6) using an ELISA reader.

In this experiment polycarbonate microtiter plates were used. Cultures were adjusted to 0.5 McFarland in LB medium. Each three wells of a non-adherence, sterile 96-well flat-bottomed plates were filled with 200 µL of bacterial suspension, covered and incubated at 37 ° C for 24 h. Subsequently, each well were aspirated, washed 5 times with sterile distilled water, air dried and biofilm formation was assayed using the crystal violet method as described above (10).

XTT tetrazolium sodium salt assay

10 isolates which produce strong biofilm by CV method were selected. 200 µL of bacterial suspension were added to each well of a microtiter plate and plates were incubated at 37 ° C for 24 hour after which each well was emptied, washed and plates were dried.

XTT (Sigma) was dissolved in phosphate-buffered saline to 1 mg/mL. Menadione (Sigma) was dissolved in acetone to 1 mM. The XTT/menadione reagent was prepared fresh prior to each assay and contained 12.5 parts XTT/1 part menadione. 100 µL XTT-menadione were added to each well and incubated at shaker incubator in 37 °C/150 r.p.m for one h. Biofilm metabolism would reduce XTT tetrazolium salt to XTT formazan by mitochondrial dehydrogenases and result in a colorimetric change. The amount of colorimetric change was measured using a microtiter plate reader at A_{505} (11). All tests were carried out in triplicates.

Statistical analysis

SPSS 16 and one way ANOVA were used to calculate the differences between microtiter plate and falcons under agitation and static conditions. Experiment was performed in triplicate. P values of ≤ 0.05 were considered as significant.

Results

The results show the ability of biofilm formation in *A. baumannii* isolates. Results of biofilm formation in falcon tubes and microtiter-plates are presented in Fig. 1. Statistical analysis was carried out following which falcon test and microtiter-plate showed significantly different results.

For easier interpretation of the results, strains may be divided into four categories: non- biofilm producer (0), weak biofilm producer (+ or 1), moderate biofilm producer (++ or 2) and strong biofilm producer (+++ or 3), based upon the previously calculated OD values: $OD \leq ODc$ non-biofilm producer; $ODc < OD \leq 2ODc$ weak biofilm producer; $2ODc < OD \leq 4ODc$ moderate biofilm producer; $OD < 4ODc$ strong biofilm producer (10). ODc means absorbance of control and OD has direct relation with biofilm formation.

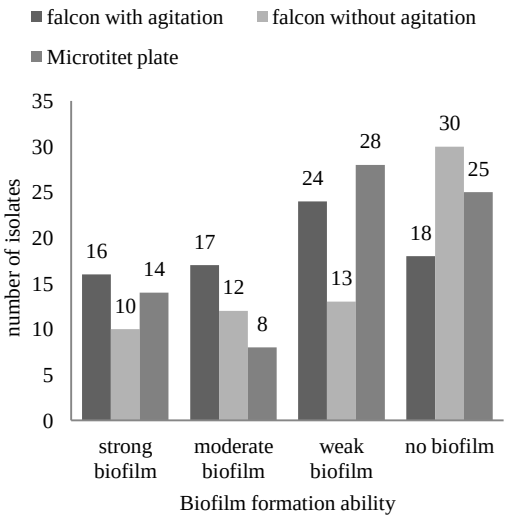

Fig 1- Biofilm formation in falcon tubes and microtiter plates

As shown in Fig 1. many isolates were able to form biofilm on polypropylene falcons with agitation.

XTT is a yellow salt that is reduced by dehydrogenase and reductases of metabolically active cells to produce a color, water-soluble formazan as an orange product, which can be assessed visually and quantified spectrophotometrically. Such assays are used to test the efficiency of antimicrobial drugs in killing or inhibiting growth of organisms. Results of XTT reduction assay confirmed that all 10 isolates produce strong biofilms which is in agreement with CV method. The average absorbances obtained from biofilms are shown in Fig. 2.

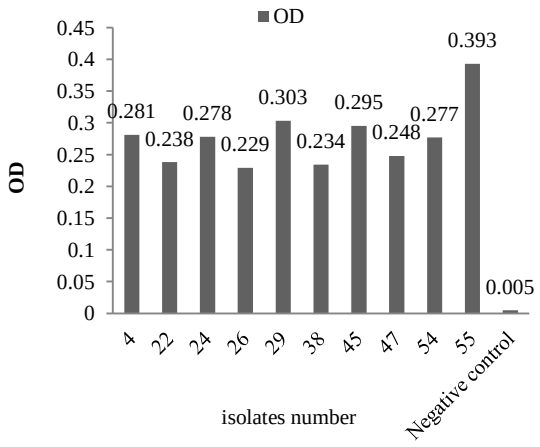

Fig 2- Absorbance of biofilms formed by isolates at A_{505}.

Discussion and conclusion

In recent decades, increasing involvement of *A. baumannii* infections in hospital and their ability to form biofilm has been an important observation. *A. baumannii* can attach to medical devices and form biofilms which cause device-related infections (6).

The growth of polypropylene in medical device applications progressed unabated throughout the last decades of the 20th century and continues strongly into the 21st (12). Moreover, polycarbonate fills a significant niche as one of the most popular engineering resins in the medical device market, possessing a broad range of physical properties that enable it to replace glass or metal in many medical products (13). In this study, we used polycarbonate plates and polypropylene falcons to assay of biofilm formation by *A. baumannii*. Because these materials are used to make medical devices, the results obtained in this study might be attributed to clinical biofilm formation abilities. Rodriguez-Bano et al. 2007 were showed more than 60% of *A. baumannii* isolates from clinical samples formed biofilm, and these isolates were associated mainly with device-related infections (14). Kazemi pour et al. 2011 demonstrated that *A. baumannii* isolates obtained from UTI can adhere to different abiotic surfaces under experimental conditions (6).

Our results obtained from polycarbonate and polypropylene materials showed association and importance of biofilms formation and surface substratum, primarily; these have been used widely for *in vitro* diagnostics in a variety of microorganisms and shown to be an excellent material for promoting adherence of cells. Secondarily, many studies on biofilms used these materials and finally these are commercially available and relatively inexpensive.

Quantitative analysis of biofilms formed by *A. baumannii* on polycarbonate and polypropylene surfaces showed that shaking conditions were suitable for biofilm formation. It seems that the possibility of cell-surface collision and subsequently biofilm formation increases.

To compare biofilm formation, it is critical to have an efficient and highly reproducible method for quantification. In this study, we used and compared two methods, and both were highly reproducible with little random error. We

used a direct staining method using CV, which is a basic dye. It binds to negatively charged extracellular molecules, including cell surface molecules and polysaccharides in the extracellular matrices in mature biofilms. Crystal violet is suitable to measure amount of biofilm and allows biofilm biomass quantification in the entire well (10) (15). XTT reduction assay measured metabolic activities of biofilm-forming cells which are based on respiration of bacteria into biofilm. The crystal violet method differs from the XTT reduction assay in that crystal violet can stain both active cells and the extracellular matrix in mature biofilms. The crystal violet staining method is widely used for measuring biofilms in bacteria. The statistical analysis showed that results from these two methods are significantly correlated (15).

Each of the two assays showed an excellent applicability for the quantification of biofilms. Moreover, assays are simple, fast and perfectly suitable for high-throughput quantification of biofilms. The XTT assay was expensive, time-consuming and showed repeatability. The Crystal violet assay is cheap, straightforward and is routinely used for the quantification of biofilms formed by microorganisms. Compared to the XTT-reduction assay, the CVstaining method was cheaper and faster but XTT is more reliable and repeatable. Peeters et al. (2008) reported that crystal violet clearly failed to give repeatable results for tested *P. aeruginosa* strains (16).

In conclusion, this study demonstrated that *A. baumannii* isolates from UTI and burn wounds have the ability to form biofilms and this is one of the main causes of device-related infections. Furthermore, it can be concluded that results of XTT-reduction assay and crystal violet method are in agreement for *A. baumannii* biofilm formation measurement.

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مقایسه دو روش برای کمی سازی تشکیل بیوفیلم *Acinetobacter baumannii*

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چکیده

مقدمه: ابزارهای پزشکی از مواد مختلفی مانند پلی‌پروپیلن، پلی‌کربنات، پلی‌استیرن، شیشه و غیره ساخته شده است. *Acinetobacter baumannii* با اتصال به این سطوح قادر به تشکیل بیوفیلم است و بنابراین، بیماری‌های عفونی وابسته به ابزار را ایجاد می‌کند. بیوفیلم‌ها اجتماعاتی از باکتری‌های متصل به سطوح اند. در این مطالعه، توانایی تشکیل بیوفیلم جدایه‌های بالینی *Acinetobacter baumannii* با دو روش بر روی سطوح متفاوت ارزیابی شده است.

مواد و روش‌ها: تشکیل بیوفیلم ۷۵ جدایه بالینی *Acinetobacter baumannii* روی سطح پلی کربنات (میکروتیتر پلیت) و سطح پلی پروپیلن (فالکون) با روش‌های کریستال ویوله و نمک XTT تترازولیوم (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt) ارزیابی شد.

نتایج: نتایج نشان می‌دهد که اغلب جدایه‌ها قادر به تشکیل بیوفیلم بودند اما تعداد بیشتری از جدایه‌ها قادر به تشکیل بیوفیلم روی سطح پروپیلن در شرایط تکان دادن بودند. روش XTT توانایی تشکیل بیوفیلم قوی ۱۰ جدایه را تایید کرد.

بحث و نتیجه‌گیری: هر دو روش سنجش، کاربری عالی را برای کمی‌سازی تشکیل بیوفیلم نشان دادند. روش کریستال ویوله ارزان و آسان است و بیشتر برای کمی‌سازی تشکیل بیوفیلم میکروارگانیسم‌ها استفاده می‌شود. اما روش XTT قابل اعتمادتر و تکرارپذیرتر است. بیشتر جدایه‌های *اسینتوباکتر* توانایی تشکیل بیوفیلم را روی ابزارهای پزشکی دارند که می‌تواند باعث عفونت‌های وابسته به ابزار شود.

واژه‌های کلیدی: *Acinetobacter baumannii*، بیوفیلم، کریستال ویوله، XTT

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