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Influence of Growth in Biofilm in the Formation of New Biofilm by Clinical Isolates of *Acinetobacter baumannii*

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The influence of growth in biofilm in formation of new biofilm by clinical isolates of *Acinetobacter baumannii* was investigated in this study. Sequential passage of *A.baumannii* isolates in biofilm culture in trypticase soy broth (TSB) resulted in gradually increased amount of biofilm production the by the isolates. On the other hand, passage of the same isolates in planktonic culture did not result in enhanced biofilm production. Passage induced enhanced biofilm production reached maximum level at passage 3 (P-3) for the strains *A.baumannii* strains AB-1 and AB-3 and passage 4 (P-4) for the strain AB-2. These values were 47 %, 40.4% and 71.8 % increased biofilm production, respectively by the biofilm passaged strains in comparison to their planktonic counterparts. Normal human serum (NHS), which enhances biofilm production by *A.baumannii* isolates was investigated to determine its effect on passage induced biofilm formation. NHS was found to further increase biofilm production by *A.baumannii* strains grown in biofilm mode, in comparison to their counterparts grown in planktonic state. Taken together, the results of this study indicate that growth of *A. baumannii* in biofilm enhances its potential to form new biofilm and biofilm formation is further increased by the presence of NHS in the growth medium.

Key words: *Acinetobacter baumannii,* Biofilm, Normal human serum, Virulence, Infection.

Acinetobacter baumannii is a gram negative, opportunistic pathogen. Over the last decade, *A.baumannii* has emerged as a major etiological agent of nosocomial infections associated with significant morbidity and mortality on a global scale[1]. It can cause a variety of infections including pneumonia, meningitis, urinary tract infection, bacteremia, osteomyelitis and wound infections[2]. Multi drug resistant (MDR) *A. baumannii* has become a threat to immunocompromised patients of all ages. Two capacities of this bacteria is considered to have contributed to its emergence as a frequent cause of outbreaks of infection and as an endemic, health care–associated pathogen are (a) remarkable capacity to acquire and disseminate antibiotic resistance and (b) the capacity of *A. baumannii* of surviving under a wide range of environmental conditions for extended periods of time[3,4]. In addition, several factors contribute towards maintaining the presence of MDR *A. baumannii* in the health care setting; these include the presence of susceptible patients, the presence of patients already colonized or infected with the organism, selective pressure from antimicrobial use and incomplete compliance with infection control procedures[5]. Despite intensive efforts, nosocomial acquisition of MDR *A. baumannii* is still a problem

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due to the organism's great ability to colonize human and environmental reservoirs[6,7].

Biofilm is a complex structure of bacterial community that can form on on solid or liquid surfaces as well as on tissues in living organisms[8]. In biofilms, bacterial communities are encased in a self-produced extracellular polymeric matrix; composed mainly of carbohydrates, proteins and nucleic acids[9,10]. In adverse growth conditions such as nutrient limitation or presence of antibiotics, biofilms serve as a survival mode of life. Bacteria transit from free floating state (planktonic state) to a surface attached state as they form biofim[11]. Bacterial cells in the biofilm exhibit genetic, physiologic and metabolic diversity; they exhibit enhanced resistance to various antimicrobial agents and clearance by the immune system The extracellular polymeric material, which encases the biofilm, functions as a diffusional barrier to antimicrobial agents[12].

Most *A. baumannii* isolates are MDR strains with high tendency of biofilm formation[13]. In a recent study, we reported that clinical isolates of *A. baumannii* produce biofilm; the potential of biofilm formation however, varies with the strain investigated[14]. Moreover, we also reported that biofilm forming potential of *A. baumannii* is influenced by normal human serum (NHS) in a differential manner. In the present study, we investigated whether growth of *A.baumannii* in biofilm modulates its potential of new biofilm formation.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A. baumannii strains were obtained from King Khaled General Hospital, Hail, Saudi Arabia. Trypticase soy broth (TSB) was used to grow the bacterial strains in liquid culture for biofilm assay. Trypticase soy agar (TSA) plates were used for culture of bacteria as needed. The clinical isolates were subjected to minimal passage prior to biofilm assay as *in vitro* passage often leads to loss of virulence attributes. The strains used in this study along with their clinical source is listed in Table-1.

Biofilm assay

Crystal violet dye binding procedure was used to determine biofilm production potential of the *A. baumannii* strains as described earlier[15]. Bacterial strains were grown overnight in TSB and the cultures were diluted in 1:100 in 3 ml of fresh TSB contained in plastic tubes. These cultures were then allowed to grow at 37°C in a static condition for 24 hours for biofilm formation. Biofilms attached to the glass tubes were washed with normal saline to remove unbound bacteria and stained with 0.1% (w/v) crystal violet solution for 10 min at room temperature. The stained biofilms were then washed (3X) with water. The bound dye was extracted with 95% ethanol. The absorbance of the dye released was determined at 570 nm. The experiments were performed in triplicates. OD 570 values for each tubes were subtracted from those of the blanks (which were uninoculated TBS) and these values represented quantitation of biofilm formation.

Effect of passage on biofilm production

To determine whether passage in biofilm of *A. baumannii* strains has any effect on new biofilm formation, the passage of bacteria was systematically carried out by growing the strains in biofilm and in planktonic states as described earlier[16]. Briefly, biofilms were allowed to be formed by the *A.baumannii* strains in glass tubes containing TSB as described above, which was considered as the starter culture. After 24 hours of incubation at 37 C, 3 new glass tubes were inoculated with 10 ul of planktonic cells from the starter culture of each strain and 3 additional such TSB broth were inoculated with one loop-full of bacteria taken from the formed biofilm of each starter culture. These tubes were marked as passage-1 (P-1). Planktonic cells and cells from biofilm of these P-1 cultures were used to generate P-2 cultures by growing them at 37 C for 24 hours in static condition as describe above. P-2 cultures were similarly used to generate P-3 and so on. Biofilm was quantitated at each passage by crystal violet dye binding procedure as described above.

Effect of normal human serum (NHS) on passage induced biofilm production

Serum samples collected from normal adult volunteers and pooled together, stored at 4°C until used (within 2 weeks of collection). As a previous study reported that 20% (v/v) NHS exerted maximal influence on biofilm formation by *A.baumannii* strains (140Shadeed et al, 2014), TSB containing 20% serum was used determine the influence of serum on passage induced biofilm

formation. Passage in biofilm and in planktonic state in TSB containing 20% (v/v) serum was carried out as described in the above section.

Statistical analysis

The experimental data were expressed as means ± S.D. of three independent experiments. Each experiment was done in triplicate. Student's t test was used for comparisons and the differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Biofilm lifestyle enables survival of *A.baumannii* strains on inanimate surfaces for long periods of time which is a prime reason for its persistence in hospital environments. A variety of bacterial and environmental factors influence the formation and dispersal of biofilm to colonize new sites. This study focused on investigations directed towards answering the question: whether formation of biofilm by *A.baumannii* strains modulates their potential to form new biofilm.

A.baumannii strains used in this study is listed in table 1. All the *A.baumannii* strains produced gradually increased amount of biofilm as they were passaged in biofilm mode of growth, i.e. inoculum taken form a pre-existing biofilm was used to start a new culture. The increase in biofilm formation ranged for 40.4% for strain AB-2 to 71.8 % for strain AB-3; which were statistically significant ($p < 0.01$) (Figure 1). This was however, not observed when the same strains were passaged in planktonic mode of growth, i.e. inoculums taken from planktonic culture was used to start a new culture. It is interesting to note that the strain AB-2, which produced highest amount of passage induced biofilm is an wound isolate and the strain AB-1 which produced least amount of biofilm following passage is a blood isolate. *A.baumannii* clinical strains are reported to produce varied amount of biofilm[17,18,19], but whether this attribute is related to clinical source of the strain is not clear at present. The finding of this study is in agreement with a previous study which showed that for gram negative pathogen *Pseudomonas aeruginosa*, growth in biofilm enhanced potential to form new biofilm and this observation was biofilm specific as the same strains when passaged in planktonic state did not reveal any enhancement in biofilm production[16] (Hossain, 2013). Inclusion of NHS (20 % v /v) in TSB growth medium further increased passage induced biofilm formation and continued increase was noted at P-4 level passage (Figure 2). Enhanced production of biofilm in presence of NHS may be a virulence attribute as this property may help the pathogen survive better *in vivo*.

In conclusion, the findings of this study demonstrate that *A.baumannii* clinical isolates exhibit passage induced biofilm formation which

Table 1. Biofilm production by the clinical *A. baumannii* isolats

Strain	Clinical Source	Biofilm Production
AB-1	Blood	1.54 + 0.38
AB-2	Wound	1.98 + 0.42
AB-3	UTI	1.42 + 0.42

The strains were grown in TSB for 24 hours and biofilm production was determined using crystal violet dye binding assay as described in the materials and methods section

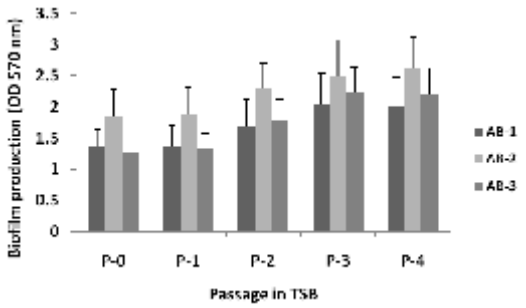

Fig. 1. Passage induced biofilm production by *A. baumannii* strains. The strains were grown in TSB and passage was carried out as described in the material and methods section

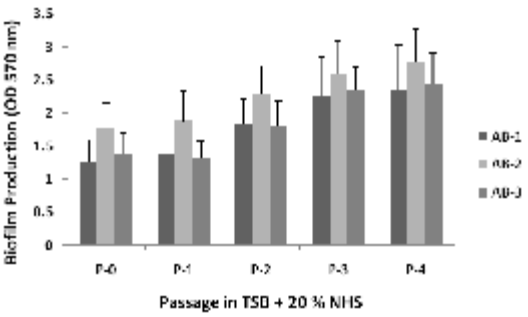

Fig. 2. Passage induced biofilm production by *A. baumannii* strains when grown in TSB containing 20 % NHS. Passage was carried as described in the materials and method section

is further enhanced in presence of NHS. These findings may have clinical relevance as dispersed bacteria from a biofilm in a clinical setting following antibiotic treatment may possess enhanced potential to populate new sites *in vivo*. Furthermore, it would be of interest to investigate whether this passage induced biofilm production is a common attribute of pathogenic microorganisms.

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