

Influence of Normal Human Serum on Production of Biofilm by *Acinetobacter baumannii*

Mamoun Shadeed¹, Mohammed S. AlMogbel¹,
Awdah Al-Hazimi² and Ashfaque Hossain^{3*}

¹Department of Microbiology, College of Health Sciences, ²Department of Physiology and Medical Education, College of Medicine³; Department of Microbiology, College of Medicine, University of Hail, Hail, Kingdom of Saudi Arabia.

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Biofilm production potential of clinical isolates of *Acinetobacter baumannii* was investigated in this study. The clinical isolates showed varied potential of biofilm production, ranging from a minimum of 1.34 ± 0.28 by a blood isolate to a maximum of 2.02 ± 0.49 by a wound isolate as determined by crystal violet dye binding assay. Biofilm production by the *A. baumannii* strains were also investigated by growing the strains in presence of different percentage (0-20 %, v/v) of normal human serum (NHS) to determine whether NHS has any effect on biofilm production. Production of biofilm by *A. baumannii* strains was increased in presence of serum in a concentration dependent manner, reaching a maximum at 20 % concentration. Strains AB-1 (blood isolate) and AB-2 (wound isolate) showed significant increase in BF production in presence of 20 %, while in the case of the strain AB-3 (UTI isolate), there was a partial reduction in biofilm production in presence of serum. Taken together, the findings of this study indicate that clinical *A. baumannii* isolates exhibit varied biofilm forming potential which is influenced by NHS in a differential manner.

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

Biofilm is a community of bacterial cells enclosed in a self-produced extracellular polymeric matrix (composed mainly of carbohydrates, proteins and nucleic acids) adhered to an inert or living surface¹. Bacteria usually form biofilm when they transit from free floating state (planktonic state) to a lifestyle in which they attach to a surface (sessile state) in response to stress such as nutrient limitation, adverse growth conditions and presence of antibiotics². Biofilm facilitates survival of bacteria in adverse environments and it exhibits

an inherent resistance to all classes of antimicrobial agents such as antibiotics, disinfectants and germicides. The extracellular polymeric material, which encases the biofilm, functions as a diffusional barrier to antimicrobial agents. Bacterial cells residing in biofilm are physiologically diverse and exhibit enhanced resistance to various physico-chemical stresses³. Production of biofilm by a pathogenic bacteria is usually considered as a virulence factor as bacteria in biofilm exhibit higher level of antibiotic resistance (10-1000 fold)^{4,5}. Moreover, bacteria in biofilm are unusually resistant to phagocytes and other components of the innate and adaptive immune system in comparison to their planktonic (free floating, not in biofilm) counterparts^{6,7}. Taken together, these properties of biofilm poses a therapeutic challenge^{8,9}.

* To whom all correspondence should be addressed.
Tel.: 966-509239719;
E-mail: a.hussain@uoh.edu.sa

Acinetobacter baumannii is a gram-negative, opportunistic, nosocomial pathogen. In recent years it has emerged as a multidrug resistant pathogen of great importance in the medical community on a global scale¹⁰. It is capable of causing a variety of infections including bacteremia, pneumonia, meningitis, urinary tract infection, and wound infection¹¹. *A. baumannii* is capable of surviving under a wide range of environmental conditions for extended periods of time which makes it a frequent cause of outbreaks of infection and an endemic, health care-associated pathogen¹². *A. baumannii* accounts for up to 20 percent of infections in intensive care units worldwide^{13,14}. In addition to its unique ability to survive for prolonged period in hospital environments, it possesses remarkable capacity to acquire and disseminate antibiotic resistance making it as one of the most notorious nosocomial bacterial pathogen to control and treat¹⁵. It is hypothesized that its ability to persist in these environments, as well as its virulence, is a result of its capacity to form biofilms¹⁶.

Previous studies on biofilm formation by *A. baumannii* strains revealed that production of biofilm is influenced by a variety of bacterial and environmental factors including subinhibitory concentration of antibiotics^{17,18,19,20}. *A. baumannii* in biofilm have altered metabolic activity in terms of UV light and acid exposure, dehydration, and phagocytosis in comparison to their planktonic cells²¹. The presence of metal cations and the expression of resistance to broad-spectrum antibiotics can also increase the ability of *A. baumannii* to adhere to, and form biofilms on a surface¹⁹. Pili assembly and production of the Bap surface-adhesion protein play a role in biofilm initiation and maturation after initial attachment to abiotic surfaces. Furthermore, the adhesion and biofilm phenotypes of some clinical isolates seem to be related to the presence of broad-spectrum antibiotic resistance¹⁹. Biofilm formation also reported to enhances survival of *A. baumannii* strains²². Although various aspects of biofilm production by *A. baumannii* investigated, the influence of normal human serum (NHS) on biofilm formation was not investigated. In this study we investigated the influence of normal human serum (NHS) on biofilm formation by fresh clinical isolates of *A. baumannii*, which extensive survey of

literature showed has not been explored so far.

We show in this work that biofilm production potential varies in clinical strains of *A. baumannii* and normal human serum exhibits a differential effect on the biofilm formation potential of the *A. baumannii* strains.

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) and trypticase soy agar (TSA) plates were used for culture of bacteria as needed.

Biofilm assay

Biofilm formation by *A. baumannii* strains was examined by crystal violet dye binding procedure as describe earlier²³. Overnight cultures of bacteria in TSB was diluted 1:100 in 3 ml of fresh TSB contained in glass tubes and allowed to grow at 37°C in a static condition for 48 hours. Biofilms attached to the glass tubes were washed to remove inbound bacteria and stained with 1% (w/v) crystal violet for 10 min at room temperature. After washing with water, the stained biofilms were dissolved in 95% ethanol and the absorbance at 570 nm was determined. The experiment was performed in triplicates. OD570 values for each tubes were subtracted from those of the blank, which were uninoculated TBS.

Effect of incubation time on biofilm production

Cultures were set up as described above and incubated at 37°C at static condition for 8, 18, 24 and 48 hours. At each time point triplicate cultures were assayed for biofilm formation as described above.

Effect of normal human serum (NHS) biofilm production

Serum was collected from adult volunteers and pooled together, stored at 4°C and used in the experiments within one week of collection. To explore the possibility whether NHS has any effect on new biofilm formation by *A. baumannii* strain, different percentage of NHS (0-20 %, v/v) in TBS was used to grow the bacterial strains. As initial experiments on biofilm production showed that biofilm production was maximal and 24 hours, the effect of serum on biofilm production was studied by growing the bacteria for 24 hours. Biofilm

formation was assayed as described above.

Statistical analysis

Data were expressed as means \pm S.D. of three independent experiment 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 formation is general attribute of bacteria and so far all organisms investigated have been found to produce biofilm³. Biofilm is an important determinant for bacterial colonization of the human host and for persistence in the hospital environment. Studies on biofilm have shown that production of biofilm is a complex process and a variety of environmental signals influence its formation^{2,5}.

Previous studies on biofilm formation by *A. baumannii* strains revealed that production of biofilm is influenced by a variety of bacterial and environmental factors^{17,18,19,20}. We show in this work that *A. baumannii* strains from different clinical sources produce varied amount of biofilm and normal human serum enhances the potential of biofilm formation in a concentration dependent manner in certain strains of *A. baumannii*.

The clinical source of the strains and their biofilm formation potential is presented in the Table 1. Maximum amount of biofilm was produced by strain AB-2 which is a wound isolate, which was followed by AB-3 (urinary tract infection isolate).

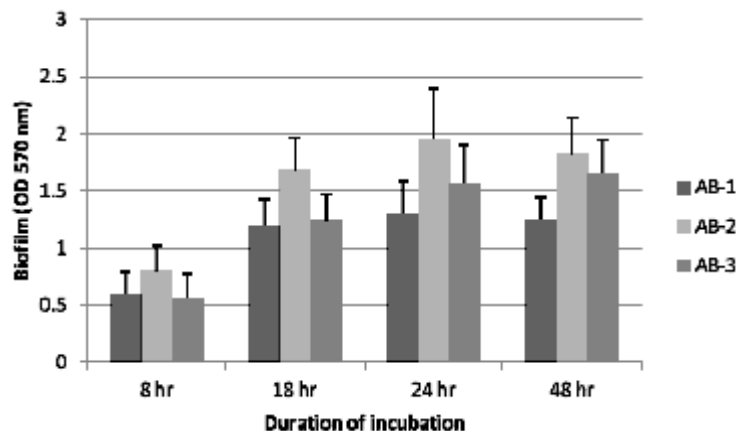
The least amount of biofilm was produced by AB-1, a blood isolate. This is in agreement with the previous findings which reported that tissue isolates of *A. baumannii* produced higher amounts of biofilm in comparison to liquid tissue isolates²⁴. Recent studies showed that there was no relationship between the degree of biofilm formation and site of sample collection^{24,25}.

Time course of biofilm formation by *A. baumannii* strains was carried out by growing the bacteria in TSB for various lengths of time and carrying out biofilm assay at different time points. As determined by crystal violet dye binding assay, biofilm production increased with the length of incubation period, with maximal production at 24 hour (Fig. 1). At 48 hour time point two strain (AB-1 and AB-2) showed a little reduction in biofilm production, while the third strain AB-3 exhibited a slight but not significant increase in biofilm production.

Table 1. Clinical source and biofilm production by *A. baumannii* strains

Strain	Clinical Source	Biofilm Production
AB-1	Blood	1.34 + 0.28
AB-2	Wound	2.02 + 0.49
AB-3	UTI	1.52 + 0.32

The strains were grown at TSB for 24 hours and biofilm assay was carried out using crystal violet dye binding assay as described in the material and methods section.



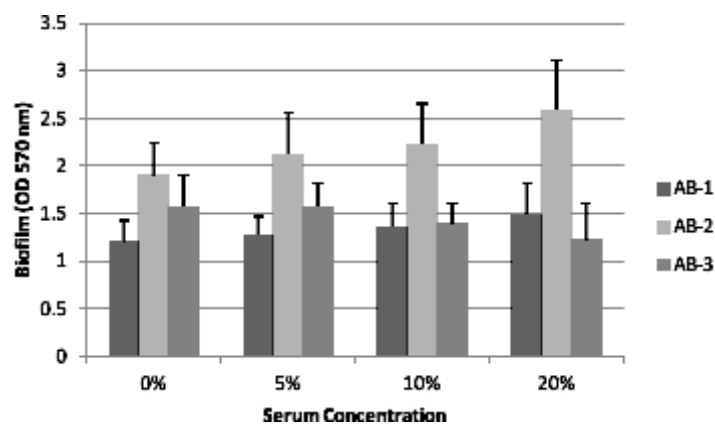
The strains were grown at TSB for different lengths of time and biofilm assay was carried out at different time points as described in the material and methods section

Fig. 1. Time course of production of biofilm by clinical isolates of *A. baumannii*

As *A. baumannii* causes systemic infection, the bacteria is exposed to various host components including serum. So, it was of interest to see if serum has any influence on biofilm formation by the clinical isolates of *A. baumannii* strains included in this study. Although many gram negative bacteria are usually susceptible to serum, *A. baumannii* clinical isolates are frequently found to be serum resistant and activate alternate pathway of complement fixation²⁶. A comparison of serum resistant and serum sensitive strains of *A. baumannii* for biofilm production showed that serum resistant strains produced relatively higher amount of biofilm in comparison to serum sensitive strains²⁶. Although production of biofilm by serum resistant *A. baumannii* strains were investigated, the effect of NHS on the production of serum was

not investigated. In this study we allowed *A. baumannii* stains to grow in different concentrations of serum for 48 hours and determined its effect on BF formation. BF formation by the strain AB-1 and AB-2 was increased when 10 % serum was used however it was not statistically significant. However at 20 % serum concentration significant increase was noted ($P < 0.05$) (Fig. 2).

Interestingly, the effect of NHS on the biofilm production by the *A. baumannii* strains were not uniform. Strains AB-1 and AB-2 showed significant increase in biofilm production in presence of 20 %, while in the case of the strain AB-1, there was reduction in biofilm production (Fig. 3). The reason for this is not apparent at present. However, it may be noted here that the



The strains were grown at TSB for 24 hours containing of different concentrations of serum and biofilm assay was carried out as described in the material and methods section

Fig. 2. Effect of normal human serum (NHS) on biofilm production by clinical isolates of *A. baumannii*

strains were from different clinical sources; AB-1 is a blood isolate, AB-2 is a wound isolate and AB-3 is an UTI isolate (Table 1). Investigation with a large number of strains from different clinical sources is warranted to delineate the mechanism of differential response of *A. baumannii* strains in producing biofilm following exposure to NHS.

Biofilm production by *A. baumannii* strain have been reported to be influenced by serum resistant trait of the strains; in addition MDR phenotype also reported to enhance biofilm production²⁶. Results of this study showing that serum also enhances biofilm production by *A. baumannii* strains adds another clinically relevant

factor that also influences biofilm production. Taken together, these findings indicate that the ability of *A. baumannii* to form biofilms is multifactorial and diverse. Understanding the molecular basis of biofilm production, which is virulence factor of this pathogen, is necessary to formulate strategies to control this pathogen. Biofilm production by pathogens poses a therapeutic challenge as bacteria in biofilm exhibit many fold increased resistance to antibiotics and enhanced resistance to clearing by immune system in comparison to their planktonic counterparts^{4,5,9}. Clear understanding of molecular basis of biofilm production by *A. baumannii* strains will help

formulation of effective strategies to develop effective therapeutic agents against this medically important bacteria.

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