Influence of normal human serum (NHS) on production of biofilm by clinical isolates of *Pseudomonas aeruginosa*

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Abstract: Biofilm production is considered as a virulence attribute of the opportunistic pathogen *Pseudomonas aeruginosa*. As *P. aeruginosa* strains causing systemic infection are exposed to various host immune factors including serum, the effect normal human serum (NHS) on biofilm forming potential of fresh clinical isolates of *P. aeruginosa* was investigated in this study. Time course of biofilm production by the *P. aeruginosa* strains showed that at 24 hour time point, biofilm production reached maximal level by all 4 strains investigated in this study. The effect of NHS on the production of biofilm was carried out by growing the strains in tripticase soy broth (TSB) containing 20 % (v/v) NHS. Two blood isolates of *P. aeruginosa* B-1 and B-2 showed enhanced production of biofilm in presence of 20 % serum, while production of biofilm by the wound isolate W-2 was partially inhibited by it. Biofilm production by the other wound isolate, W-1, was not effected by 20 % serum. Taken together, the findings of this study show that NHS has a differential effect on biofilm production by fresh clinical isolates of *P. aeruginosa*.

[Hossain, A. **Influence of normal human serum (NHS) on production of biofilm by clinical isolates of *Pseudomonas aeruginosa.*** *Life Sci J* 2013;10(10s):151-154] (ISSN:1097-8135). http://www.lifesciencesite.com**.** 23

Key Words: Biofilm, *Pseudomonas aeruginosa,* Serum, Clinical isolate

1. Introduction

Biofilm is a community of bacterial cells adherent to a biotic or abiotic surface, enclosed in a self-produced extracellular polymeric matrix (EPS), which is composed mainly of carbohydrate, protein and DNA (Lopaz *et al,* 2010; Hall-Stoodley and Stoodley, 2009). Bacteria form biofilm when they transit from free floating (planktonic) state to a lifestyle in which they attach to a surface (Flemming and Wingender, 2010). 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) (Parsek and Singh, 2003; Lewis, 2001) and are unusually resistant to phagocytes and other components of the innate and adaptive immune system (Leid, 2009; Meyle *et al,* 2010) in comparison to their planktonic (free floating, not in biofilm) bacteria. According to a report from National Institute of Health (NIH), USA, biofilm accounts for 60-70% percent of microbial infections in human (NIH, 2002), which highlights the importance of biofilm in treatment of infectious diseases.

Pseudomonas aeruginosa is gram negative an opportunistic bacterial pathogen that can cause a variety of infections in the human host, especially in immunocompromised hosts and individuals with cystic fibrosis (Costerton *et al,* 1999). It also causes wound infections and bacteremia (Fergie et al., 1994; Harrison- Balistra *et al,* 2003) and biofilm that the pathogen forms *in vivo* contributes to chronicity of infection (Lebeaux *et al,* 2013). Various physical and chemical factors, such as growth conditions and culture media composition influence the production of biofilm by *P. aeruginosa* (Ma *et al,* 2009). The persistence of chronic *P. aeruginosa* in lung infections in cystic fibrosis (CF) patients is due to biofilm-growing mucoid (alginate-producing) strains (Moskowitz *et al,* 2004). It has been reported that that growth of *P. aeruginosa* in biofilm enhanced its potential to form new biofilm, presumably indicating that passage in biofilm induces gene expression cascade which results in increased amount of biofilm formation (Hossain, 2013). We show in this work that *P. aeruginosa* strains from different clinical sources are able to produce biofilm and normal human serum exerted a differential effect on the biofilm formation potential of different strains of *P. aeruginosa* .

2. Materials and Methods

Bacterial strains and culture conditions: *P. aeruginosa* 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 *P. aeruginosa* strains was quantitated by crystal violet staining procedure as describe earlier (Moskowitz *et al,* 2004). Overnight cultures of bacteria in TSB were diluted 1:100 in 3 ml of fresh TSB contained in glass tubes and allowed to grow at 37^0 C in a static condition for 24 hours. Biofilms attached to the glass tubes were washed to remove unbound 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 100% ethanol and the absorbance at 570 nm was determined using a spectrophotometer.

Effect of incubation time on biofilm production: Cultures were set up as described above and incubated at 37^0 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) on biofilm production: Serum was collected from healthy, adult volunteers pooled together, stored at 4^0 C and used in the experiments within one week of collection. To explore the possibility whether NHS exerts any effect on biofilm formation by *P. aeruginosa* strains, different percentage of NHS (0-20 %, v/v) in TBS was used to grow the bacterial strains and allowed to form biofilm. As initial experiments on biofilm production showed that the maximum amount of biofilm was produced at 24 hour time point, the effect of NHS on biofilm production was studied by growing the bacteria for 24 hours. Biofilm formation was assayed as described above.

3. Results and Discussion

P. aeruginosa causes a variety of infections in the immunocompromised host including bacteremia. Clinical *P. aeruginosa* strains causing bacteremia must withstand the bactericidal action of serum, phagocytosis and other components of both innate and adaptive immune system. Most strains of *P. aeruginosa* are resistant to killing in serum alone, but the addition of polymorphonuclear leukocytes results in bacterial killing. Killing is most efficient in the presence of type-specific opsonizing antibodies, directed primarily at the antigenic determinants of LPS, indicating that normal human serum is usually devoid of any specific antibodies (Vitkauskiene *et al,* 2005).

P. aeruginosa is one of the most extensively studied bacteria for biofilm production. Biofilm is an important determinant for colonization of the human host by

Table-1. Production of biofilm by clinical isolates of *P. aeruginosa.* The results represent mean± standard deviations of three independent experiments.

Strain and source	Biofilm production
B-1(Blood)	0.87±0.19
B-2 (Blood)	0.72±0.21
W-1 (Wound)	1.09±0.25
W-2 (Wound)	1.34±0.31

P. aeruginosa and it facilitates its survival *in vivo* that results in chronic infection (Costerton *et al,* 1999). Previous studies on biofilm formation by *P. aeruginosa* strains revealed that its production is influenced by a variety of bacterial and environmental factors (Janjua *et al,* 2012; Drenkard E, Ausubel, 2002). As the pathogen can cause systemic infection in immunocompromised host and also as *P. aeruginosa* clinical isolates are frequently found to be serum resistant (Vitkauskiene *et al,* 2005), it was of interest to investigate whether normal human serum has any influence on biofilm formation.

Table1 shows the clinical source of the strains and their biofilm formation potential. Maximum biofilm was produced by strain W-1 which is a wound isolate; which was followed by another wound isolate, W-2. The two blood isolates B-1 and B-2 produced relative lesser amount of biofilm. This is in agreement with the previous findings which reported that tissue isolates of *P. aeruginosa* produced higher amounts of biofilm, in comparison to liquid tissue isolates such as those isolated from blood (Sanchez *et al,* 2013).

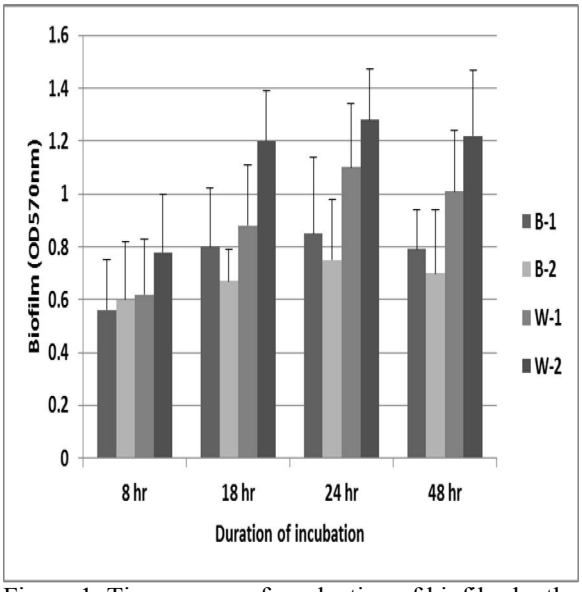

Figure 1. Time course of production of biofilm by the *P. aeruginosa* strains. The results represent mean± standard deviations of three independent experiments.

Time course of biofilm formation by strains were carried out by growing the bacteria in TSB for various lengths of time and carrying out biofilm assay at each time point. As determined by crustal violet dye binding assay, biofilm production increased with the length of incubation period, with maximal production at 24 hour time point (Figure 1). At 48 hour time point, no significant change in biofilm production was noted for two strains (B-1

and W-2), while the other two strains (B-2 and W-2) showed a slight but not significant reduction in biofilm production. So 24 hour time point was used to investigate the effect of serum on production of biofilm. Use of different concentrations of serum (0-20 % volume / volume in TSB) showed that biofilm formation by strains B-1 and B-2 was enhanced by NHS in a concentration dependent manner, reaching a maximal level by 20 % serum, which was statistically significant ($P < 0.05$) for both the strains in comparison to biofilm production when no serum was used. On the other hand, formation of biofilm by the wound isolate W-2 was partially inhibited by 20 % serum and which however, had no effect on the biofilm production by the W-1 (Figure 2). It is interesting to note here that *P. aeruginosa* strains B-1 and B-2 are blood isolates, whereas strain W-1 and W-2 are wound isolates. Why blood isolates showed increased production of biofilm in presence of NHS, while reduced amount of biofilm was produced by the wound isolate W-1 is not clear at clear at present. It is probable that as blood isolates are constantly exposed to various bactericidal blood components, these may have acted as trigger to induce increased biofilm production as a survival mode of these strains.

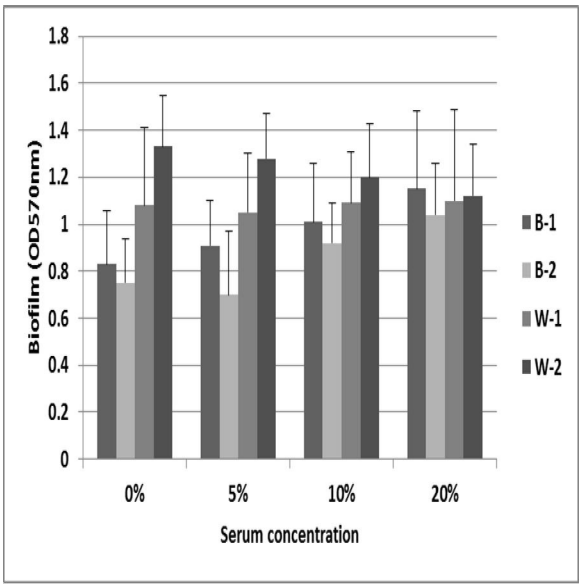

Figure 2. Influence of normal human serum (NHS) on production of biofilm by *P. aeruginosa* strains. The results represent mean± standard deviations of three independent experiments.

Hammond *et al* (2010) investigated the effect of adult bovine serum (ABS) and bovine serum albumin (BSA), adult human serum (AHS) and adult human plasma (AHP) on biofilm formation by the *P. aeruginosa* strain PAO-1. They found that ABS, BSA, AHS and AHP inhibited formation of BF by *P. aeruginosa*. This finding is partial disagreement with the finding of the present study. Here 2 out of 4 strains in this study showed enhanced BF formation in presence of normal human serum (NHS); while one stain exhibited reduction in biofilm production and biofilm formation by the other strain was not affected. This apparent disagreement can be explained both in terms of strain and growth media used. In this study fresh clinical strains were used whereas Hammond *et al* (2010) used *P. aeruginosa* strain PAO-1, a widely used laboratory adapted strain. Genomic plasticity of *P. aeruginosa* endows a variety of characteristics in different strains and growth media is also reported to play a significant role in different phenotypic properties of *P. aeruginosa* including biofilm production (Drenkard and Ausubel, 2002). Use of M-9 media by Hammond et al (2010) and TSB in the present study may also have contributed to the apparent different findings in this study and that of Hammond *et al* (2010). Another important point here is that clinical strains of PA exhibit wide range of variation in terms of virulence (Janjua *et al*, 2012). It may be noted here that NHS has been reported to exert an inhibitory effect on the biofilm production by gram positive pathogenic bacteria *Staphylococcus aureus* (Abraham and Jefferson, 2010), while NHS enhanced biofilm production by unicellular fungal pathogen *Candida albicans* (Samaranayake *et al*, 2013). However, none of these studies described the molecular basis of inhibition or enhancement of biofilm production by serum.

The finding of this study highlights the wide variation in the phenotypic characteristics of the fresh clinical strains of *P. aeruginosa* isolated from different types of infections. Studies with a larger number of *P. aeruginosa* stains from different clinical sources is warranted to delineate the apparent differential response of *P. aeruginosa* strains in biofilm production in presence of NHS.

Acknowledgements:

The generosity of Dr. Awdah Al-Hazimi, Dean of the College of Medicine and Dr. Mohamed Mogbil, Dean, College of Health Sciences, University of Hail, Hail, Saudi Arabia for providing laboratory materials for this study is gratefully acknowledged.

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9/2/2013