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Original article

Determination of Biofilm Formation between Different Strains of *Propionibacterium Acnes* Using Biofilm Assay

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Abstract

Propionibacterium acnes (P. acnes) a member of the normal flora of the skin has constantly been associated with deep tissue infections especially during medical processes. *P. acnes* have been isolated in deep tissues and are believed to be an aetiological agent in these infections, contributing to the progression of some of these diseases. The biofilm formation ability between different strains of *P. acnes* was determined. Ten (10) *P. acnes* clinical isolates were considered, two (2) from acne vulgaris and eight (8) [two (2) per *recA* types 1A1, 1B, II and III] from lumber herniation tissues. Semi quantitative biofilm analysis using the microtiter plate assay was used with some modification. The semi quantitative biofilm assay was done in triplicates. The result obtained from the biofilm triplicates from 4 days' incubation using 3 days' culture showed that isolates 17(IB), 82(IB) and 55(II) showed very high biofilm production for 2 replicates which implies that they are real biofilm producing isolates. Using overnight cultures, higher biofilm production was witnessed with isolates 1(III), Lesion 7 and 84 (IA1) being the highest biofilm producers. Although with 3 days' culture, isolate 1(III) could easily be discarded as a-non biofilm producer, while lesion 7 and 84 (IA) has been associated to biofilm formation in 3 days' culture. The production of biofilms by isolates supports the theory that the ability of *P. acnes* to form biofilms enables it to attach to medical implants hence causing deep tissue infections.

Keywords: ***Propionibacterium acnes, Biofilm, Biofilm assay, Strains, Medical implants.***

Introduction

Propionibacterium acne (P. acnes) is an aero tolerant, anaerobic, Gram- positive, non-motile, pleomorphic rod belonging to the phylum *Actinobacteria* and class *Propionibacteriales* (Patrick and McDowell, 2012). *P. acnes* is a member of the normal flora inhabiting and colonizing the human gut, skin, intestinal tract, external ear canal and the conjunctiva (Cogen *et al.*, 2008) and it often is non-pathogenic (Portillo *et al.*, 2013) although it has been hypothesized to have a major contribution in the infection of deep tissues. The haemolysin and repair and maintenance (*recA*) of DNA genes was used in grouping strains of *P. acnes* from different infections into types I, II and III using the whole genome sequencing (McDowell *et al.*, 2005: McDowell *et al.*, 2008). Types IA IB, II and III of *P. acnes* being highly evolutionary and distinct linage of *P. acnes* were identified using the multilocus sequence typing (Lomholt and Kilan, 2010). McDowell *et al.* (2012) isolated antibiotics resistance strain of *P. acnes* from a patient of acne vulgaris described as type 1C cluster.

The virulence of *P. acnes* can be improved by its ability to adhere/attach to the human skin, mostly on sebaceous body sites leading to infections of deep tissue via bacteria seeding through surgical incisions, inadequate antiseptics or aseptic techniques (Gallo *et al.*, 2003; Grice *et al.*, 2009). During or after a medical device implant, *P. acnes* can enter the bloodstream by contaminating the haematogenous or exogenous skin microbiota (Sohail *et al.*, 2009). The ability of *P. acnes* to travel to an implant site where conditions favorable to its growth and multiplication can be found once it is seeded is termed ''the race for the surface'' as termed by Gristina *et al.*, (1988).

A polysaccharide matrix, bacteria cells or substratum holding bacteria cells in a particular metabolic state is a biofilm (Coneye *et al.*, 2007). *P. acnes* are able to produce biofilms in vivo (on beads) and in vitro (in acne and within follicles) (Jahns and Alexeyev, 2014). A mature *P. acnes* biofilm is first seen in vitro from 18 - 96 hours (Ramage *et al.*, 2003: Qi *et al.*, 2008). Recent studies suggest that the cause of the chronic skin infection acne vulgaris may be

influenced by the formation of biofilms by *P. acnes* (Burkhart and Burkhart, 2003a; Coenye *et al.*, 2007: Jahns *et al.*, 2012). Biofilm pathogenesis theory has been strengthened by the tissue-invasive patterns and macro-colonies on the walls of the follicles witnessed through the direct visualization of *P. acnes* cells using immunofluorescence microscopy (IFM) and fluorescence in situ hybridization (FISH) (Alexeyev *et al.*, 2012), as defined by Parsek and Singh 2003. Bacteria are able to adhere to the host extracellular matrix proteins after an implant and the granulocytes may be unable to destroy them (Kristian *et al.*, 2008). Changed phenotypes in respect to bacterial growth, gene expression and production of protein is observed in sessile *P. acnes* cells as compared to their planktonic phase and antibiotic resistance among strains can develop as a result of this change (Archer *et al.*, 2011). In a study by Holmberg *et al.* (2009), it was discovered that deep tissue infections isolates of *P. acnes*, especially those relating to foreign materials produced more biofilms in vitro than *P. acnes* isolates found on the skin of healthy persons. Bacterial biofilms contain mostly carbohydrates (Bruggermann *et al.*, 2004). Glycosyl transferases and UDP-N-acetylglucosamine-2-epimerase (enzymes that utilize activated sugars), thought to be involved in biofilm formation have genes encoding for them in *P. acnes* (Burkhart and Burkhart, 2003b: Burggemann *et al.*, 2004). The increased lipase activity of sessile *P. acnes* cells is able to attract neutrophils, these host immune cells suffer frustrated phagocytosis hence lysing and adding to the biofilms exopolymeric substances (Lee *et al.*, 1982). Due to the difficulty in treating biofilm related *P. acnes* infections, surgical removal of the foreign material mostly serves as the cure. Biofilm forming ability of *P. acnes* is an important virulence factor for deep tissue infections and medical implant (Holmberg *et al.*, 2009)

Materials and Methods

Eleven (11) *P. acnes* isolates (10 clinical infection isolates and NCTC 737 a type I reference strain, {biotype 3}) were selected at random for this experiment, with no two (2) samples coming from the same source (patient). The sample size covered two (2) isolates per *recA* genotype ($1A_1$, 1B, II, III). Lumber disc herniation provided eight (8) of the isolates while two (2) came from acne lesions. The used isolates were 71($1A_1$), 84($1A_1$), 17(IB), 82(IB), 24(II), 55(II), 1(III), 64(III), acne lesion 1, acne lesion 7 and NCTC 737.

Chemicals/Reagents: Brain heart infusion agar and broth, purchased from Oxoid, United Kingdom, 0.1% Crystal violent, 30% acetic acid.

Materials: Petri dishes, 96-well microtitre plates, 96-well flat bottom microtitre plates, Pasteur pipette, colorimeter (Corning 253), Elisa auto reader (Tecan, sunrise plate reader).

Overnight cultures preparation

Brain heart infusion (BHI) agar and broth (Oxoid) was prepared according to the Manufacturer's prescription. BHI agar plates were used to anaerobically grow *P. acnes* cultures (by streaking) and incubated at 37˚C for 4 days (96 hours). Overnight cultures were made from these pure *P. acnes* colonies by anaerobically growing them in sterile broth at 37˚C.

Biofilm formation assay

Microtiter plate biofilm assay (Semi quantitative analysis)

Semi quantitative biofilm analysis using the microtiter plate assay as described by Merritt *et al.*, (2005) was used with some modification. Briefly, bacteria (*P. acnes* isolates of interest), were cultivated in BHI broth for 3 days and then standardised to an optical density of 0.1 at a wave length of 590nm with a colorimeter (Corning 253) using some sterile broth. 200µl of these standardised samples were inoculated directly to sterile 96- well microtiter plates, using 12 wells (replicates) per sample. Assay plates were covered with lids and incubated at 37°C for 4 days. After growth, planktonic bacteria were removed by briskly shaking off the microtiter dish into a disposal box. Microtiter plates were washed by using a Pasteur pipette to add drop wise, sterile water to each well two times. This was discarded and the plates were stained for 10 minutes with 250µL of 0.1% crystal violent solution by adding to each well at room temperature. The crystal violent was washed off using water and as much liquid as possible after every wash. Each plate and wells were drained out, each microtiter plate was inverted and allowed to air dry. 250µL of 30% acetic acid was solubilised and added to each stained well and allowed to solubilise by incubating them at 37°C for 15 minutes on the desk. The content of each well was mixed for a short time by pipetting up and down. From each well to a separate well in an optically clear flat bottom 96-well plate in 12 replicates for every strain 125µL of the crystal violent/acetic acid solution was transferred. NCTC 737 was the positive control for biofilm formation. An Elisa auto reader (Tecan, sunrise plate reader) was used to measure the absorbance of each of the wells containing the 125µL solution at 595nm. The biofilm experiment using 3 days old culture was done in triplicate. An overnight culture of *P. acnes* isolate was also prepared and centrifuged to get a 0.1 optical density. This was grown and stained following the above method as to identify which culture yielded high biofilm formation. The triplicate (3) biofilm 3 day's culture upon standardisation, were inoculated into two different microtiter plate. Inoculated microtiter plates were incubated anaerobically and grown at 37˚C for 4 days.

Statistics

Completely Randomized Design (CRD) as described by Gomez and Gomez (1984) was used with three replication and data obtained were analyzed statistically by Analysis of Variance (ANOVA) according to Gomez and Gomez (1984).

Result

Semi quantitative biofilm formation analysis

Biofilm formation was compared in a group of 10 *P. acnes* isolates from clinical infections. The result obtained from the biofilm triplicates from 4 days' incubation using 3 days' culture showed from the highest biofilm producer to the least that.

- Replicate one (1) showed greater biofilms being produced by the isolates 82(IB), 24(II), 55(II), 64(III) and the NCTC.
- Replicate two showed greater biofilm production by isolates 17(IB), 82(IB), 55(II), Lesion 7, 71(IA1), 24(II) and the NCTC.
- Replicate three showed higher biofilm production from isolates 71(IA1), 24(II), 55(II), 17(IB), 82(IB), Lesion 7, 84(IA1), Lesion 1 and the NCTC. Isolates 17(IB), 82(IB) and 55(II) showed very high biofilm production for 2 replicates which implies they are real biofilm producing isolates. The mean absorbance for each isolate was

calculated as each of the isolates used was tested in triplicates on three different occasions, maintaining the procedure.

From the result obtained, higher biofilm production was witnessed with the use of overnight cultures than the 3 days' old culture. Isolates 1(III), Lesion 7 and 84 (IA1) were the highest biofilm producers. Although with 3 days' culture, isolate 1(III) could be judged as a-none biofilm producer but lesion 7 and 84 (IA) has been associated to biofilm formation in 3 days' culture.

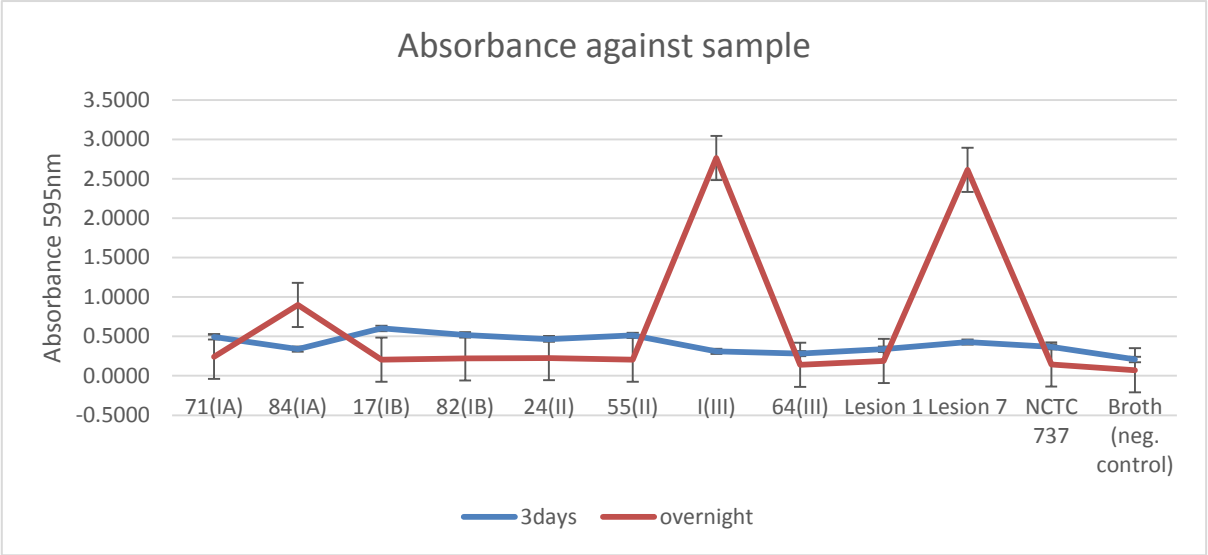

Figure 1: Comparing the biofilm forming ability between triplicates of *P. acnes* isolates from 3 days old culture to an overnight culture against their absorbance upon 4 day's incubation.

Using ANOVA, *P. acnes* samples, P= 0.54599. P is greater than 0.05. For the biofilm test, P = 0.394056. P is also greater than 0.05. There is a significant difference between the use of a direct overnight culture and the use of a 3days culture although both were standardized to the same optical density.

Confirming the reproducibility of this method, the same standardized *P. acnes* culture was used and inoculated on to two different microtiter plates, under the same conditions. It was observed that there was still a slight difference to the result with 3A recording a very high biofilm in isolate 82(IB) with above 0.5nm absorbance while the highest biofilm produced by 3B was in isolate 71(IA1) with about 0.3nm. This showed that this method cannot be said to be 100 percent reproducible.

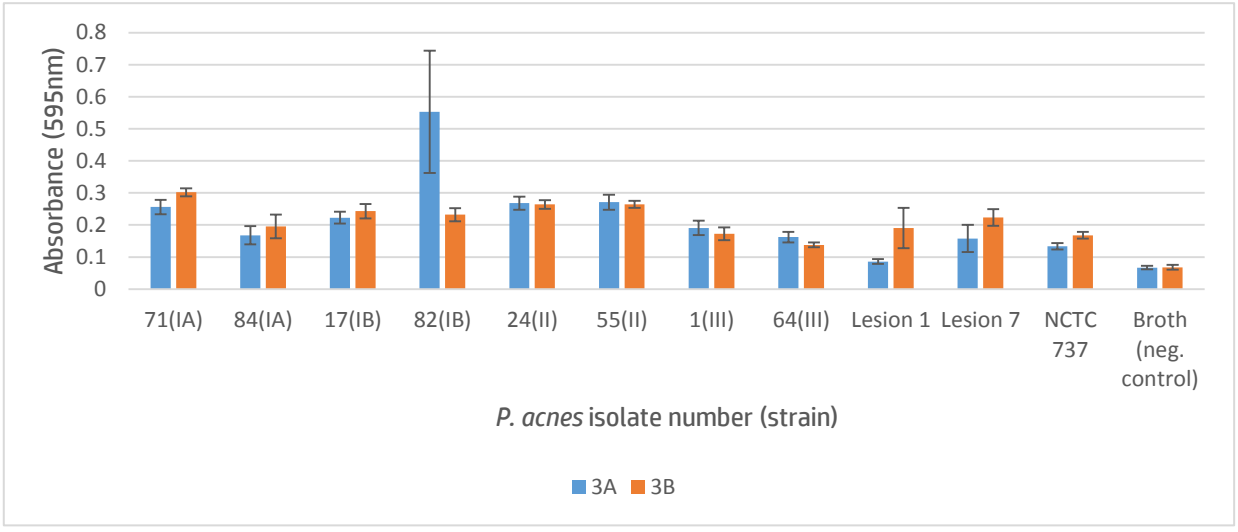

Figure 2: Replicate 3 of *P. acnes* biofilm, comparing reproducibility of method from 4 day's incubation.

Discussion

A vital virulence strategy of most bacteria infecting foreign materials studied is their ability to form biofilms (O'Gara, 2007). In this study different isolates of *P. acnes* produced biofilms at different rates however, the regulation of biofilm formation and the difference in biofilm formation by different *P. acnes* isolates is not very well understood (Holmberg *et al.*, 2009). An important aetiological agent of invasive infections related to foreign materials is *P. acnes* even though it is a skin commensal (Lutz *et al.*, 2005: Zeller *et al.*, 2007). The fact that *P. acnes* can form biofilms have been fully established, (Ramage *et al.*, 2003; Bayston *et al.*, 2007; Conye *et al.*, 2007: Qi *et al.*, 2007). Holmberg *et al.* (2009) indicated that the environment influences regulation of genes

encoding components of *P. acnes* forming biofilms. It was demonstrated that production of biofilm is not affected only by the site of the isolation but also by the human plasma and the composition of growth medium.

This result from this study showed higher biofilm production among all the lumber disc herniation isolate than the negative control which is same as the work of Holmberg *et al*. (2009) which showed that more biofilm is produced in vitro by deep infections isolates related to foreign material than healthy individuals skin isolate. This shows that biofilm formation is vital for infections of foreign materials, lumbar disc herniation and joint prostheses and biofilm forming ability is a virulence factor of *P. acnes* (Holmberg *et al*., 2009). The number of prosthetic joint infections recorded to have been caused by *P. acnes* might have been greatly underestimated, due to the availability of modern bacteria dictating techniques and many cases thought to have been of 'aseptic' prosthesis loosening were true infections of *P. acnes* (Tunney *et al*., 1999).

The type of nutrient received via haemolysis, composition of growth medium, site of bacteria isolation and human plasma all have been shown to affect the production of biofilms (Holmberg *et al*., 2009). Carbohydrates make up most bacterial biofilms, glycosyl transferases and UDP-N-acetylglucosamine-2-epimerase (enzymes that utilize activated sugars), thought to be involved in biofilm formation have genes encoding for them in *P. acnes* (Burkhart and Burkhart, 2003b: Burggemann *et al.*, 2004).

The result of this study shows no variation was seen in the method but the semi quantitative biofilm analysis cannot be said to be a reproducible method. The absorbance of each well measured were considered as an index of bacteria adhering to surface and forming biofilms. This may support the report of most investigations that have assumed that biofilm formation on a foreign material can be facilitated by a preconditioned film of protein coat (O'Gara, 2007). Hence, most researchers use plasma to pre-treat the foreign material of their studies. The plasma proteins treatment effect on biofilm formation is complicated. This is because the initial step in the formation of biofilms is adhesion and every protein has different effect on adhesion of bacteria cells to foreign materials (Holmberg *et al*., 2009). For instance, cell adhesion and biofilm production is inhibited by plasma and albumin (Kinnari *et al*., 2005). In vivo formation of biofilm does not compulsory equal its' in vitro formation (Holmberg *et al*., 2009). Although, most variations seen in biofilm formation can be due to in the method and not biological (Holmberg *et al*., 2009).

In this study, it was established that *P. acnes* isolates from lumbar disc herniation could produce biofilms which is highly clinically relevant and the pathogenesis of deep infections with *P. acnes* (Holmberg *et al*., 2009). A vital tool in differentiating a bacterial contamination and a true bacterial infection is testing for biofilms which is due to the relationship between an infection site and biofilm formation (McLorinan *et al*., 2005: Corgen *et al*., 2008). Ramage *et al*. (2003) were able to show that *P. acnes* strains had the ability to form very resistant biofilms in various biomaterials, supporting the formation of *P. acnes* biofilm in lumber disc herniation as shown by this research. Although this research was conflicted by Takemura *et al*. (2004) which proved that formation of biofilms by *P. acnes* in dental filling gutta-percha points was not possible. Three different cluster of genes encoding for enzymes involved in extracellular polysaccharide biosynthesis were identified in the whole genome sequencing of *P. acnes* (Burkhart and Burkhart, 2003b: Bruggemann *et al*., 2004). This implies the capability of *P. acnes* to form necessary biofilm extracellular matrix (Conye *et al*., 2007). Many cell adhesion related proteins were identified (Burkhart and Burkhart, 2003b: Bruggemann *et al*., 2004).

The genes responsible for *P. acnes* biofilm formation is still unknown at present, so also the chemical composition of the biofilm (Holmberg *et al*., 2009). In 2009, Holmberg *et al*. hypothesized that when *P. acnes* biofilms are exposed to plasma (during a skin cut or during blood transfusion), *P. acnes* cells are able to respond to this by converting to a planktonic state of growth. Spread of bacteria to distant body sites or even to a new host is facilitated by this change in growth phase (Holmberg *et al*., 2009). This also is a virulent strategy if proven. This deserves more investigation since the formation of biofilms by *P. acnes* is important to the invasiveness of infections.

Some isolates showed high biofilm production for replicates with 3 days' culture, this implies they are real biofilm producing isolates. In the overnight culture, these isolates expressed biofilm formation but not as the highest biofilm producers. Also an isolate which showed very low biofilm using the 3 days' culture produced the highest biofilm with the overnight culture. A major factor responsible for difference in strain biofilm formation is the phase variation affects adhesion expression like PPA2210 and dermatan-sulfate adhesin PPA2127 (Holmberg *et al*., 2009: Brzuskiewicz *et al*., 2011). The differences in biofilm assay results from overnight culture and three days old culture can be hypothesized to be due to the fact that biofilm genes may be produced at a particular growth phase. Although the particular genes for biofilm formation are not known (Holmberg *et al*., 2009), it is hypothesized that they are expressed in the *P. acnes* exponential growth phase. Lee *et al*. (2010) showed that supernatant of ATCC 6919 culture a *P. acnes* strain had high proteolytic activity with a casein substrate in the exponential growth phase. Identification of about 20 proteins secreted at the mid-exponential growth phase of *P. acnes* was done using in vitro proteomic investigation. Putative adhesins are believed to contribute to *P. acnes'* ability of binding /attaching as aforementioned (Holland *et al*., 2010). Hence the high biofilm expression in the overnight culture might be because the *P. acnes* cells in overnight culture is in the exponential phase where biofilm formation genes are expressed. As to the three days old culture, even though it was standardized and sterile broth added to it, the bacteria cells at this stage might be said to be in stationary stage or the death phase. Therefore, even in the presence of fresh nutrients, *P. acnes* cells will still need more time to stabilize and return to their logarithmic growth stage where biofilm genes are hypothesized to be primarily expressed.

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Conflict of interest

There was no conflict of interest between the authors.

Ethical Approval

The Coventry University Ethics committee approved this study with project reference number P42435.

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