Research Article

The Effect of Various Growth Parameters on the Strengths of Biofilm Produced by Otits Media Causing Bacteria

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ABSTRACT

Otitis media (OM) is one among the common infections inflicting human population from ancient times. Even though many viral and fungal agents have been reported to cause OM, the majority of the cases have been attributed to bacterial pathogens. The ability of the bacteria invading the middle ear to produce extracellular polymeric layer called as biofilms contribute to their success in causing the infection. In general, the studies on the effect of various growth parameters on the intensity of biofilm produced in medically relevant isolates is comparatively rare and so his study aims at gaining a better understanding regarding bacterial biofilms and the effect of various growth parameters on the intensity of the biofilm produced.

Keywords: Otitis media, bacteria, biofilm, parameters, pH, temperature.

INTRODUCTION

Otitis media (OM) or ear infection is a common ailment inflicting a vast majority of the population, especially children. This infection of the middle ear is characterized by purulent discharge, feeling of fullness inside the ear, irritation and pain. In case of recurrent infections, complications such as otorrhoea, rupture of the tympanic membrane and mastoiditis may arise. OM may be classified into four types based on the symptoms experienced; CSOM, OME, AOM and Adhesive OM. The onset of the disease, mainly during childhood occurs as an acute infection of the middle ear (Acute Otitis Media, AOM). As the disease progresses, it becomes more resistant to antibiotic therapy and continues to progress, thus giving rise to complications like hearing loss and this stage of the disease may be termed as Chronic Suppurative Otitis Media (CSOM). The prevalence of this infection in children is mainly due to the anatomy of their Eustachian tube [1]. Case studies points to this infection as one which has been highly neglected mainly due to its self limiting nature and also due to the ignorance regarding its intra and extra cranial complications like facial nerve paralysis, brain abscess etc. Postauricular edema with acute mastoiditis may be regarded as the first sign of the oncoming complications [2]. Such complications may even be fatal to the patients even though the incidences are rare [3]. The number of people worldwide who has never experienced middle ear infection is extremely low and a good number of people infected with CSOM have had lasting damages done to the infected ear. Although the main causative agents of otitis media are bacteria, many viruses and fungi have been reported to cause the infection. Majority of the viruses responsible for the infection comes from the infection in the upper respiratory tract or from the nasopharynx[4].

Biofilms are aggregates of microbes which help them adhere effectively to various surfaces by producing an Extracellular Polymeric Substance (EPS) around them [5]. This sheath helps them resist unfavorable conditions and substances [6]. The formation of biofilms has become a major problem in the medical field, where these harbor in the indwelling implants and catheters, which may even prove fatal to the patients.

MATERIALS AND METHODS

Biofilm formation is the ability of certain bacteria to produce an extracellular polymeric layer, generally made of polysaccharide or proteins around themselves, which facilitates better attachment to the growth surfaces due to the production of exopolysaccharide like Polycellular Intracellular Adhesin (PIA) especially in case of *Staphylococcus sp* [7].

A total of 100 bacterial strains were isolated from the ear swabs of otitis media patients and these were identified by staining, microscopy and various biochemical tests as described in Bergey's Manual of Systemic bacteriology.

Detection of Biofilm Formation

Congo red Method (CRA)

CRA plates were prepared by adding 0.08% of autoclaved Congo Red to sterile BHIB (Brain Heart Infusion Broth) supplemented with 1% Glucose and to this 24 hour old isolates were streaked and incubated at 37^{O}C for 24 to 48 hours aerobically. The formation of black color colonies on the streak lines was regarded as a positive result [8].

Tube Method (TM)

Sterile Brain Heart Infusion broths supplemented with 1% Glucose were taken in test tubes and to this 24 hour old culture of the isolates were inoculated and the tubes were incubated at 37°C for 48 hours. After incubation, the

tubes were decanted and washed twice with Phosphate Buffer Saline (PBS) and left for drying. After drying, the tubes were stained with 0.1% Crystal Violet for 20 min, washed with distilled water and air dried. After drying, the tubes were resuspended in 95% ethanol and OD was taken using a UV-Visible Spectrophotometer (Elico SL 159) at 580 nm.

Tissue Culture Plate Method

Sterile 96 well microtitre plate was used for detecting biofilm formation using this method. To the sterile microtitre wells, 100 µl of sterile Brain Heart Infusion broth supplemented with 2% Glucose was added and to this 100 µl of 24 hour old culture of the isolates were added and incubated at 37^{O}C for 48 hours. After incubation, the broths in the wells were decanted and the wells were washed twice with Phosphate Buffer Saline (PBS) and dried. After drying, the wells were stained with 0.1% Crystal Violet for 20 min, washed with distilled water and air dried. After drying, the wells were checked for the intensity of blue color and based on the intensity, the strength of the biofilm formed was determined as strong, moderate or weak [9].

Checking the Effect of Various Parameters On Biofilm Formation

Biofilms are exopolymeric layers produced by microorganisms, which help them in resisting unfavorable conditions effectively. *Staphylococcus aureus and Pseudomonas aeruginosa* were the two pathogens constituting the majority of the isolated bacteria that produced strong biofilms and hence these isolates were used to study the effect of various growth parameters on the strength of biofilms produced.

Temperature

Sterile Brain Heart Infusion broth added with 2% glucose was taken in two sets of 4 test tubes each and to one set, 24 hour old cultures of the isolated *Staphylococcus aureus* and was inoculated and to the other, *Pseudomonas aeruginosa* were added and incubated at different temperatures (4 $^{\circ}$C, 28 $^{\circ}$C, 37 $^{\circ}$C and 47 $^{\circ}$C) for 48 hours. After incubation, the tubes were decanted, washed twice with Phosphate Buffer Saline and left to dry. After drying, the tubes were stained with 0.1% Crystal Violet for 20 minutes, washed with distilled water and left for drying. After drying, the tubes were resuspended in 95% ethanol and OD was taken using a UV-Visible Spectrophotometer at 580 nm [10].

pH

Sterile BHIB with 2% glucose was taken in test tubes and the pHs of the tubes were adjusted to 4, 5, 6, 7, 8 and 9 in two sets. To each set, 24 hour old cultures of one the two isolates were added and incubated at 37 ^{O}C m for 48 hours. After incubation, the tubes were decanted, washed twice with Phosphate Buffer Saline, dried and stained with 0.1% Crystal Violet for 20 minutes, after which these were washed with distilled water and left for drying. After drying, the tubes were resuspended in 95% ethanol and OD was taken using a UV-Visible Spectrophotometer at 580 nm.

Glucose Concentration

Two sets of Brain Heart Infusion broth was prepared in test tubes and to these varying concentrations of Glucose was added (0%, 1%, 2%, 3% and 4%) and the tubes were sterilized at 15 lb pressure at 121^{O}C. To one set, *Staphylococcus aureus* isolates ware added and to the other set, *Pseudomonas aeruginosa* isolates were added and the tubes were incubated at 37 ^{O}C for 48 hours. After incubation, the tubes were decanted, washed twice with Phosphate Buffer Saline and left to dry. After drying, the tubes were stained with 1% Crystal Violet for 20 minutes, washed with distilled water and left for drying. After drying, the tubes were resuspended in 95% ethanol and OD was taken using a UV-Visible Spectrophotometer at 580 nm.

Salt concentration

Brain heart infusion broth was prepared by mixing the constituents of the medium separately so that the Sodium Chloride concentration can be varied. The concentration of Sodium Chloride was adjusted to 0%, 0.5%, 1%, 2% and 3% in each of tubes two sets of tubes. The isolated *Staphylococcus aureus* was added to one set of test tubes *and Pseudomonas aeruginosa* was added to the other set. As above said, the tubes were incubated at 37 $^{\circ}$C m for 48 hours. After incubation, the contents of the tubes were poured out and the tubes were washed with Phosphate Buffer Saline, after which these were left for air drying. Further staining was done for 20 minutes using 0.1% Crystal Violet. After drying, the tubes were resuspended in 95% ethanol and OD was taken using a UV-Visible Spectrophotometer at 580 nm.

Glucose vs Sucrose

Glucose and Sucrose are the common sugars used along with BHI broth for detecting biofilm formation. The effect of both these sugars on biofilm formation was studied to understand which supports the growth of biofilms better. Brain Heart Infusion broths were prepared in two sets. To one 2% Glucose was added and to the other, 2% Sucrose was added. This was dispensed into two sets of testtubes and sterilized. To one tube of each set, isolated culture of *Staphylococcus aureus* was inoculated and the other set was inoculated with *Pseudomonas aeruginosa.* The tubes were incubated at 37 ^{O}C m for 48 hours. After incubation, the contents of the tubes were poured out and the tubes were washed with Phosphate Buffer Saline, after which these were left for air drying. Further staining was done for 20 minutes using 0.1% Crystal Violet. After drying, the tubes were resuspended in 95% ethanol and OD was taken using a UV-Visible Spectrophotometer at 580 nm.

Time of Incubation

Sterile BHI broth added with 2% glucose was prepared in 5 sets of test tubes and inoculated with 24 hour old

cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates. These tubes were then incubated at 37 ^{O}C for varying time durations; 24, 48, 72, 96 and 120 hours. After incubation, the contents of the tubes were poured out and the tubes were washed with Phosphate Buffer Saline and air dried. Further staining was done for 20 minutes using 0.1% Crystal Violet. After drying, the tubes were resuspended in 95% ethanol and OD was taken using a UV-Visible Spectrophotometer at 580 nm.

RESULTS

Detection Of Biofilm Formation

Congo Red Method (CRA)

Based upon this method, the isolates that produced black colored colonies were considered as biofilm producers. Out of the 90 isolates studied, only 13 isolates gave black colored colonies (Fig. 1).

Tube Method (TM)

After staining using Crystal Violet, 43.3% of the isolates were identified as strong biofilm formers and 32.2% were found to be moderate biofilm formers. The rest were classified as weak or no biofilm formers (Fig 2).

Tissue Culture Plate Method

After the wells were stained with Crystal Violet, 44.4% were identified as strong biofilm formers and 34.4% were found to be Moderate Biofilm formers. The rest were weak or no biofilm formers (Fig 3).

Checking the Effect of Various Parameters On Biofilm Formation

Temperature of Incubation

On studying the biofilm formation at various temperatures, it was found that the optimum temperature for biofilm formation was 37 ^{O}C for both *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates (Graph 1).

pH

The best pH for the growth of *Staphylococcus aureus* biofilms was 6 whereas 7 was the ideal pH for *Pseudomonas aeruginosa* isolates (Graph 2).

Glucose Concentration

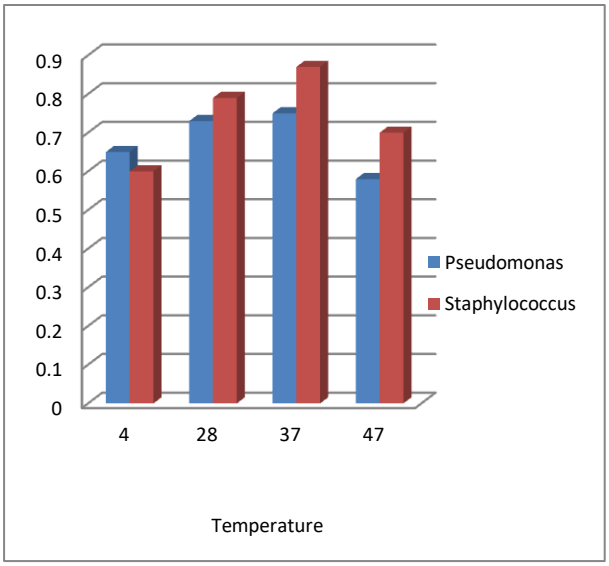
The two isolates had the same optimum glucose concentrations. The biofilm production was highest when 2% Glucose was added (Graph 3).

Salt Concentration

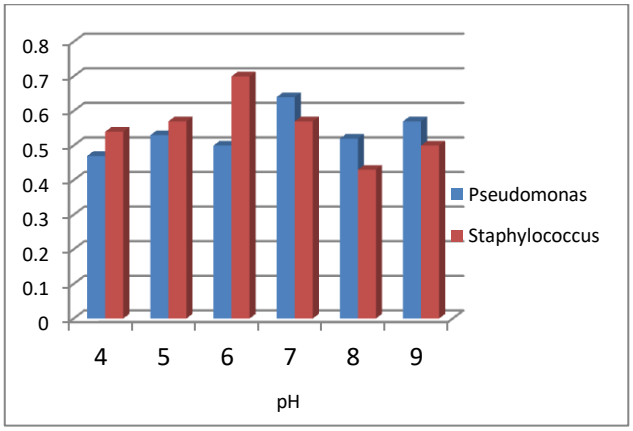
The requirement of Sodium chloride was different for the two isolates. Biofilm formation by *Pseudomonas aeruginosa* was highest at 1% NaCl concentration whereas *Staphylococcus aureus* produced stronger biofilms at 2% NaCl concentration (Graph 4).

Time of Incubation

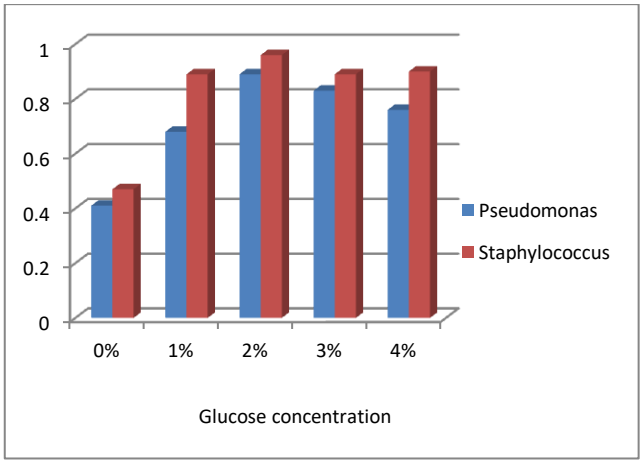
On incubating the isolates for 24, 48, 72, 96 and 120 hrs, the strongest biofilm was produced by *Staphylococcus aureus* after 96 hours of incubation whereas the biofilms produced by *Pseudomonas aeruginosa* were highest after 120 hours of incubation (Graph 5).



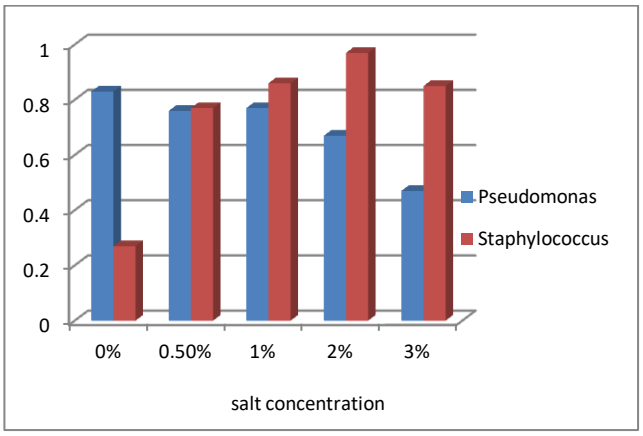
Graph 1: Effect of Incubation temperature on biofilm formation of isolates



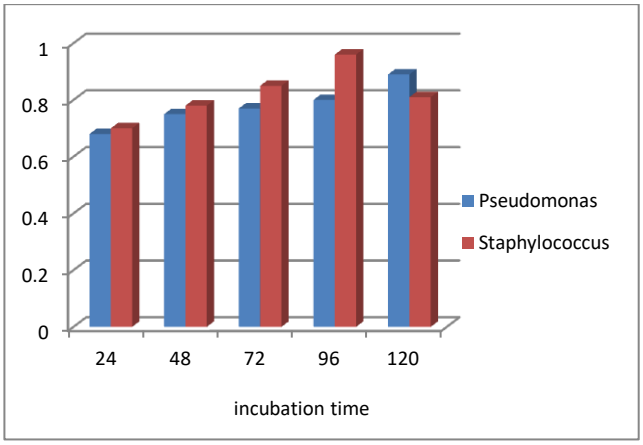
Graph 2: Effect of pH on biofilm formation of isolates



Graph 3: Effect of Glucose concentration on biofilm formation of isolates



Graph 4: Effect of Salt concentration on biofilm formation of isolates



Graph 5: Effect of Incubation time on biofilm formation of isolates

DISCUSSION

Bacteria embedded in an organic polymeric layer of bacterial origin are known as biofilms. It acts as a protective layer around the bacteria which help them adhere better to various external surfaces and also offers them protection against the action of various antibacterial agents [11]. The detection of biofilm was done using three methods namely Congo Red agar method (CRA), Tube method (TM) and Tissue Culture Plate (TCP) method and of these three methods, TCP method was found to be the better method as it was effective in detecting the majority of the biofilm formers. This result is in accordance with that obtained by Hassan *et al.* [12] but they contradict the result by Ruzicka *et al.* [13], which states that TM is more efficient in detection of biofilm formers than TCP method.

In the present study, only 14.4% of biofilm formers were identified by Congo Red agar plate method and this method was the least reliable as compared to the other two methods. Similar results were reported by Ruzicka *et al.* and Knobloch *et al.* [14]. However there are studies whose results starkly contradict the above said reports. A study by Subramanian *et al.* says that 90% of biofilm producers were detected by CRA method against only 83% being detected by TM method. Results obtained by Jain and Agarwal (2009) [15] also supports the use of CRA for detection of biofilm formers. Tube method detected 43.3% as strong biofilm formers and 32.2% as moderate biofilm formers and TCP method 44.4% as strong biofilm producers and 34.4% as moderate biofilm formers in our study. In the study by Hassan *et al.*, [12] similar results were reported with 25% and 21% detected as strong biofilm formers by TCP and TM methods and 4% by CRA method. TCP method has been reported in various other studies to have detected 57.1 % [14] 45.6% [16] and 47.3% [8] of the biofilm formers. But there are other report that says that 75% [17] and 84.3% [18] of biofilm producers were detected by TM method.

As stated above, the studies on the effect of various growth parameters on the strength of biofilm produced in medical isolates is less and so this part of the study is helpful in gaining a better understanding regarding the same. therefore this study case of the parameters studied, the increase in biofilm formation at neutral or near neutral pH is in accordance with the study by Chaieb *et al.* (2007) [19] but the results obtained by Prabhakar *et al.* (2012) [20] indicates that there was no biofilm production upto pH 6 and above this level, the biofilm production gradually increased and was found to be the maximum at pH 9 and 10. The same study also reports maximum biofilm formation after incubation time of 72 hours whereas the current study has obtained highest biofilm production after 96 hours of incubation for *Staphylocccus aureus* and 120 hours of incubation for *Pseudomonas aeruginosa*. A salt concentration of 2% was most suited for *Staphylocccus aureus* whereas *Pseudomonas aeruginosa* produced biofilm best at 1% NaCl levels and glucose supplementation of 1% helped in the production of highest biofilms in case of both the isolates. However *Serratia* and *Enterobacter* biofilms thrived at a salt concentration of 0.5% and the same concentration of media ingredients was best for *Enterobacter* biofilms but *Serratia* sp produced high intensity biofilms when the media ingredients of LB broth was kept constant at 1%.

CONCLUSION

This study reveals that, of the three methods commonly followed for biofilm detection, the Tissue Culture Plate method is the best method for identifying biofilm formers as the other two methods followed fails to detect biofilm producers as effectively as TCP method. The effects of the growth parameters like pH, salt concentration, glucose concentration etc. on the intensity of biofilm produced by the bacteria causing otitis media has been studied and this can go a long way in gaining a better understanding regarding the mechanism of biofilm formation.

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