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Detection of Biofilm Producers Causing Otitis Media: A Comparative Analysis of the Three Major Methods

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ABSTRACT

Otitis media is the infection of the middle ear, caused as a result of the infection of the fluid built up in the tympanic cavity, mainly by bacteria even though viral and fungal otitis media has been reported. The bacterial isolated from the otitis media patients are known to be biofilm producers and hence the success in causing recurrent ear infections and so the detection of biofilms becomes essential as only then can effective treatment strategy be planned. As such, this paper is dedicated to finding the best method for the detection of biofilm producers in the case of otitis media causing bacteria.

Keywords: Middle ear, otitis media, biofilm, treatment, detection.

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INTRODUCTION

The ear may be divided into three main parts, the outer ear, middle ear and inner ear. The sound waves pass through the outer ear cavity into the inner ear, thus hitting on the tympanic membrane, which vibrates and these vibrations are passed on to the three small bones into the inner ear and transformed as electric impulses and these impulses are carried by the auditory nerves to the brain and thus sound is identified and so any infection in the inner parts of the ear has a direct effect on the hearing ability of a person. One of the most common diseases of the ear worldwide is Otitis media. It may be defined as the inflammation of the fluid buildup in the otherwise air filled space of the middle ear. It is one of the most common infections in every age group but the incidences are more in infants and children. As the infection cures on its own, [1] generally, cases of otitis media go unnoticed and are not treated. But in such cases reoccurrences may be expected. Even though the symptoms clear up on its own, in chronic cases, irreversible side effects like slight to complete impairment of hearing, tinnitus, mastoiditis etc. are seen. Otitis media may be caused by bacteria, viruses [2] or fungi [3] even though bacterial otitis media is most common. The main entry of this pathogen is from the upper respiratory tract and nasopharynx. It has been

seen that patients with upper respiratory infections has been diagnosed with otitis media in many cases.

Antibiotic therapy is the treatment of choice but even then the incidences of recurrence of the infection in most cases are high. The main reason behind the success of the pathogen is their ability to form biofilms in the middle ear. Biofilms are the communities of bacteria that exists together by producing a polymeric matrix around themselves, [4] thus protecting them from adverse conditions and resisting the effects of antibiotics [5]. These are capable of attaching to various biotic surfaces (in patients with cystic fibrosis, peridontitis etc) [6] as well as abiotic surfaces (on the surface of implants like urinary catheters, dental implants etc.). [7] Once produced, their removal is extremely difficult even with antibiotic drugs. The ability of the bacteria to produce biofilms has been pointed as one of the reasons for the development of drug resistance because the biofilms offer better protection to the bacteria to overcome the action of the antibiotics. [8] This highlights the necessity to detect the presence of such biological films so that proper treatment strategies may be adopted for their removal.

Mainly for the detection of biofilms, three main methods are used: Congo Red agar plate method, Tube method and Tissue Culture plate method. The first method is based on the ability of the isolated biofilm producing bacteria to give black colored colonies on Congo Red medium whereas the other two methods evaluate the biofilm strength using their adherence to glass tubes and polyvinyl microwells respectively. In addition to understanding if a bacteria possess the capacity to produce biofilm, this paper also is aimed at checking the efficiency of the biofilm detection by these conventional methods.

MATERIALS AND METHODS

Sample Collection

Ear samples were collected directly from patients showing symptoms of otitis media using sterile cotton swabs and these were sent to the laboratory without delay and processed. Apart from this collection, otits media swabs were obtained from hospitals ad these were transported to the laboratory in transport medium.

Identification of the bacteria

The swabs were inoculated into sterile Brain Heart Infusion agar and also MacConkey agar and the plates were incubated at 37°C for 24 hours for the growth of the bacterial pathogens. After incubation, the growth on the plates were subjected to Gram's staining and microscopically observed. Based on the gram staining result, the isolates were subjected to biochemical analysis such as catalase and coagulase for gram positive and IMViC, TSI and Urease for gram negative bacteria. Based on these results the isolates were identified.

Detection of Biofilm Formation

The isolated organisms were checked for biofilm formation using Crystal violet assay by the following three methods.

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°C for 24 to 48 hours aerobically. The formation of black color colonies on the streak lines was regarded as a positive result. [9]

Tube Method (TM)

Sterile Brain Heart Infusion broths supplimented 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. [10]

Tissue Culture Plate Method

Sterile 96 well microtitre plate was used for detecting biofilm formation using this method. To the sterile microtitre wells, 100µl of sterile Brain Heart Infusion broth supplemented with 2% Glucose was added and to this 100µl of 24 hour old culture of the isolates were added 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. [11]

RESULTS

Identification of the bacteria

Based on the results obtained by colony morphology, gram's staining and biochemical analysis, the organisms isolated were identified as *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli, Klebsiella, Salmonella* and *Proteus.*

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. Others gave pale pink or off white colored colonies (Fig. 1).

Tube Method (TM)

After staining using Crystal Violet, 43.3% of the isolates were identified as strong biofilm formers (OD> 8.0) and 32.2% were found to be moderate biofilm formers (4.0<OD<8.0). The rest were classified as weak or no biofilm formers based on the absorbance measured (OD<4) (Fig. 2).

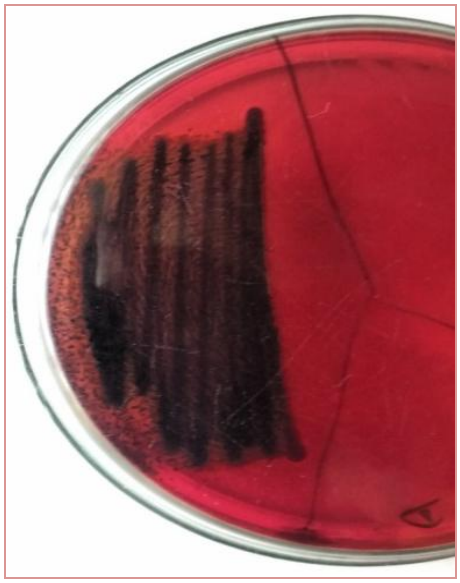

Fig. 1: CRA method

Fig. 2: Tube method

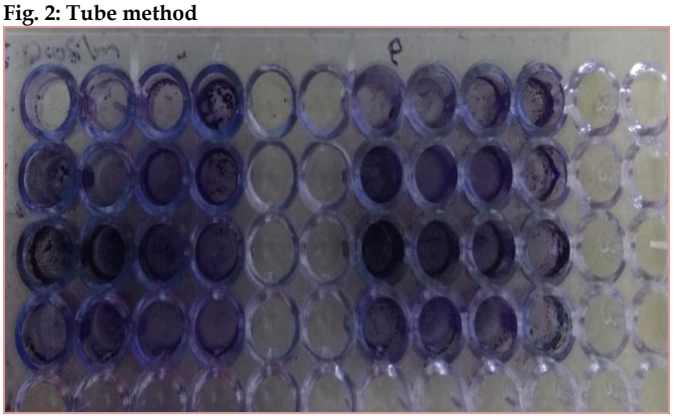

Fig. 3: Tissue Culture Plate method

Tissue Culture Plate Method

After the wells were stained with Crystal Violet, 44.4% were identified as strong biofilm formers with the high intensity of blue color and 34.4% were found to be Moderate Biofilm formers by the lower intensity of blue coloration. The rest were weak or no biofilm formers as the intensity of blue color in the wells were very pale or even nil in some cases (Fig. 3).

DISCUSSION

Biofilm formation is a major problem in the healthcare industry due to the difficulty in the eradication of such bacteria and hence the detection of biofilms is a necessity. In this study, the detection of biofilms was done by three different methods namely Congo Red agar method (CRA), Tube method (TM) and Tissue Culture Plate method (TCP). The results suggest that Tissue Culture Plate method is the most efficient method in detecting biofilm formers as the maximum number of biofilm producers were detected by the method. This result is in agreement with the results obtained by [12] and [13] which TCP method has detected the highest number of biofilm producers in the case of uropathogens. However works by [14] reports that TM method is best at detecting biofilm whereas [15] supports CRA method for biofilm detection. The modification of amount of Congo red dye and sucrose to the Congo red agar has been seen to increase the efficient of this method in detecting Staphylococcal biofilms. [16] However, most of the studies agree that the CRA method cannot be relied upon for the detection of biofilm formers and the best method would be Tissue Culture Plate method as in this method, the majority of the biofilm producing bacteria were detected by this method.

A wide study through the various reports regarding biofilm it is seen that the best method of biofilm detecting is TCP method as compared with the other two methods. This method has, in most cases, succeeded in detecting most of the biofilm formers. However it needs to be noted that the efficiency of detection of biofilms by TCP and TM methods differs only by a small margin. This may be because of the fact that biofilm formation is more on plastic surfaces than glass [17-18] and so the more intensity in the biofilm produced enhance the visualization.

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