

Original Research Article

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Detection of Bio-film Production among the Most Frequent Bacterial Isolates in Cases of Chronic Suppurative Otitis Media: A Cross-sectional Study

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ABSTRACT

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To detect biofilm production among the most common micro-organisms isolated from CSOM cases. Five hundred consecutive patients with a diagnosis of CSOM were enrolled in the study. Organisms were isolated using standard culture methods and biochemical reactions. Antibiotic sensitivity pattern was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Biofilm production was detected using Tube method and microtitre plate method. The most common organism isolated was *Pseudomonas aeruginosa* in 220 cases(44%) followed by *Staphylococcus aureus* in 110 cases (22%). Out of 186 isolates tested for biofilm production, 149 isolates (80%) showed biofilm production. Out of the moderately positive and strongly positive biofilm producers, 90% isolates were from unsafe CSOM. High proportion of isolates from CSOM cases shows biofilm production. Biofilm production may account for antibiotic resistance, recurrence of CSOM and failure of surgical procedures in CSOM cases.

Introduction

Chronic suppurative otitis media (CSOM) is defined as chronic inflammation of middle ear and mastoid cavity that presents with recurrent ear discharge of more than three months duration through a perforated tympanic membrane (WHO, 2004).CSOM is a major health problem in developing countries causing serious local damage and

life threatening complications. It is an important cause of preventable hearing loss in developing countries. The infection may begin in childhood as a complication of untreated or inadequately treated Acute Suppurative Otitis Media (ASOM) or may be chronic in onset. The bacteria may gain entry to the middle ear through a chronic perforation (Afolabi *et al.*, 2012). The spread of micro-organisms to the adjacent structures

of the ear may cause local damage or intracranial and extra cranial complications, ranging from persistent otorrhoea, mastoiditis, labyrinthitis, facial nerve palsy, meningitis, intracranial abscess or thrombosis and sepsis (Kumar *et al*, 2015).The most common microorganisms found in isolates from ear discharge in CSOM are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Escherichia coli*, *Aspergillus spp.*, *Candida spp.* and these vary in different geographical distributions (Yeo *et al.*, 2007).Biofilms are adherent communities of bacteria contained within a complex matrix.

Host immune responses to planktonic species have been relatively well characterized and also how they modulate anti-bacterial effector mechanisms when organized in this protective milieu (Hank *et al.*, 2012).Biofilms act as non selective physical barriers that obstruct antibiotic diffusion and hinder the cellular and humoral immune responses (Marchant *et al.*, 2013). They have a characteristic physiology and architecture that form the basis of biofilm resistance to many antibiotics and mechanisms of host defense (Otto, 2013).

Bacteria may communicate with each other through diffusible molecules within the milieu of a biofilm through a mechanism known as quorum sensing (Li and Tian, 2012).The purpose of this study was to determine the prevalence of biofilm producing bacteria among the most common bacterial isolates in cases of CSOM.

An epidemiological knowledge of the prevalence of biofilm production in CSOM is necessary as biofilm production may account for recurrence of CSOM after treatment, failure of surgery and resistance to antibiotic therapy. Also, we may be able to institute such therapies which may inhibit biofilm production (Rabin *et al.*, 2015).

Materials and Methods

This prospective study was conducted in the Department of Microbiology in association with Department of Otorhinolaryngology in a tertiary care university hospital in Bangalore, India.

Study period

The study was conducted for a period of 18 months from January 2017 to June 2018.

Study population

500 patients with clinical diagnosis of CSOM attending OPD and IPD of ENT department of our institute who satisfied the inclusion criteria were enrolled for the study.

Ethical consideration

Approval was obtained from the Institutional ethics committee before the commencement of the study. Informed consent was obtained from all the patients who participated in the study.

Statistical analysis

Data was entered on Microsoft Excel 2016 for analysis. Data was represented in form of numbers and percentages. Analysis was done using descriptive statistics.

Sample size estimation

All ear discharge samples from cases of CSOM with a minimum of 500 consecutive non-duplicate isolates will be included. Sample size has been calculated to be 500 depending upon previous years statistics.

Inclusion criteria

Patients diagnosed as CSOM after thorough

clinical evaluation i.e. the patients having ear discharge of more than 3 months.

Patients who were not on antibiotic (both systemic and topical) treatment for minimum of 24 hours prior to sample collection.

Exclusion criteria

Patients suffering from CSOM who are on systemic antibiotics in the past 7 days of presentation.

Patients who are on topical medications to the ear.

Patients having ear discharge due to some traumatic or neoplastic condition.

Patients having ear discharge arising from otitis externa.

Sample collection

The ear discharge was collected using sterile swabs under aseptic precautions with the aid of an aural speculum, prior to the instillation of any topical medication. Using standard sterile technique, two swabs were taken.

Specimen processing

Direct gram stain

Shows possible pathogens present in sample

KOH mount

Detection of fungal elements.

Interpretation of bacterial culture

The swab on reaching the laboratory was inoculated on the following culture media

5% Sheep Blood agar plate
MacConkey agar plate
Thioglycollate broth

After overnight incubation at 37°C aerobically, the plates were examined for growth and culture characteristics were identified. The isolates were identified by Grams stain morphology, motility, culture characteristics and biochemical reactions by the standard techniques. The isolated colonies depending on the Gram reaction were subjected to following biochemical tests for identification:

If gram positive cocci were found, then catalase test was done. For catalase positive isolates, tube coagulase was done -Tube coagulase positive were identified as *Staphylococcus aureus* whereas tube coagulase negative were Coagulase negative staphylococcus aureus (CONS). The identification of *Staphylococcus aureus* is shown in Figure 1.

If Gram negative bacilli were seen, the colonies were subjected to the following tests and biochemical reactions using standard microbiological techniques.

Catalase test
Oxidase test
Nitrate reduction test
Hugh-Leifson's Oxidation Fermentation test
Indole test
Methyl red test
Voges Proskauer test
Simmon's Citrate utilization test
Christensen's Urease test
Mannitol motility
Triple sugar iron agar

1% Sugar fermentation tests Glucose, Sucrose, Maltose, Mannitol.

Lysine decarboxylase, Ornithine decarboxylase and Arginine dihydrolase test. Isolates that were Gram Negative bacilli, catalase positive, oxidase positive and motile by hanging drop were identified as *Pseudomonas aeruginosa*.

The culture characteristics of *Pseudomonas aeruginosa* are shown in Figure 2.

Antibiotic susceptibility

The isolates were subjected for antibiotic susceptibility testing by employing Kirby-Bauer standard disc diffusion method on Muller- Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S24).

Biofilm production

Biofilm production among *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolates was tested by following methods:

Tube method

Tubes and incubated at 37 °C for 48 hrs. Content decanted and tubes washed with Normal saline and left for air dry, then stained with crystal violet(0.1%w/v) and rotated to ensure uniform staining, again washed with distilled H₂O and dried in inverted position.

Scoring of biofilm production

The biofilm production was scored in a qualitative manner comparing the test sample with positive and negative controls as depicted in figure 3. The grading was done as: 0-absent,1-weakly positive,2-moderately positive,3-strongly positive.

Microtitre plate method

Pseudomonas aeruginosa and *Staphylococcus aureus* cultures were inoculated in 3-5 ml BHI broth and incubated for 24hrs at 37 °C,cultures diluted 1:100 in BHI and 100 ml of each diluted culture was pipetted in each well of 96 well flat bottom microtiter plate, covered and incubated at 37 °C for 48 hrs., washed with Normal saline, 125 ml of 0.1 %

crystal violet(w/v) solution put to each well, stain removed, plate washed with distilled water, left for air dry. Optical Density (OD) of each well was measured at 630 nm and performed in duplicate.

Characterization of isolates based on biofilm production was done based on cut-off OD (OD_{CUT}) measured as:

$OD_{CUT} = OD_{avg} \text{ of negative control} + 3(SD) \text{ of OD of negative control}$.

Where OD_{avg}– Average Optical Density , SD – Standard Deviation

The interpretation of biofilm production using microtitre plate method is shown as in Figure 4 and Figure 5.

Results and Discussion

500 patients were included in the study. 63% of the patients were males (n=315) while 37% of patients were females (n = 185). Highest incidence of CSOM was found to be among patients in the age group of 20-29 years (32.8%) while the lowest incidence was found in patients ≥ 60 years age (3.2%). The most common presenting complaint was ear discharge for more than 3 months (93%), followed by decreased hearing (66%). Upper respiratory tract infection was noted in 30% cases and allergic history was noted in 12% cases. 25% cases had a past history of modified radical mastoidectomy with tympanoplasty (Type 2). Tonsillitis and Deviated nasal septum was noted in 5% and 3% respectively. Tuberculosis and adenoids were noted in 2% cases. Stroke(1%), Miller-Fischer syndrome(1.6%), Extrapulmonary tuberculosis(0.4%) and nasal carcinoma (0.4%) were the other co-morbidities among the patients. The most common organism isolated was *Pseudomonas aeruginosa* in 220 cases(44%) followed by *Staphylococcus*

aureus 110 cases (22%). Methicillin resistant *Staphylococcus aureus*(MRSA) was isolated in 15 cases. CONS were isolated in 40 cases (8%). Fungi were isolated in 6% cases. In mixed culture growth, the most common isolates were *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (2.8%) followed by *Pseudomonas aeruginosa* and *Escherichia coli* (2.6%).

Attico-antral type of CSOM was found in 150 cases (30%) while 350 cases (70%) were diagnosed with tubo-tympanic type of CSOM. Among the cases with attico-antral CSOM(Unsafe type), the most common organism isolated was *Pseudomonas aeruginosa* (54.6%) followed by *Staphylococcus aureus*(32%) and *Klebsiella* species(6.6%).

Among *Pseudomonas aeruginosa* isolates, highest sensitivity was noted for Piperacillin-tazobactam (97%), Meropenem (95%), Cefoperazone (93%) and Piperacillin (93%). Only 68% isolates of *P.aeruginosa* were found to be sensitive to Ciprofloxacin.

100% of *Staphylococcus aureus* isolates were found to be sensitive to Linezolid and Teicoplanin. 87% of *S.aureus* isolates were Cefoxitin sensitive (MSSA). 15% of *S.aureus* isolates were found to be Cefoxitin resistant (MRSA). Highest resistance among *S.aureus* isolates was found for Penicillin(81%) followed by Ciprofloxacin (65%), Ampicillin(63%) and Amoxicillin-Clavulanate(54%).

Biofilm production in *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Out of 186 isolates tested for biofilm production, 149 isolates (80%) showed biofilm production. 50% of biofilm producing isolates were moderately positive, 47% were weakly positive while 3% were strongly

positive. Out of the moderately positive and strongly positive biofilm producers (n =79), 90% isolates were from unsafe CSOM (n=71) out of which 57(80%) were *Pseudomonas aeruginosa* while 14 (20%) were *Staphylococcus aureus*. 86% isolates (n=12) of *Staphylococcus aureus* were MRSA while 14% were MSSA (n =2).

All these patients with unsafe CSOM had a history of recurrent CSOM and were on antibiotic therapy for many years (mean time on antibiotic treatment – 8 years). 60 patients (84.5%)with unsafe CSOM with detection of moderately positive or strongly positive biofilm production had underwent modified radical mastoidectomy (MRM) with type 2 tympanoplasty in the past. The results of biofilm production are summarized in figure 6.

Chronic Suppurative Otitis Media(CSOM) is considered as a major public health problem in the developing world and India is one of the countries with high prevalence (WHO, 2004).It is a persistent disease with risk of irreversible complications and is an important cause of preventable hearing loss in adults and children (Berman, 1995).

Analysis of bacterial profile of the present study showed that Gram negative bacilli outnumbered Gram positive cocci among the isolates from the cases of CSOM which is similar to previous studies (Naheed *et al.*, 2003 and Kulchal *et al.*, 2010) The predominant pathogen was *Pseudomonas aeruginosa* (44%) followed by *Staphylococcus aureus* (22%), *Acinetobacter* (4%), *Klebsiella pneumoniae* (6%), *Escherichia coli*(4%), and *Proteus mirabilis*(3%). Similar findings have been noted in previous studies by (Shyamala *et al.*, 2012).

The microbial profile may vary between

different regions based on patient population and geographical distribution and hence necessitates the need for frequent analysis and update of the microbial profile in every region.

Antibiotic susceptibility pattern of *P.aeruginosa* revealed 100% susceptibility to Colistin, while 97% of the isolates were sensitive to Piperacillin-tazobactam, 95% were sensitive to Meropenem, 93% isolates were sensitive to Piperacillin, 93% to Cefoperazone, 90% to Imipenem, 81% to Amikacin, 75% to Gentamicin and 68% to Ciprofloxacin. *Pseudomonas aeruginosa* isolates were found to be more sensitive to Amikacin than Gentamicin and this finding is similar to previous studies in Sharma et al¹⁶ who reported 83.9% sensitivity to Amikacin and 70.5% sensitivity to Gentamycin.

Similar findings were documented in previous studies(Saini *et al.*, 2005 and Wario *et al.*, 2006). Fluoroquinolones inhibits the bacterial DNA gyrase or the topoisomerase II thereby inhibiting DNA transcription and replication. They have a broad range of activity and found to be active against *Pseudomonas aeruginosa*.

In the present study 52% of Gram negative isolates and 68% of *P.aeruginosa* were found to be sensitive to ciprofloxacin which is similar to the sensitivity reported in previous studies (Kumar R *et al.*, 2015 and Kumar S *et al.*, 2012).

100% isolates of *Staphylococcus aureus* were sensitive to Linezolid and Teichoplanin. 65% of *Staphylococcus aureus* isolates were resistant to Ciprofloxacin and 54% were resistant to Amoxicillin-Clavulanate. This indicates high level of resistance among *Staphylococcus aureus* for β -Lactams and Fluoroquinolones. In a previous study, out of 181 isolates, 48% of isolates were *Staphylococcus aureus* and all were found to

be methicillin sensitive (Prakash *et al.*, 2013). Biofilm production by bacteria has been implicated in the pathogenesis of many chronic infections including CSOM.

Biofilm production is considered as one of the mechanisms which may contribute to the bacterial resistance to antibiotics and other antimicrobial compounds (Stewart and Costerton, 2001). Biofilms are difficult to eradicate and may cause recurrent infections (Donelli and Vuotto, 2014).Biofilms attach firmly to damaged tissue, such as exposed osteitic bone and ulcerated middle ear mucosa, or to otological implants such as tympanostomy tubes (Wang *et al.*, 2014).

The critical first event in formation of biofilm is adherence to host tissue or to foreign body surfaces. During insertion of a device such as a tympanostomy tube, there may be inoculation of bacteria from the skin and mucous membranes of the host tissue. An amorphous extracellular material composed of bacterial products, such as teichoic acids, proteins, polysaccharides, and extracellular DNA (eDNA) and host products, encases the bacterial agglomerates within a biofilm (Schommer *et al.*, 2011).

Biofilms may be formed on the abiotic surfaces of medical devices or on biotic surfaces, such as host tissue (Becker *et al.*, 2014). The development of a biofilm requires a combination of adhesive forces and disruptive forces. Adhesive forces are required for the colonization of surfaces and for cell-cell interaction.

Disruptive forces are needed for nutrient delivery to all biofilm cells through fluid filled channels and also for the detachment of cell clusters from the biofilm, which facilitates the dissemination of infection (Otto, 2013).

Biofilm is formed as a result of various steps which are summarized below:

Attachment of bacteria to biotic or abiotic surface.

Multiplication and accumulation in multilayered cell aggregates.

Growth and maturation of the the biofilm into a thick, structured layer.

Dissociation and disseminate via the bloodstream.

To mediate attachment to abiotic surfaces or host tissues, proteinaceous and non-proteinaceous adhesins are required. Proteinaceous adhesins of bacteria have been classified into covalently surface-anchored proteins, also called cell wall anchored (CWA) proteins and noncovalently surface-associated proteins which include the autolysin/adhesin family, and the membrane spanning proteins (Becker *et al.*, 2014).

Among the non-proteinaceous adhesins are the polysaccharide intercellular adhesin (PIA) (also known as poly-N-acetylglucosamine [PNAG]) as well as wall teichoic and lipoteichoic acids (Becker *et al.*, 2014). Within a mature biofilm, multiple fluid-filled channels are formed which ensure the delivery of oxygen and nutrients to the bacterial cells located within the deeper layers of the biofilm (Costerton *et al.*, 1999).

After adhesion, the bacteria proliferate and accumulate into multilayered cell clusters. This process is mediated by the polysaccharide intercellular adhesin (PIA). The genes which are responsible for PIA formation are located on the ICA (intercellular adhesion) operon.

The ICA operon consists of two genes i.e. *icaR* (regulating) gene and *icaABCD* (biosynthetic) genes (Longauerova, 2006). Upon biofilm maturation a variety of

extracellular enzymatic activities or phenol soluble modulins (PSMs) may cause dissociation of bacteria or bacterial clusters and may lead to dissemination of the infection into the blood stream (Becker *et al.*, 2014).

In our study biofilm production was seen in 30% of unsafe CSOM cases. In a study, biofilm production was noted in 75% cases of unsafe CSOM and was significantly more common in unsafe CSOM than safe CSOM (Aronto *et al.*, 2015). In another study, biofilms were present in 60% patients with CSOM as compared with 10% of controls who had ear surgeries for other causes (Lee *et al.*, 2009).

The present study evaluated 186 isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* for biofilm production. We found out of 186 isolates tested for biofilm production, 149 isolates (80%) showed biofilm production. 50% of biofilm producing isolates were moderately positive, 47% were weakly positive while 3% were strongly positive.

Out of the moderately positive and strongly positive biofilm producers, 90% isolates were from unsafe CSOM out of which 57(80%) were *Pseudomonas aeruginosa* while 14 (20%) were *Staphylococcus aureus*.

86% isolates of *Staphylococcus aureus* isolated from unsafe CSOM showed Methicillin resistance and these patients had a past history of MRM with Type 2 tympanoplasty, presented with recurrences and were on antibiotic treatment since many years.

Biofilm formation may explain the etiopathogenesis of recurrence and resistance pattern in unsafe CSOM. Determining the type of bacteria that form biofilm in unsafe CSOM will help in better management in recurrent CSOM cases.

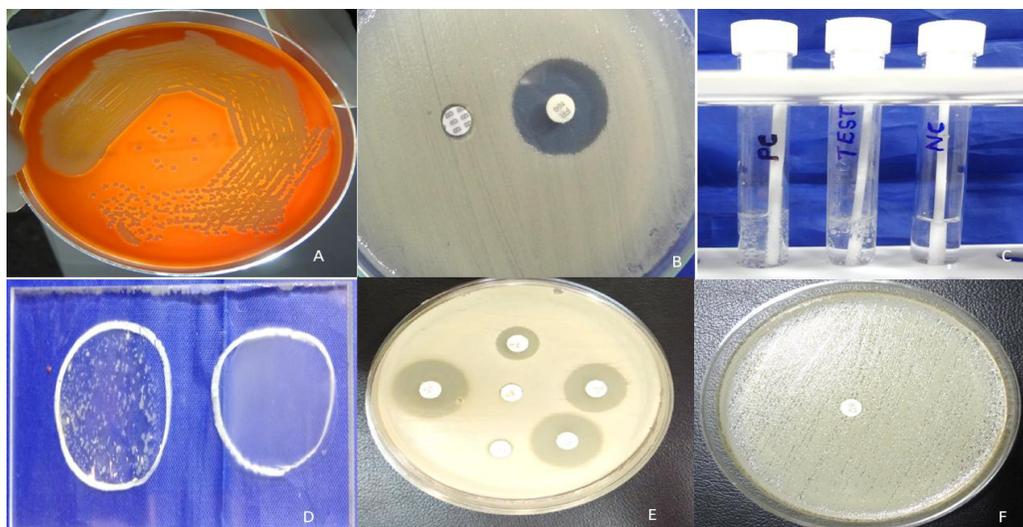


Figure.1 Characteristic features of *Staphylococcus aureus*. (A) Colonies of *Staphylococcus aureus* on blood agar. (B) Susceptibility to bacitracin and Furazolidone is seen in *Staphylococcus aureus*. (C) *Staphylococcus aureus* shows positive catalase test. (D) *Staphylococcus aureus* shows positive slide coagulase test. (E) Antibiotic susceptibility test using Kirby-Bauer standard disc diffusion method on Muller- Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S24). Methicillin sensitive *Staphylococcus aureus* (MSSA) is noted. (F) Antibiotic susceptibility test using Kirby-Bauer standard disc diffusion method on Muller- Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S24). Methicillin resistant *Staphylococcus aureus* (MRSA) is noted



Figure.2 Culture characteristics of *Pseudomonas aeruginosa*. Colonies of *Pseudomonas aeruginosa* on Nutrient agar (A), MacConkey agar(B) and Blood agar(C). (D) Antibiotic susceptibility test using Kirby-Bauer standard disc diffusion method on Muller- Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S24). Among *Pseudomonas aeruginosa* isolates, highest sensitivity was noted for Piperacillin-tazobactam (97%), Meropenem (95%), Cefoperazone (93%) and Piperacillin (93%). Only 68% isolates of *P.aeruginosa* were found to be sensitive to Ciprofloxacin

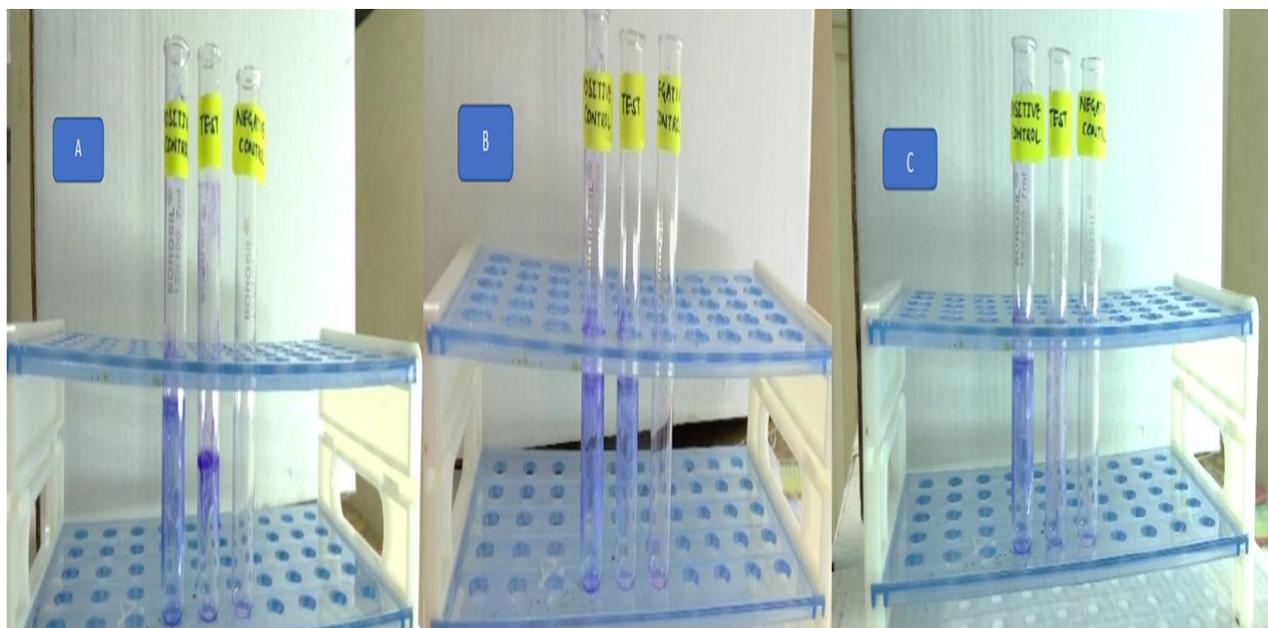


Figure.3 Biofilm detection using tube method. Comparison of test sample is made with positive and negative controls. (A) Strongly positive biofilm production. (B) Moderately positive biofilm production. (C) Weakly positive biofilm production

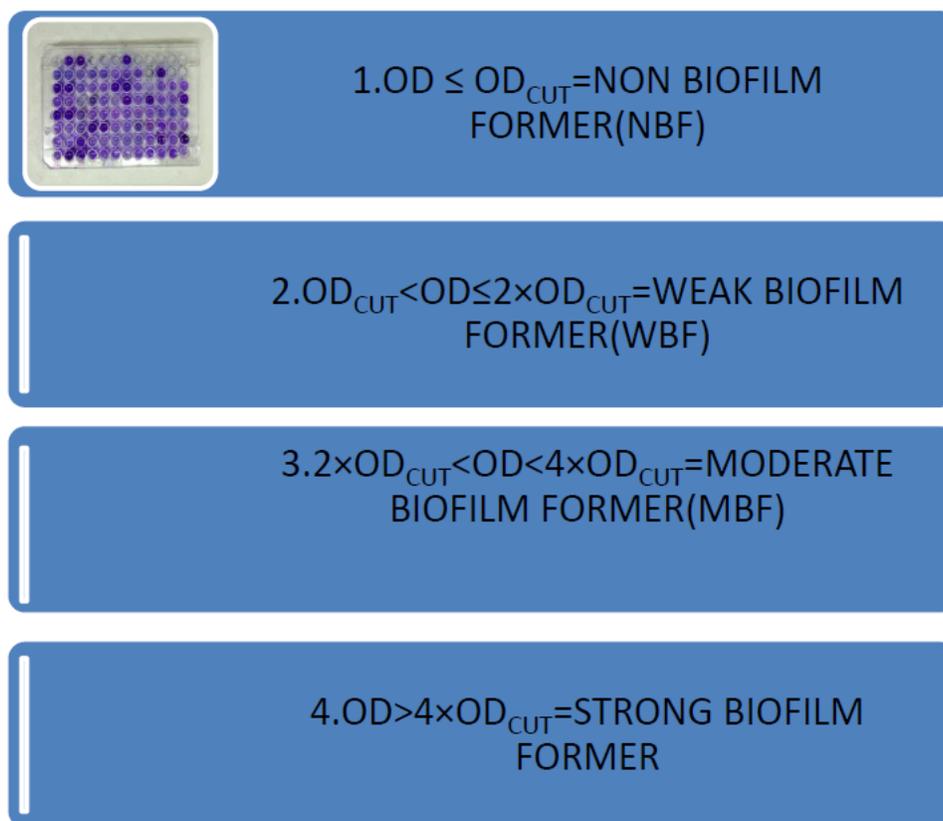


Figure.4 Interpretation of biofilm production using micro-titre plate method

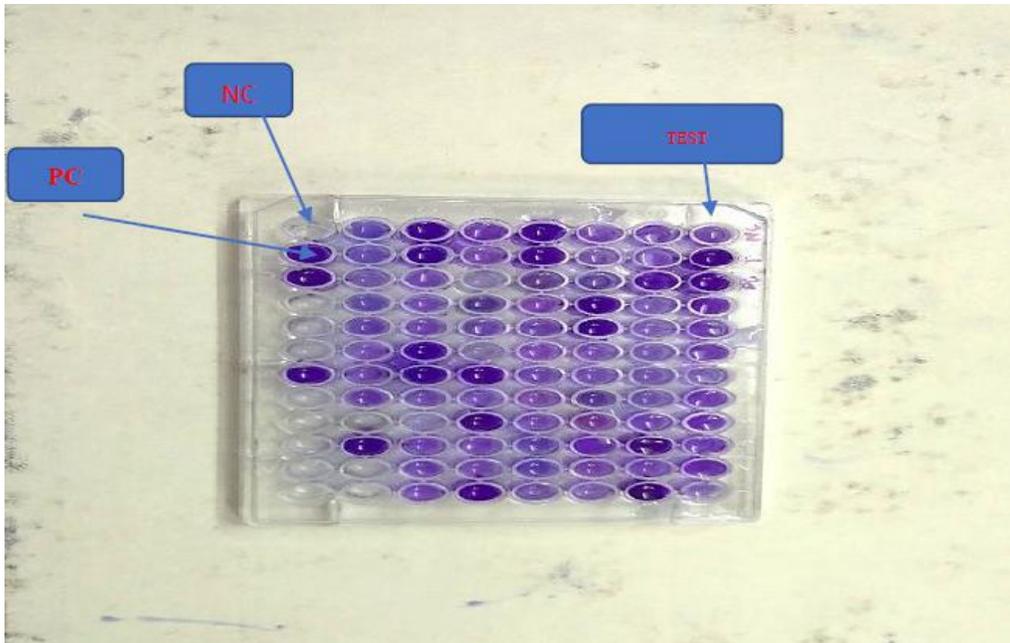


Figure.5 Biofilm production tested using microtitre plate method. The optical density (OD) of the test, positive control and negative control is measured and biofilm production detected using the criteria elucidated in Figure 4

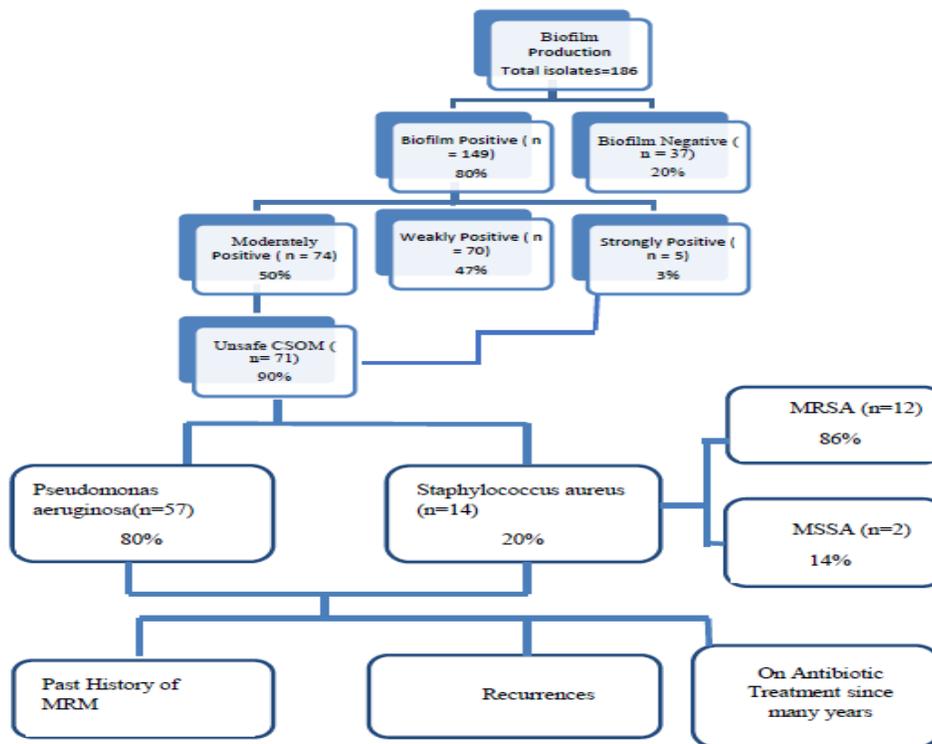


Figure.6 Flowchart showing a summary of results of Biofilm Production by *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Pseudomonas aeruginosa and *Staphylococcus aureus* are the most common bacterial pathogens isolated from the cases of CSOM. Biofilm production may be seen in a very high percentage of isolates and may contribute to resistance to antibiotic, recurrent CSOM and failure of surgical procedures such as modified radical mastoidectomy. Since biofilm production appears to play a major role in pathogenesis of CSOM, it may be necessary to develop and apply in clinical practice such agents which may inhibit the production of biofilm and help in better management of CSOM.

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