DETERMINATION THE ABILITY OF BACTERIA ISOLATED FROM OTITIS MEDIA PATIENTS TO FORM BIOFILM

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ABSTRACT:

(100) ear swabs were collected from (96) patients with otitis media who visited Basra General Hospital in Basra province, Iraq. For the period from November 2019 to February 2021. (92) Patients were unilateral infection and (4) patients were bilateral. Males account for 51% (49 cases), while females account for 49% (47 cases). Patients' ages vary from 1 to 72 years, with patients aged > 51 having the greatest infection rate. (88) Cases had positive growth, compared to (12) cases who had negative growth. (81) Swabs showed bacterial growth, six samples revealed fungal growth, and one sample showed both bacterial and fungal growth. Through the study, it was found that (73.2%) % of the bacterial cultures were pure and 26.8% were mixed. Bacterial species, including gram-positive and gram-negative bacteria, were isolated and identified throughout the research, the results showed the predominance of Staphylococcus aureus (23.3%) as the main and important cause of otitis media followed by Pseudomonas aeruginosa (19.4 %), Klebsiella pneumoniae 17.5%, Proteus mirabilis 16.5%, Staphylococcus epidermidis 11.7%, Staphylococcus hemolytic 4.9%, Escherichia coli 2.9 % Enterobacter cloacae 1.9% and Pseudomonas stutzeri 1.9 %. These bacterial isolates were tested for biofilm formation using the Congo Red Agar and Tissue Culture Plate methods and the results were as follows: The biofilm formation was identified (100%) in all isolates of Staphylococcus spp., Pseudomonas spp., and Enterobacter cloacae on both techniques; on the other hand, Biofilm formation was seen in 88.9% of Klebsiella pneumonia isolates, 88.2% of Proteus mirabilis isolates, and 66.7 % of Escherichia coli isolates.

Keywords: Otitis Media Patients, Pseudomonas Aeruginosa, Enterobacter Cloacae.

I. INTRODUCTION

Otitis media (OM) is one of the most prevalent pediatric diseases and a leading cause of morbidity in kids. The adaptive and native immune systems, Eustachian tube dysfunction, viral and bacterial load, genetic and environmental variables all have a role in the pathogenesis of OM (Rovers et al., 2004). Although OM can resolve without problems, it can be associated with hearing loss and long-term consequences. Many classifications may be used to categorize OM. Acute OM lasts 3 weeks or less; subacute OM 3 weeks - 3 months; and chronic OM lasts more than 3 months (Jung et al., 2017). OM may be caused by bacterial or viral infections. During 'colds,' the virus can spread to the Eustachian tube, reaching the middle ear, and making bacterial infection more likely (Protasova et al., 2017). Early bacterial colonization of the nasopharynx greatly raises the likelihood of OM episodes in the future (Faden et al., 1997). Bacterial biofilms (colonization of bacteria embedded in the extracellular matrix and adhering to a surface) have been seen in the middle ears of patients with Chronic suppurative otitis media (Lampikoski et al., 2012), which have been shown to protect bacteria from antibiotic treatment and the host's immunological response (Garcia-Cobos et al., 2014).

II. MATERIALS AND METHODS

Between November 2019 and February 2021, this study was conducted at Basra General Hospital in Basra province, southern Iraq, with approval from Basra Health Directorate – Al Razi Center for Training and Development. All patients between the ages of 1-72 years who had ear discharge due to OM were eligible. A complete history was taken which included the following information: name, sex, and date, time since diagnosis, laterality, and antibiotics use.
Swabs were taken by a specialist doctor after used alcohol with a concentration of 70% to wipe and clean the ear, samples were transferred to the laboratory and inoculated on culture media (Blood, MacConkey, Mannitol, and Nutrient agar) and cultured aerobically at 37°C for 24-48. Gram stain and some biochemical tests were used for the initial diagnosis of bacteria and the final diagnosis was by using the Vitek-2 system.

**Biofilm Formation**

**Congo Red Agar (CRA)**

CRA is a specifically produced medium consisting of a brain heart infusion agar (BHI) of 37 g/l, Sucrose (50 g/l). Congo red dye 0.8 g/l, suspending the materials with 1000 ml of distilled water. Heat to boiling to dissolved the medium completely and sterilized by autoclaved at 121 °C for 15 minutes (Nabajit, 2014). Biofilm producers formed black colonies on CRA, whereas strains that formed a pink or white color are intermediate in their ability to produce biofilm, and biofilm non-producers form red colonies (Deka, 2014).

**Tissue Culture Plate (TCP)**

All isolates were evaluated for their ability to form biofilm using the TCP technique defined by (Mathur et al., 2006). Overnight cultures of bacteria from nutrient agar plates were inoculated into Tryptic soy broth with 1% glucose, 20 μl of inoculums were placed into 96 wells of a flat bottom microtitre layer, and 180 μl of TSB was used to make the dilution; wells containing only TSB served as a negative control. In an aerobic and moist environment, the plate was covered and incubated at 37°C for 24 hours. Following incubation, the wells were decanted and washed three times with 200 μl Phosphate buffer saline. The wells were then filled with 200 μl methanol for 15 minutes. the wells were decanted and colored for 15-20 minutes with 160 μl crystal violet and then the well decanted and cleaned with distilled water, The optical density (OD) of each well in a microtiter plate was measured at 595 nm using an electronic ELISA reader The biofilm formation (BF) was calculated using the formula: BF = AB- CW, where AB indicates the optical density of a well containing stained adherent bacteria at 595 nm and CW, the optical density at 595 nm of the stained control wells contained medium that is free of bacteria. The samples were divided into three categories: strong (>0.240), moderate (0.120-0.240), and negative (<0.120). (Mathur et al., 2006).

**III. RESULTS**

A total of 100 swabs from (96) patients with otitis media were collected for this research. 49 males and 47 females. In 92 cases, one ear was infected, while both ears were infected in four others. Gram-negative bacteria accounted for 62/103 (60.2 %) of all isolates and were the most frequent etiological agent of OM, whereas Gram-positive bacteria accounted for 41/103 (39.8 %) isolates. Figure 1 shows the distribution and percentage of bacteria isolated from ear swabs cultures of individuals with OM illness. *Staphylococcus aureus* predominated (23.3%) among the organisms isolated from the OM, followed by *Pseudomonas aeruginosa* (19.4 %), *Klebsiella pneumoniae* 17.5%, *Proteus mirabilis* 16.5%, *Staphylococcus epidermidis* 11.7 %, *Staphylococcus hemolyticus* 4.9%, *Escherichia coli* 2.9 %, *Enterobacter cloacae* 1.9%, *Pseudomonas stutzeri* 1.9 %.

![Figure 1. Distribution of microorganisms isolated from ear cultures of patients with OM illness](image-url)
According to the results, single bacterial infections were more prevalent than mixed infections. The frequency of a single infection was (73.2%) (60/82), whereas the frequency of mixed infection was (26.8%) (22/82). The ability of the isolated bacteria to form biofilm was assessed using two different methods. The first technique, which used Congo red agar media, was qualitative, while the second technique, which used tissue culture plates, was quantitative. The results indicated that all isolates of *Staphylococcus* spp., *Pseudomonas* spp., and *Enterobacter cloacae* formed biofilm on both techniques (100%); on the other hand, Biofilm formation was seen in 88.9% of *Klebsiella pneumonia* isolates, 88.2% of *Proteus mirabilis* isolates, and 66.7% of *Escherichia coli* isolates (table 1).

### Table 1. Ability to form biofilm in bacteria isolated from patients with OM

<table>
<thead>
<tr>
<th>Bacterial isolates (No.)</th>
<th>Biofilm formation</th>
<th>% of BF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strong</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (24)</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (20)</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> (18)</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> (17)</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em> (12)</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td><em>Staphylococcus hemolyticus</em> (5)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (3)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em> (2)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><em>Pseudomonas stutzeri</em> (2)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total (103)</strong></td>
<td>69</td>
<td>29</td>
</tr>
</tbody>
</table>

![Figure 2. CRA plates showing formation of biofilm in bacterial isolated from OM](image1)

![Figure 3. Crystal violet stained TCP showing formation of biofilm in bacterial isolated from OM](image2)

**IV. DISCUSSION**

In undeveloped nations, OM is a prevalent disease. The impact of microbiological factors such as otopathogen colonization (*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus epidermidis, Staphylococcus hemolyticus, Escherichia coli, Enterobacter cloacae and Pseudomonas stutzeri*) and biofilms forming will have to be determined in this research. The current investigation showed significant differences in the frequencies and kinds of otitis media etiology. *Staphylococcus aureus* was responsible for 24 cases (23.3%), 9 cases were the sole cause of the disease and 15 cases were common with...
other pathogens. This indicated that S. aureus is the most prevalent cause of otitis media, accounting for the greatest proportion of cases among other pathogenic bacteria. This is due to two factors: first, it may enter the middle ear as a naturally occurring flora from the external canal when the tympanic membrane is destroyed, and second, S. aureus innate ability to generate antibiotic-resistant strains (Ayal, 2019). The current study was consistent with (Mahmoud et al., 2019), who found S. aureus in 41% of the total infections from OM patients admitted to Samarra Hospital, and also with (Deyno et al., 2017), who found In their research of the sequence of infection rates of the bacterial species that cause otitis media, the prevalence of S. aureus infection was 28.2%. Pseudomonas aeruginosa was the second type of bacteria isolated from OM, and it was found to be the direct cause of 20 cases (19.4%), though it was only found to be common with other pathogens in (2) cases. This finding was predicted for several reasons, the first of which is that P. aeruginosa is the most common secondary invader when the middle ear's resistance is reduced. Second, it spreads rapidly by external canal from stable carriers or environmental locations to vulnerable patients. Third, P. aeruginosa has a higher incidence of general antibiotic resistance than gram-positive strains, and fourth, its resistance to phagocytosis and opsonization is mediated by the production of a large variety of extracellular products, including alkaline protease, elastases, and exotoxin A, which may cleave IgA (Rocha et al., 2019), the current study results in line with (Shaima, 2017) who found P. aeruginosa came in second place in middle ear infection (from CSOM patients) after S. aureas (58% and 21%, respectively). It was also consistent with (Shumi et al., 2017) who isolated this bacteria with a percentage of 22.83% from CSOM patinas in Rajshahi, Bangladesh.

After S. aureus and P. aeruginosa, K. pneumoniae proved to be the third most frequent pathogen, with infection rates of 17.5 % (18) cases, and it was the primary cause of 13 infections and shared with the other causes in 5 others, also this study was compatible with (Shaima, 2017). Proteus mirabilis became a secondary invader from the external canal when the middle ear's resistance was lowered, the present study results showed Proteus mirabilis constituted 16.5 % of the population. According to (Joshi, 2019), patients with COM accounted for 16 % of the population. According to other study (Piet & Eric, 2002), P. mirabilis bacteria was the main cause of this disease, followed by P. aeruginosa and S. aureus.

Despite the fact that S. epidermidis is an undesirable bacteria that lives on the eardrum membrane's skin and plays a key role in infection when the body's resistance diminishes, it becomes opportunistic and causes illnesses, and these bacteria are not pathogens (Tannock et al., 2012). The number of isolates of this bacteria in this investigation was 12 (11.7 %), which is consistent with (Onifade et al., 2018), where the infection rate of this bacterium for otitis media was 9%. In this investigation, five isolates of S. hemolyticus were collected. Furthermore, S. hemolyticus' capacity to adapt to the hospital environment and medical equipment makes it a key component in nosocomial infections (Saber et al., 2017). The percentage of E. coli was 2.9 %, which is similar with the findings of (Shumi et al., 2017), who detected this bacteria in 3.2 % of OM isolates. E. cloacea and P. stutzeri had the lowest rates (1.9%) to each of them. This is in agreement with (Mahmood et al., 2019), who identified E. cloacea with a proportion of 1% from CSOM patients. In terms of gender, the findings revealed that the incidence of male infection is similar to that of female infection, with 49 cases (51%) of male infections and 47 cases (47%) of female infections (49%). This was verified by the study of (Lafi et al., 2020), which found no significant variations in the numbers of male and female patients in their study of otitis media patients in al- Ramadi province, where they scored 46.0% males and 54.0% females. The samples of cases were divided into six age categories when it came to the distribution of instances by age's groups. The age groups 51 and higher were determined to be the most prepared for infection, as they accounted for (25) cases with percentage of 26.1 %. However, the results were similar for the age groups (11-20) 20 cases and (31-40) 19 cases, respectively. Bacterial and viral inflammation, , seasonal allergies, group childcare facilities, smoking, obesity, anatomy-physiological abnormalities of the Eustachian canal and nasopharynx, immaturity and immune system deficiencies, formula feeding, sex, race, and age. Are all factors that play a role in OM infections (Jung et al., 2021).

As a virulence factor, biofilm formation plays a key role in pathogenicity. Biofilm-forming bacteria have been related to chronic and recurring illnesses and are more resistant to environmental changes including pH, antibiotics, disinfectants, oxygen radicals, and phagocytosis (Kostakioti et al., 2013).

In this study, the results were consistent between the two method CRA and TCP, which agrees with (Eftekhar et al., 2011), who found that both of these procedures had the same sensitivity and that the results in biofilm production were equivalent 91 %.

Many studies have focused on the importance of biofilm formation since it is a source of many chronic illnesses, including chronic pneumonia, bone inflammation, bladder inflammation, and others. The accumulation of bacterial cells and the producing of toxins cause this diseases. On CRA, colonies looked black, indicating the formation of a biofilm, according to the CRA test results. The interaction of the pigment Congo red with the polysaccharide, which is confirmation of biofilm formation, the flat red color suggests that the bacteria cannot form polysaccharides, indicating that they are not biofilm formation. While the formation of a mucous pink color indicate the bacteria's ability to be few since it began as an external capsule is demonstrated by the formation polysaccharide production is slow, so the dye was not stained black in terms of

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biofilm formation. While the (TCP), a quantitative method for identifying biofilm formation, has been proven to have good sensitivity and specificity, the positive and negative results are evaluated by an Elisa reader (Mathur et al., 2006; Zegaer, et al., 2014).

The findings of the current investigation revealed that 100% of S. aureas formed biofilm. This is similar with the findings of (Abdul-Hassan and Zaki, 2018), who discovered that after 24 hours of incubation, S. aureus isolates developed a biofilm, and the results showed biofilm formations (100%) of S. epidermidis isolates. These findings agree with the findings of (Vadyvaloo and Otto, 2008) who showed these bacteria formed biofilms. On the other hand, the results revealed that (100%) of Pseudomonas aeruginosa formed biofilm. This conclusion is largely similar to the findings of (Vishnu et al., 2009; El-Mahdy and El-Kannishy, 2019), who showed that (80%, 77.5% respectively) of P. aeruginosa formed biofilm. This study performed well in terms of biofilm formation. Spectrophotometry and Fourier transform infrared spectroscopy were used to analyze the extracellular polymeric substances produced by strain XL-2. Extracellular protein concentration had a strong positive association with biofilm development, but no link with exopolysaccharide content. K. pneumoniae was shown to have a major role in the development of biofilm in this study (88.9%). This is similar with the findings of (Madam et al., 2008), who detected that 62.5% of K. pneumoniae isolates formed biofilm in this study. In addition, the result identified (88.2%) of P. mirabilis for their ability to produce biofilms in vitro. This was confirmed by (Abd Al-Mayahi, 2017), who discovered that 89.5% of P. mirabilis isolated developed biofilm. In this study, Only two isolates out of three isolates of (coli), were biofilm forming this agree with (Niba et al. 2007) who detected recently by Using genomic methods such as DNA microarray analysis, several novel Escherichia coli genes associated to biofilm formation.

In (Kim et al., 2012) study investigated at the ability of clinical isolates of Enterobacter cloacae to form biofilms by studying the mRNA expression of curli genes, and the morphologies of curli fimbriae and related with biofilm formation. So in this study detected all isolates of E. cloacae formed biofilm.

V. CONCLUSION

The current study concluded that 95.1% of bacterial species isolated from OM had ability to form biofilm.

REFERENCES


