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ORIGINAL ARTICLE

Prevalence of Bacteria-inducing Biofilm in Otitis Media With Effusion in Children

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Abstract

Background: A number of chronic upper respiratory tract infections have been linked to biofilms. The significance of bacterial biofilms in the development of chronic rhinosinusitis has been extensively researched. Its influence on the emergence of middle ear effusion, however, is still up for debate.

Aim: To assess the prevalence of bacteria that may be present in otitis media with effusion in children and its ability to induce biofilm.

Patients and methods: This is a prospective clinical study of 50 children with secretory otitis media (SOM) who fulfill our inclusion and exclusion criteria and attended the ENT Department of Ahmed Maher Teaching Hospital during the period from February 2022 until August 2022. The study group suffered from persistent otitis media with effusion and was planned for myringotomy and tests to identify isolated bacteria and organisms that can cause biofilms were scheduled at the Department of Microbiology and Immunology, Faculty of Medicine, Al-Azhar University, Cairo.

Results: From the middle ear effusion, *Staphylococcus aureus*, *Pseudomonas*, *Candida albicans*, and *Klebsiella pneumoniae* were found to be four isolated pathogenic organisms. Strongly positive biofilm-producing organisms made up 5.9 % of the numbers, while nonproducers made up 23.5 %. The kind of organism and the presence of comorbidity had a strong positive connection.

Conclusion: The middle ear effusion contained biofilm-producing organisms that were challenging to treat due to their complexity and rising antibiotic resistance.

Keywords: Bacteria, Biofilm, Otitis media

1. Introduction

Otitis media with effusion (OME) is a middle ear disorder marked by chronic inflammation that lacks the typical signs of an acute infection. Fluid buildup in the tympanic cavity and conductive hearing loss are the disease's hallmarks. OME is among the most prevalent illnesses in childhood. By the time they turn 3 years old, two-thirds of kids have experienced at least one OME episode. One-third of them will experience the attack without being aware of it, which is why it is known as 'silent'

otitis media and can covertly compromise their hearing.¹

There are two major hypotheses about the pathophysiology of OME. The conventional theory suggests that Eustachian tube malfunction plays a significant role, while Politzer's 'ex-vacuo' theory suggests that middle ear fluid accumulation is caused by persistent middle ear negative pressure.²

The middle ear mucosa has an inflammatory reaction caused by bacteria; this releases inflammatory mediators, which then upregulate the mucin genes, causing the formation of a mucin-rich effusion.

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Long-term induction of the inflammatory response and inadequate mucociliary clearance caused the middle ear fluid to persist, which resulted in the clinical manifestation of OME.³

There have been numerous reports of bacterial involvement in OME, and there are numerous ways to identify pathogens from middle ear effusions, including traditional culture methods and PCR. The most prevalent pathogens in OME have been identified using traditional culture-based techniques as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus epidermidis*. However, only 20–30 % of individuals with chronic OME (duration 3 months) respond well to middle ear fluid cultures.⁴

In the natural world, biofilms, or sessile communities of bacteria, are common. These communities form structures that are more resistant to environmental variables and are morphologically and physiologically distinct from free-living bacteria. The idea of disease based on the presence of bacterial biofilm explains why it may be challenging to get positive cultures in a chronic bacterial infection and explains the relative failure of treating OME with antibacterial medications.⁵

The mucosal biofilm theory also explains the finding that tympanostomy with ear drainage is the most successful treatment for OME. An ideal setting for the development of a bacterial mucosal biofilm is the middle ear, which has inadequate ventilation.⁶

This work aimed to study the prevalence of bacteria that may be present in OME in children and their ability to induce biofilm.

2. Patients and methods

This is a prospective clinical study including 50 secretory otitis media (SOM) children who fulfilled the inclusion and exclusion criteria and attended the ENT clinic at Ahmed Maher Teaching Hospital between February 2022 and August 2022. The research group was scheduled for myringotomy with or without the insertion of a ventilatory tube because they had persistent OME. Inclusion criteria include patients with chronic OME for 3 months, and their age is up to 15 years old. Patients with one or more of the following criteria were excluded from the study: patients with signs of acute otitis media or respiratory tract infection; patients on antibiotic therapy; children with previous myringotomy with or without grommet insertion; and patients aged more than 15 years.

The patients who were not improved on medical treatment were subjected to surgical intervention

(myringotomy with or without ventilation tubes placement), adenoidectomy in children with adenoidal hypertrophy, and tonsillectomy in children with chronic tonsillitis.

The fluid of the middle ear was collected by a sterile plastic cannula (16 G) combined with a sterile plastic syringe (3 cm) after cutting the upper part of the syringe to adapt the size of the suction hose. Aseptic collection of samples, preservation, and sending them for microbiology examination and biofilm detection (Fig. 1).

2.1. Microbiological methodology

The samples were collected and delivered to the Microbiology Department, Al-Azhar University (Cairo-Boys), on the same operation day. Full criteria of the ear fluid were reported regarding color, content, volume, consistency, and turbidity. The samples were inoculated via the streaking method over different agar plates with specific media. After 48 h of incubation at 37 °C, full microbiological identification was estimated.

2.2. Biofilm assay

In this investigation, biofilm development was carried out using a semiquantitative binding assay on Nunclon TM (use F, PS, 'not issue-culture treated'; Nunc GmbH & Co., Wiesbaden, Germany) 96-well tissue culture microtiter plates.⁷ According to McFarland's turbidity of 0.5, the bacterial isolates and inocula have been created.⁸ After being isolated from overnight cultures, the test organisms were infused into 10 ml of Trypticase soy broth that had been fortified with 1 % glucose. The mixture was then incubated at 37 °C for 24 h. The bacterial suspensions were placed into a U-bottomed 96-well microtiter plate with a total volume of 200 l. The negative control was sterile TSB alone. After that, the microtiter plates were incubated for 20–22 h at 37 °C with 150 rpm of agitation. The planktonic suspension and nutrient solution were also taken out at the conclusion of the incubation period. The plates were gently tapped out of the contents to eliminate the nonadherent bacterial cells, then washed three times with 300 l of a sterile saline solution (0.85 %). Finally, the plates were dried. Plates were stained with 200 l of 1 % (wt/vol) crystal violet (Merck, Darmstadt, Germany) for 5 min after adherent biofilms were preserved with 150 l of 95 % ethanol. After being cleaned with running water, the stain was allowed to dry for 2 h. Since the biofilms



Fig. 1. Case presented with obstructive sleep apnea and hearing loss; tympanometry was bilateral type (b); lateral ear otoscopic examination showed SOM and grade 4 adenoid hypertrophy in radiograph preoperative. SOM, secretory otitis media.

could be seen as purple rings on the well, the findings were estimated. By dissolving the well stains with 200 l of 33 % (v/v) glacial acetic acid (Merck), the associated cell (biofilm) was measured. After that, an ELISA reader (BioTek, 800 TSUV, Santa Clara, California, United States) was used to examine and measure the stain's optical density at 570 nm. The isolates were divided into four groups: nonbiofilm producers (0), weak biofilm producers (1+), moderate biofilm producers (2+), and strong biofilm producers (3+).

2.3. Motility detection (swimming and swarming)

Thermo Fisher Scientific's (Basingstoke, Hants, UK) Oxoid Luria broth (LB) plates [supplemented with 1 % (w/v) glucose and 0.5 % (w/v) agar from Tianjin Kemio Chemical Reagent Co. Ltd., Tianjin, China] were used to inoculate the collected bacterial isolates and estimate the results of the swarming test. The plates were then kept at 37 °C for a further 24 h. As previously reported, this technique identified the region centered around the bacterial inoculation point's morphology and assessed its diameter.⁹

2.4. Pyocyanin production estimation

Using methods from Zhu et al.,¹⁰ a quantitative chemical experiment was performed to ascertain the synthesis of pyocyanin. After being adjusted to an OD600 of 1.0 ml in LB medium, the overnight cultures were cultivated for 24 h at 37 °C. The cultured samples were spun at 10 000 g for 15 min before being filtered through sterile 0.22 mm filter paper. 0.2 M HCl was added to 1 ml. By combining the bacterium with chloroform in a 3 : 2 ratio, the pyocyanin was isolated. Using an ELISA reader (BioTek, 800 TSUV, USA), the pyocyanin in the supernatant was measured at OD520 on a 96-well plate.

2.5. Ethical consideration

The aim and nature of the study were explained to parents of children before inclusion. An informed written consent was obtained before enrollment.

2.6. Statistical analysis

The obtained data was coded, processed, and analyzed using Microsoft Excel software and covered the history, essential clinical examination, laboratory tests, and outcome measures. The Statistical Package for the Social Sciences (SPSS Inc., version 27.0, Basingstoke, Hants, UK) was used to import the data. The qualitative data were presented as numbers and percentages, depending on the kind. The data were also quantitatively represented as a group by mean \pm SD, and the following tests were used to determine whether any differences were statistically significant: logistic regression for predictors and the difference and association of qualitative variables by χ^2 test. The *P* value was set at 0.05 for outcomes that were significant and at 0.001 for those that were highly significant.

3. Results

Only 67 samples total, of which 33 were unilateral (21 left and 12 right) and 17 were bilateral, were taken from the 50 participants. Only 15 of the samples that were grown on various types of particular microbiological media exhibited any growth, and the remaining 35 showed only slight growth.

Microscopically and through biochemical assays, the isolated organisms were recognized as *Pseudomonas aeruginosa* (six), *Staphylococcus* sps. (five), and *Klebsiella pneumoniae*, *Candida albicans*, and *Escherichia coli* (two).

Only eight of the isolated organisms displayed multiple drug resistance, four had

extended-spectrum beta-lactamases, three were vancomycin-sensitive, and two were resistant to all antifungal drugs.

Participants' ages in the current study ranged from 3 to 15 years, with a mean age of 6.7 years. Male individuals make up 52 % (26) of the sample as a whole (Table 1).

Bronchial asthma (20 %) was the most prevalent comorbidity in the clinical history, followed by diabetes mellitus type 1 (6 %), as indicated in Table 2.

The majority of cases exhibit several auditory, nasal, or oropharyngeal symptoms, which are compiled in Table 3.

Regarding the microbiological examination, only 15 (30 %) cases showed signs of microbial growth on various culture mediums, 35 (70 %) cases had no growth or growth that was negligible, and 10 (58.8 %) cases primarily originated from the left ear.

P. aeruginosa six (35.3 %), *Staphylococcus* five (29.4 %), and *Staphylococcus* two (11.8 %) for *K. pneumoniae*, *C. albicans*, and *E. coli* were the isolates (Fig. 2).

Table 1. Demographic data of the studied group.

	Range	Mean	SD
Age (years)	3–15	6.7	2.9
		<i>n</i> (%)	
Sex	Female	24 (48)	
	Male	26 (52)	

Table 2. Frequency of comorbidity among the studied group.

	<i>n</i> (%)
Diabetes mellitus	3 (6)
Bronchial asthma	10 (20)
Cleft palate	1 (2)
G6PD	1 (2)

Table 3. Clinical presentation of the studied group.

	<i>n</i> (%)
Ear	
Hearing loss	32 (64)
Accidental discovered type B tympanometry	7 (14)
Recurrent otitis media	8 (16)
itching	6 (12)
Accidental discovered SOM	1 (2)
Nasal	
Snoring	22 (44)
Mouth breathing	21 (42)
Recurrent rhinosinusitis	10 (20)
Nasal obstruction	10 (20)
Nasal discharge	3 (6)
Allergic rhinitis	12 (24)
Other	
OSA	7 (14)
Chronic tonsillitis	11 (22)
Kissing tonsils	6 (12)

SOM, secretory otitis media.

Regarding the isolated organisms' antimicrobial sensitivities, two (11.8 %) were sensitive to all anti-fungal medications, three (17.6 %) were sensitive to vancomycin, four (23.5 %) were extended-spectrum beta-lactamases, and eight (47.1 %) were multiple drug resistance.

Regarding biofilm production and detection for the isolated organisms (Fig. 3 and Table 4) show that none of the *P. aeruginosa* isolates were unable to produce the biofilms shown in Fig. 3 and Table 4. Out of the six isolates, one (33.3 %) showed strong biofilm production, two (33.3 %) had moderate biofilm production, and three (33.3 %) were weak.

Out of five *S. aureus* isolates, only one (20 %) produced a moderate amount of biofilm, two (40 %) produced just a little amount of biofilm, two (40 %) produced no biofilm, and none produced a significant amount of biofilm.

The two isolates of *K. pneumoniae* produced a moderate amount of biofilm. One isolate of *E. coli* produced weak biofilms, and the other did not produce any. Finally, none of the isolates of *C. albicans* could grow biofilm (Fig. 4 and Table 4).

4. Discussion

Bronchial asthma was the most prevalent comorbidity in our study (20 %), and when we observed the relationship between comorbidity present and growth features, the only significant relationship we identified was between the type of organism and comorbidity presence.

The most prevalent lower airway condition in children is asthma. In the USA, between 8.3 and 9.3 % of children aged 0–17 have asthma.¹¹ Asthma affects 6.27–7.39 % of Korean children aged 4–12.¹² Patients with asthma frequently also have inflammatory conditions of the upper airways, such as rhinitis.^{13,14} A united airway disease was proposed to explain the co-occurrence of airway disorders based on the continuity of the respiratory mucosa and the inflammatory response.¹⁵

The influence of diabetes mellitus on patients with external otitis, malignant external otitis, otitis media, abrupt sensorineural hearing loss, and slowly progressive hearing loss is the main emphasis of the material available on the importance of diabetes mellitus for ear illnesses.¹⁶

The findings suggest that bacteria may play a role in the etiopathogenesis of OME by demonstrating viable bacteria in 30 % of OME samples when culture and biofilm are combined. Along with the usual three bacteria investigated in the past (*S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*), a wide range of other bacteria were also found.^{4,17}

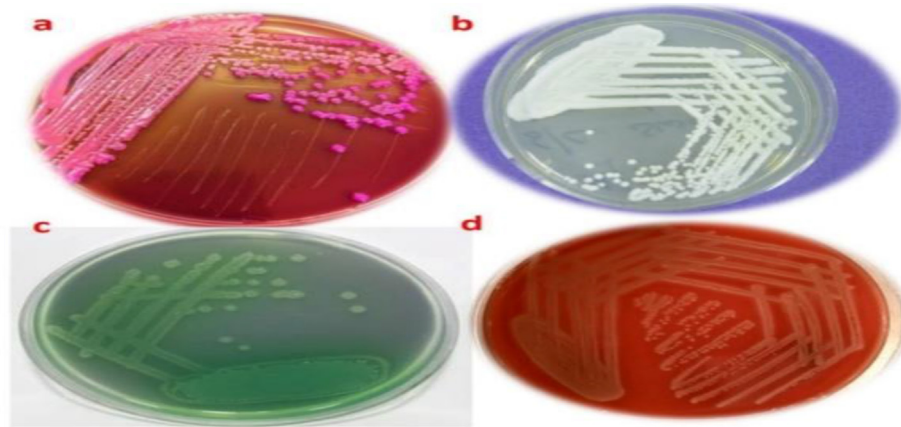


Fig. 2. The isolated organisms from various specific media were shown to be cultivable. Examples include (a) *Klebsiella pneumoniae* on MacConkey agar; (b) *Candida albicans* on nutrient agar; (c) *Pseudomonas aeruginosa* on nutrient agar with exo-pigment formation; and (d) *Staphylococcus aureus* on blood agar with fully developed hemolytic colonies.

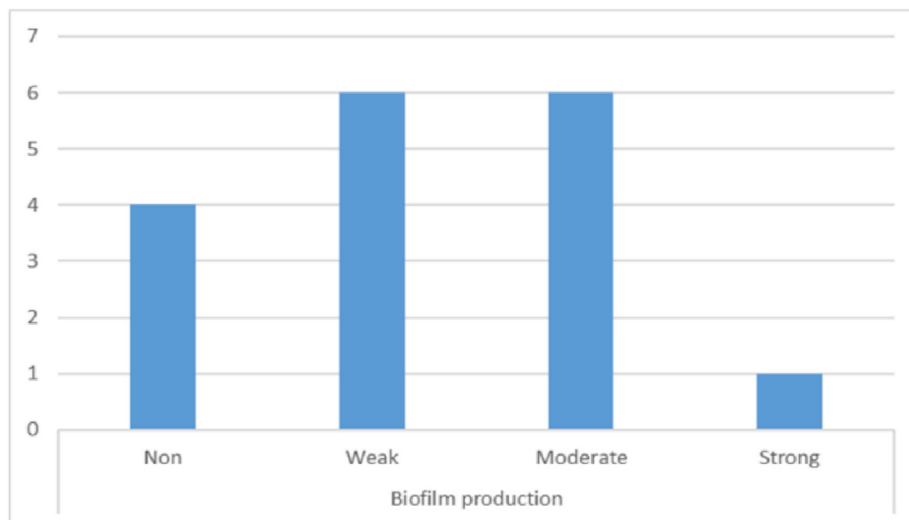


Fig. 3. Biofilm production.

Six *P. aeruginosa* isolates were used in the study, and six were shown to develop biofilm to varying degrees (16.6 % produced strong biofilm, 33.3 % moderate biofilm, 33.3 % weak biofilm, and none could not form biofilm). Using qualitative biofilm formation assays, Rehman et al.¹⁸ discovered that every isolate of *P. aeruginosa* and *S. aureus* was an effective biofilm builder.

Sharma and Chaudhary,¹⁹ on the other hand, observed that 72.34 % of *P. aeruginosa* received from various sources were strong, 10.63 % were intermediate, 7.14 % were weak, and 10.7 % were nonbiofilm producers after screening the bacteria for their phenotypic biofilm production. They claimed that ear swab isolates were more likely to produce a strong

biofilm. Furthermore, according to Firouzi-Dalvand and Pooladi,²⁰ isolates from ear and throat swabs formed more biofilm than those from urine samples.

Regarding the *S. aureus* isolates, 20 % of the isolates produced moderate amounts of biofilm, 40 % produced weak amounts of biofilm, 40 % produced no biofilm, and none produced significant amounts of biofilm. A phenotypic ability to build biofilm was reported in 37.5 % of all *Staphylococcus* spp. strains, according to Niedzielski et al.⁶ The microtiter plate approach, on the other hand, was used by Nasr et al.²¹ to report that *S. aureus* isolates generated biofilms to varied degrees; 26 % of the isolates were strong producers, 12 % were moderate producers, and 8 % were weak producers.

Table 4. Classification of isolates according to biofilm production.

Sample numbers	Laterality	Organism	Motility	Culture/sensitivity	OD	Biofilm production
1	Left	<i>Klebsiella pneumoniae</i>	–	MDR	0.224	+2 ^a
2	Left	<i>Staphylococcus aureus</i>	–	Vancomycin sensitive	0.214	+2
6	Left	<i>Pseudomonas aeruginosa</i>	+	MDR	0.226	+2
7	Left	<i>Candida albicans</i>	–	Sensitive to all antifungal	0.068	0
8	Right	<i>Staphylococcus aureus</i>	–	MDR	0.107	+1
11	Left	<i>Pseudomonas aeruginosa</i>	+	MDR	0.122	+1
13	Right	<i>Staphylococcus aureus</i>	–	Vancomycin sensitive	0.152	+1
14	Left	<i>Candida albicans</i>	–	Sensitive for all antifungal	0.043	0
16	Right	<i>Pseudomonas aeruginosa</i>	+	MDR	0.216	+2
17	Right	<i>Escherichia coli</i>	+	ESBL	0.057	0
19	Left	<i>Staphylococcus aureus</i>	–	MDR	0.002	0
19	Right	<i>Pseudomonas aeruginosa</i>	+	MDR	0.1036	+1
22	Left	<i>Escherichia coli</i>	+	ESBL	0.126	+1
23	Right	<i>Klebsiella pneumoniae</i>	–	ESBL	0.183	+2
25	Left	<i>Pseudomonas aeruginosa</i>	+	MDR	0.144	+1
25	Right	<i>Pseudomonas aeruginosa</i>	+	ESBL	0.377	+4
28	Left	<i>Staphylococcus aureus</i>	–	Vancomycin sensitive	0.023	+2

ESBL, extended-spectrum beta-lactamases; MDR, multiple drug resistance.

^a Nonbiofilm producer (0), weak biofilm producer (1+), moderate biofilm producer (2+), strong biofilm producer (3+).

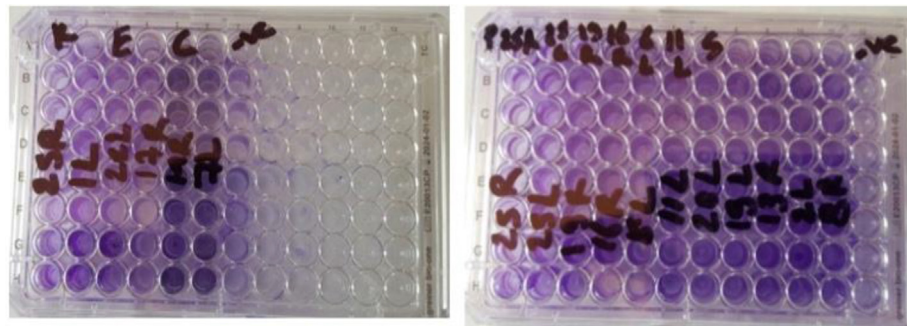


Fig. 4. Biofilm formation by different clinical isolates.

4.1. Conclusion

Infections caused by biofilms are challenging to treat because of their complexity and rising drug resistance. As surface colonization is the first step in the creation of biofilms, it is essential to prevent it in order to limit biofilm development.

Conflicts of interest

There is no conflict of interest.

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