Correlation of bacterial biofilm profile based on optical density cut-off with clinical severity in patients with chronic suppurative otitis media tubotympanic type

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ABSTRACT

Introduction: Chronic suppurative otitis media (CSOM) is a middle ear infection with a high incidence in ear cases, is often recurrent, and causes hearing impairment. Bacteria in the CSOM frequently form biofilms, which enhance antibiotic resistance and contribute to disease progression. The aim of this study was to determine the correlation of bacterial biofilm profiles based on optical density cut-off with the clinical picture of patients with tubotympanic type CSOM.

Materials and Methods: This was a cross-sectional study using a descriptive analytical design. The study was conducted at the tertiary teaching hospital of Hasanuddin University and the network hospital in Makassar, Indonesia, from July 2023 to July 2024. The study population consisted of patients with the CSOM tubotympanic type who met the inclusion criteria. Bacterial cultures and biofilm examinations were performed using the tissue culture plate method. Data were analyzed using SPSS® version 28.

Results: A total of 53 patients with the CSOM tubotympanic type were included in this study. The mean age of the patients was 30±14 years. Pseudomonas aeruginosa was the most dominant bacterium (32.1%), with 20 other bacteria, and all these bacteria formed biofilms with either weak or moderate strength. There was a significant association between biofilm formation and nature of secretion (r=0.395, p=0.003). The chronicity of the disease (r=0.407, p=0.002) and the degree of hearing impairment (r=0.294, p=0.032) were also significant. A significant positive association was found between total clinical score and biofilm formation (r=0.429, p=0.001).

Conclusion: All bacteria found in the tubotympanic CSOM formed biofilms. The correlation analysis revealed a significant positive relationship between several clinical variables and biofilm formation. The substantial formation of biofilms may account for the fact that patients with elevated scores frequently experience infections that are challenging to manage with conventional antibiotic treatments.

KEYWORDS:
Biofilm, chronic suppurative otitis media, tubotympanic type, clinical characteristics

INTRODUCTION

Globally, CSOM often occurs, especially in developing countries, affecting between 65 to 330 million people. It is estimated that there is an annual incidence of 31 million new cases, a fifth of which occur in children under the age of 5.[1] *Staphylococcus aureus* is the most commonly identified microorganism is *Staphylococcus aureus* (including MRSA). Other similar bacteria, including *Pseudomonas aeruginosa, Proteus spp., Klebsiella spp., Bacteroides spp.,* and *Fusobacterium spp.*, can cause this disease.[2]

Bacteria are generally considered microorganisms that live individually; however, most form complex organizations and adhere to surfaces. This phenomenon is known as biofilm formation.[3] Biofilms have been shown to play a significant role in otitis media and have been identified in direct biopsy specimens from the middle ear. Many bacteria commonly found in CSOM exist in biofilm states under favorable conditions.[4] Bacteria within biofilms are 10–1,000 times more resistant than when they are not in biofilms.[5] Numerous studies have reported the presence of biofilm bacteria in CSOM, highlighting their impact on bacterial persistence and infection treatment. The presence of biofilm bacteria may explain the occurrence of resistance to systemic and topical antibiotics.[6]

MATERIALS AND METHODS

This was a cross-sectional study that was conducted at the Ear, Nose and Throat (ENT) clinics of Hasanuddin University Hospital and Network Hospital in Makassar from July 2023 to July 2024. The study population consisted of patients with tubotympanic-type CSOM who underwent treatment at an outpatient clinic.

The inclusion criteria were as follows: patients with tubotympanic type CSOM who were over the age of 17 years

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and had never undergone ear surgery. Exclusion criteria included CSOM patients accompanied by immunocompromised disorders, such as malignancy, malnutrition, diabetes mellitus, and CSOM tuberculosis.

The study's protocol involved patients with CSOM attending the outpatient clinic, where history-taking and physical examination were conducted. This included assessment of the severity and duration of the disease and hearing examination using pure tone audiometry. CT scan examinations serve as supplementary examinations that complement ENT assessments to establish a diagnosis of tubotympanic CSOM and to eliminate the possibility of cholesteatoma. Following the satisfaction of the inclusion criteria, the patient was deemed eligible for further consideration to be enrolled in the study.

The next stage of the process involved the collection of ear secretion samples for culture and bacterial identification. These samples were then analysed using the Microtiter Plate Assay method to detect biofilm.

An optical density (OD) examination of the biofilm was performed with a micro ELISA autoreader (Model 680, Bio-Rad, UK) at a wavelength of 620 nm. The results were then interpreted based on the Stepanovic criteria. The threshold value (ODc) was calculated from the mean OD of the negative control plus 3×SD. The presence of biofilms was classified as weak (<2×ODc), moderate (2-4×ODc), and strong (>4×ODc).

Data were obtained from patient histories, physical examinations, audiometric results, bacterial cultures, and bacterial biofilm examination. The data were analyzed using SPSS® software version 28 with Chi-square and Spearman tests and are presented in the form of tables and diagrams.

RESULTS

In this study, an analysis was conducted on 53 patients with tubotympanic CSOM. The results of the data analysis included the distribution of sex, age, types of bacteria, biofilm profiles, and the relationship between clinical severity and biofilm profiles. The majority of respondents were female (64.15%), while males accounted for only 35.8%. The average age of the patients was 35 years (median, 17 – 77 years). The clinical picture of patients with CSOM tubotympanic includes mucopurulent secretions in 27 (50.94%) of cases and mucoid secretions in 26 (49.05%) of cases. The intensity of otorrhea is predominantly categorized as intermittent in 43 (81.1%) patients and continuous in 10 (18.8%) patients. Regarding the volume of secretions, 36 (67.9%) patients experience slight otorrhea (limited to the middle ear) and 17 (32.07%) experience significant otorrhea (extending to the ear canal). In terms of chronicity of the illness, 30 (56.6%) patients have suffered for more than 3 years, 15 (28.3%) for between 1 to 3 years, and 8 (15.09%) for less than one year. Regarding the type of hearing impairment, 42 (79.2%) patients suffered from conductive hearing loss, 8 (15.09%) from mixed hearing loss, 1 (1.88%) from sensorineural hearing loss, and 2 (3.77%) with no hearing impairment. The degree of hearing impairment is predominantly moderate in 35 (66.03%) patients, mild in 9 (16.9%) , severe in 4 (7.54%) , very severe in 3 (5.66%), and with no hearing impairment in 2 (3.77%).

Table I shows the bacteria responsible for the tubotympanic type of CSOM. Among the 21 identified bacterial species, 11 (52%) were gram-negative and 10 (48%) were gram-positive. *Pseudomonas aeruginosa* was the most dominant pathogen (32.1%), followed by *Staphylococcus aureus* (11.3%). Other identified bacteria included *Proteus mirabilis* (7.5%), *Achromobacter xylosoxidans* (5.7%), and several other species, indicating the involvement of a diverse range of pathogens.

The relationship between the bacterial species and biofilm formation was classified into three categories: weak, moderate, and strong. The majority of bacteria (81.1%) formed weak biofilms (43 isolates), whereas 18.8% formed moderate biofilms (10 isolates), with no samples exhibiting strong biofilm formation. The bacteria responsible for moderate biofilm formation include *Pseudomonas aeruginosa, Achromobacter xylosoxidans, Burkholderia cepacia, Staphylococcus epidermidis,* and *Corynebacterium amycolatum*. (Table I).

In terms of discharge characteristics, the weak biofilm group was predominantly associated with mucoid discharge (60.47%), whereas the moderate biofilm group was mostly associated with mucopurulent discharge (90.00%). This difference was statistically significant (p=0.004), suggesting that moderate biofilms were more likely to produce mucopurulent discharge (Table II).

In terms of the intensity of otorrhea, the majority of patients in both the weak (83.72%) and moderate biofilm (70.00%) groups experienced intermittent otorrhea; however, no significant difference was observed between the two groups (p=0.318). Regarding the volume of discharge, both the weak and moderate biofilm groups predominantly exhibited small amounts of discharge (69.77% and 60.00%, respectively); however, the difference was not statistically significant (p=0.551) (Table II).

Regarding disease chronicity, the weak biofilm group exhibited varied disease duration, with 18.60% of cases having a duration of less than one year, 34.88% between 1 and 3 years, and 46.51% with a duration of ≥3 years. In contrast, all patients in the moderate biofilm group had a disease duration of ≥3 years. Pearson's correlation test showed a moderate correlation (r=0.515, p<0.001), confirming that stronger biofilms were associated with a longer disease duration (Figure 1).

Regarding the severity of hearing loss, both weak and moderate biofilm groups were predominantly associated with moderate hearing loss (65.12% and 70.00%, respectively). Pearson's test revealed a low correlation between biofilm strength and hearing loss severity (r=0.281, p=0.015), indicating that stronger biofilms were associated with more severe hearing loss (Figure 2).

Based on Chi-Square test, most patients in both the weak (81.40%) and moderate (70.00%) biofilm groups had

Table I: Bacteria responsible for CSOM tubotympanic type

	Bacterial Species	Biofilm form		n (%)
		weak	moderate	
Gram- Negative	Pseudomonas aeruginosa	12	5	17 (32.1)
	Proteus mirabilis	4	0	4 (7.5)
	achromobacter xylosoxidans	1	2	3 (5.7)
	Pseudomonas Putida	2	0	2 (3.8)
	Acinetobacter baumannii	2	0	2 (3.8)
	Eschericia coli	2	0	2 (3.8)
	Klebsiella pneumoniae	2	0	2 (3.8)
	Providencia stuartii	1	0	1 (1.9)
	Citrobacter freundii	1	0	1 (1.9)
	Burkholderia cepacia	0	1	1 (1.9)
	Pandoraea ssp	1	0	1 (1.9)
Gram-Positive	Staphylococcus aureus	6	0	6 (11.3)
	Staphylococcus haemolyticus	3	0	3 (5.7)
	Arcanobacterium haemolyticum	1	0	1 (1.9)
	Staphylococcus hominis	1	0	1 (1.9)
	Staphylococcus cohnii	1	0	1 (1.9)
	Staphylococcus epidermidis	0	1	1 (1.9)
	Streptococcus Agalactiae	1	0	1 (1.9)
	Gemella morbillorum	1	0	1 (1.9)
	Corynebacterium Striatum	1	0	1 (1.9)
	Corynebacterium amycolatum	0	1	1 (1.9)
TOTAL		43	10	53 (100)

Table II: Correlation of biofilm profiles with discharge characteristics and type of hearing impairment in patients with CSOM tubotympanic type based on chi-square test

	Variable	Biofilm Profile		Total n (%)	p-value
		Weak n (%)	Moderate n (%)		
Type	Mucoid	26 (60.47)	1 (10.00)	27 (50.94)	0.004
	Mucopurulent	17 (39.53)	9 (90.00)	26 (49.06)	
Intensity	Intermittent	36 (83.27)	7 (70.00)	43 (81.13)	0.318
	Continuous	7 (16.28)	3 (30.00)	10 (18.87)	
Volume	Slight (Middle ear only)	30 (69.77)	6 (60.00)	36 (67.92)	0.551
	plenty (Ear canal)	13 (30.23)	4 (40.00)	17 (32.08)	
Type of hearing impairment	Normal hearing	2 (4.65)	0 (0.00)	2 (3.77)	0.165
	Conductive	35 (81.40)	7 (70.00)	42 (79.25)	
	Sensorineural	0 (0.00)	1 (10.00)	1 (1.89)	
	Mixed	6 (13.95)	2 (20.00)	8 (15.09)	

Chi Square Test

conductive hearing loss. However, no statistically significant difference was observed in the type of hearing loss between the two groups (p=0.165) (Table II).

A significant relationship was found between bacterial biofilm profiles and clinical severity in the CSOM tubotympanic type. According to the graph, the correlation between biofilm strength and overall clinical presentation was 0.373, representing a low but statistically significant correlation (p=0.002). This indicates a positive relationship between biofilm strength and clinical presentation, in which stronger biofilms are linked to more severe clinical manifestations. This significant biofilm formation may help explain why patients with higher clinical scores often present with infections that are difficult to treat with standard antibiotic therapies (Figure 3).

DISCUSSION

The results of this study provide significant insights into the clinical severity influenced by biofilm profiles in patients with tubo tympanic CSOM. In the analysis of the 53 CSOM isolates, 21 bacterial species were identified. The variety of bacterial species indicates that despite the dominance of certain pathogens, CSOM infections may be caused by a broad spectrum of microorganisms. Among the identified bacteria, 52% were gram-negative and 48% were gram-positive. This finding may serve as a reference for the choice of antibiotic therapy in patients with tubotympanic type CSOM before the results of microbial culture are obtained.

In terms of microbiological findings, *Pseudomonas aeruginosa* was identified as the most dominant pathogen (32.1%), underscoring its significance as a major pathogen in chronic ear infections (CSOM). This bacterium is known for its ability to form biofilms, which contributes to resistance to a variety of antibiotics.[7] Biofilms formed by *Pseudomonas aeruginosa* act as protective barriers against the host immune system and enhance antibiotic resistance, making infection difficult to treat. These findings align with those of Ha et al. (2018), who showed that biofilms formed by *Pseudomonas aeruginosa* in chronic infections play a significant role in treatment

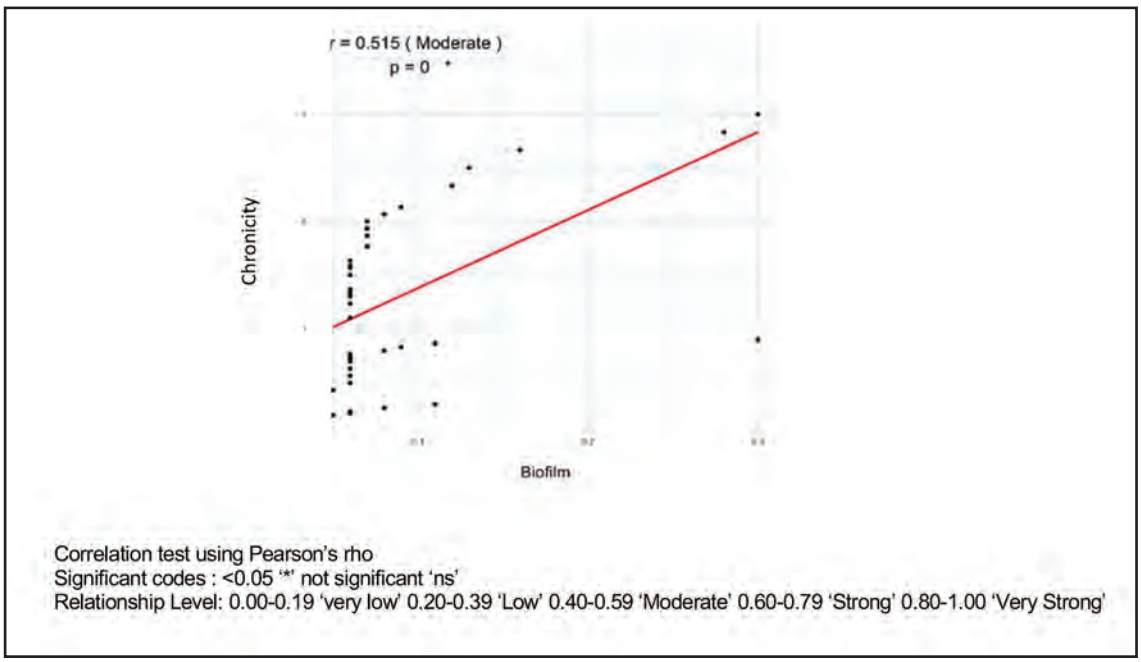

Fig. 1: Correlation of biofilm profiles with disease chronicity in patients with chronic otitis media tubotympanic based on Pearson's Test

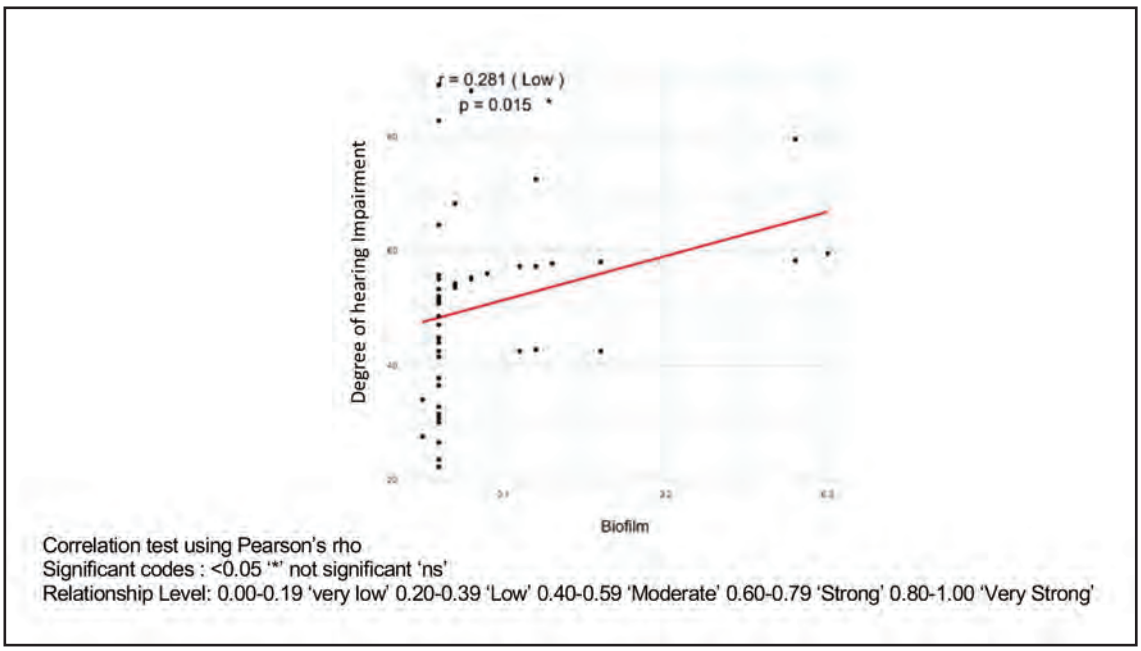

Fig. 2: Correlation of biofilm profiles with degree of hearing impairment in patients with chronic otitis media tubotympanic based on Pearson's Test

resistance and are associated with poorer clinical outcomes. *Staphylococcus aureus* has long been recognized as a pathogen involved in chronic infections, particularly because of its ability to form biofilms that protect the bacteria from antibiotic therapy and immune responses.[8] However, in the present study, the biofilm formed by *Staphylococcus aureus* was weak. Becker et al. found that biofilms formed by *Staphylococcus aureus* in chronic infections enhance inflammation and prolong healing. In the context of CSOM, these biofilms may exacerbate clinical symptoms such as otorrhea and tympanic membrane perforation.[9]

Among the various bacterial species responsible for CSOM, all are capable of biofilm formation. Ten isolates formed moderate biofilms, including *Pseudomonas aeruginosa* (5 out of 17 samples, 29.4%), Achromobacter xylosoxidans (2 out of 3 samples, 66.6%), and other species such as *Burkholderia cepacia*, *Staphylococcus epidermidis,* and *Corynebacterium amycolatum,* which also exhibited severe clinical severity. Biofilm formation plays a crucial role in persistence of chronic infections. Although some bacteria form weak biofilms, their presence remains a significant factor in exacerbating infections. In vitro studies have shown that TSB

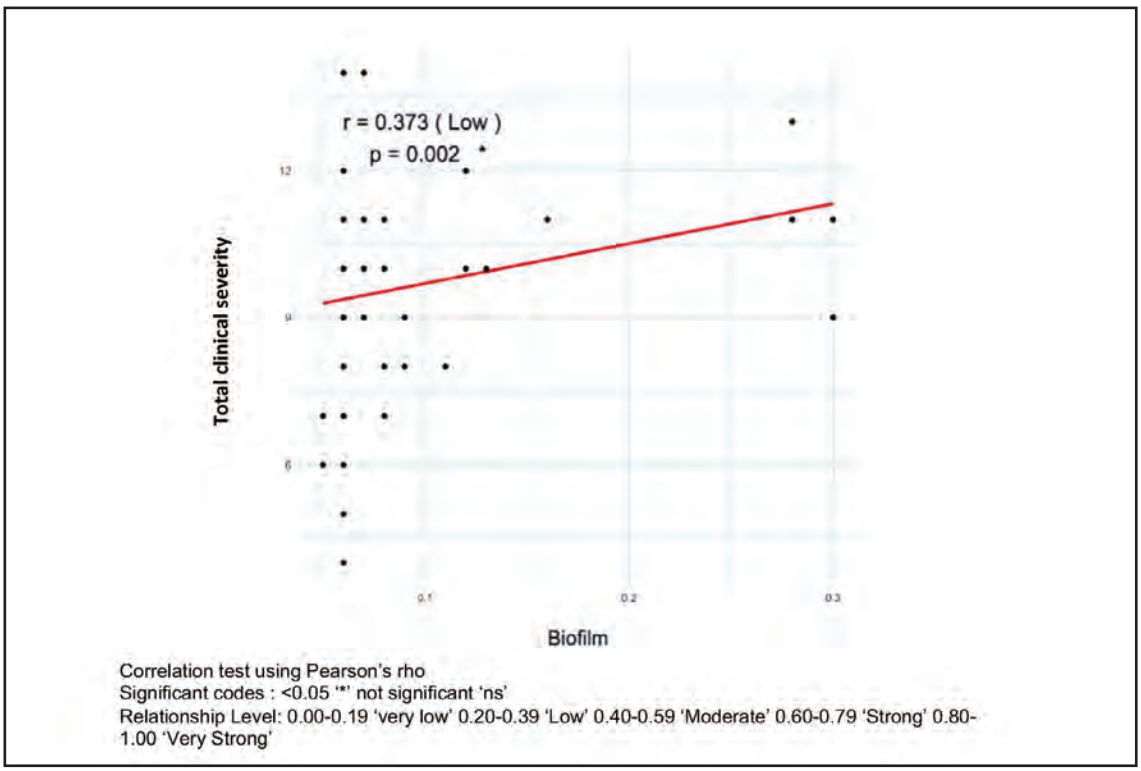

Fig. 3: Correlation between bacterial biofilm profile and total clinical severity in patients with chronic suppurative otitis media tubotympanic type based on Pearson's test

supplemented with glucose can support strong biofilm formation, which can be reproducibly quantified.[10] Moreover, weak biofilms still affect therapeutic outcomes and worsen infections.

The intensity of otorrhea and quantity of discharge did not show a significant relationship, suggesting that these factors may not be influenced by the biofilm profile. Biofilm formation, whether weak or moderate, enables bacteria to survive for extended periods at the infection site, leading to persistent clinical symptoms. Otorrhea, often caused by ongoing inflammation and secretion in the ear, may be associated with the ability of biofilms to create an environment conducive to recurrent infections. Hall-Stoodley et al. demonstrated that biofilms enhance bacterial resistance to the immune system, making it difficult to resolve inflammation and fluid production. Additionally, tympanic membrane perforation, commonly observed in CSOM, may be linked to the ability of bacteria within the biofilm to damage local tissues. Biofilms protect bacteria by enabling them to continue producing enzymes or toxins that can harm tissue structures, slow the natural healing process, and cause further damage to the tympanic membrane.[11] This is in line with findings by Jensen et al. who showed that bacteria within biofilms produce proteases and other bioactive components that can damage local tissues and exacerbate the infection.[12]

The most statistically significant finding was the relationship between disease chronicity and biofilm formation ($p<0.001$), indicating that stronger biofilms correlated with longer disease duration. This is consistent with the research by Edward and Novianti (2023), which emphasizes the crucial role of biofilm formation in maintaining chronic infections and impeding the response to treatment.[13]

The degree of hearing loss also showed a correlation coefficient ($r=0.281$), indicating low correlation strength, but the correlation was statistically significant ($p=0.015$). This suggests that stronger biofilms are associated with more severe hearing impairment. Individuals with more severe hearing loss may have this condition because of the chronic nature of the disease, which is influenced by biofilm formation. This finding is consistent with reports by Triola et al. state that chronic otitis media is a major cause of hearing loss, especially when the infection persists. In chronic infections, biofilms facilitate bacterial colonization of the middle ear structures, causing chronic inflammation that disrupts normal auditory function.[14] Alhede et al. (2019) found that bacteria within biofilms are frequently resistant to conventional antibiotics, resulting in persistent hearing loss due to uncontrolled inflammation in the region.[15]

In this study, two patients did not exhibit hearing loss, which could be related to the fact that eight patients had a disease duration of less than one year. Additionally, this could be associated with excellent hearing levels. Therefore, when tympanic membrane perforation occurs due to CSOM, there is a reduction in hearing function, but it has not yet reached the level of mild hearing loss. Furthermore, one patient was found to have sensorineural hearing loss, which warrants further investigation to determine whether there are diseases or other disorders besides CSOM that cause this condition. Overall, the results of this study indicate a significant

relationship between the clinical symptoms of patients with tubotympanic type chronic suppurative otitis media and bacterial biofilm profiles. Biofilms formed by various types of bacteria in these patients contribute to the persistence and exacerbation of the clinical symptoms. Generally, the primary symptoms of CSOM, such as otorrhea, hearing loss, and chronicity, can be worsened by the presence of biofilms, which protect bacteria from both the body's immune response and antibiotic treatment. The clinical severity of CSOM tubotympanic type is strongly associated with the presence of bacterial biofilms. Biofilms not only prolong the infection and increase resistance to treatment but also play a crucial role in exacerbating the clinical manifestations of the infection, making it difficult to treat and often requiring more aggressive and prolonged therapies.[16] In this study, participants who were selected were those who did not have comorbid conditions such as immunocompromised disorders, in order to avoid bias caused by other factors that could affect the severity of the disease aside from pathogens and biofilms

The results of this study indicate that all bacteria are capable of forming biofilms, highlighting the importance of considering additional therapeutic strategies for managing CSOM. Ear irrigation with antiseptic solutions, as well as the use of adjunctive anti-biofilm therapies and quorum sensing inhibitors, are believed to enhance the success of treatment in CSOM. Ear irrigation can help to clear debris, pus, and microorganisms, thereby improving the effectiveness of topical medications. Antiseptic solutions such as 2% acetic acid and hydrogen peroxide are commonly used. Acetic acid inhibits the formation and eradication of *Pseudomonas aeruginosa* biofilms in CSOM.[6] Hydrogen peroxide (H_2O_2) is used as an antiseptic irrigation solution because of its ability to generate reactive oxygen species, which can damage bacterial cell walls and biofilm structures, making it effective in killing microorganisms and disrupting biofilm integrity.[17]

Several studies have shown that certain agents can inhibit biofilm formation or destroy the existing biofilms. Blasi and Page (2016) demonstrated in vitro that N-acetylcysteine has antibacterial properties and can inhibit biofilm formation. N-acetylcysteine blocks the production of exopolysaccharides, a major component of the biofilm matrix, thus preventing biofilm formation and accelerating biofilm degradation.[18] In addition, N-acetylcysteine induces oxidative stress in bacterial cells, contributing to cell death and biofilm disruption.[19] Ethylenediaminetetraacetic acid (EDTA) can chelate divalent cations such as calcium and magnesium, which stabilize the biofilm matrix. The removal of these ions weakens the biofilm structure and increases its permeability, making the bacteria more susceptible to antimicrobial agents.[20,21] The combination of N-acetylcysteine and EDTA synergistically disrupted the biofilm matrix and enhanced the effectiveness of antimicrobial therapy. However, clinical use must consider potential side effects and compatibility with other therapeutic agents.

Quorum sensing (QS) is a form of bacterial communication that allows bacteria to collectively coordinate gene expression, including genes involved in biofilm formation and virulence factor production. Nur et al (2006) explored several agents that inhibit enzymes involved in the production of QS signaling molecules known as autoinducers.[22] Specific enzymes, such as halogenated furanones from *Delisea* pulchra algae, N-(heptylsulfanylacetyl)-L-homoserine lactone from *Allium sativum*, and flustramine from the bryozoan *Flustra foliacea*, can inhibit and degrade autoinducers, thereby preventing gene expression and activation of quorum sensing pathways. These findings highlight the importance of understanding the clinical and microbiological characteristics of patients with CSOM and the relationship between these factors and biofilm formation. This understanding could inform the development of more effective treatment strategies, taking into account the interaction between bacterial pathogens and the clinical conditions of patients. Further research is needed to explore the underlying mechanisms of this relationship and to assess its impact on treatment and patient prognosis.

CONCLUSION

This study successfully analyzed the clinical and microbiological characteristics of patients with tubotympanic type chronic suppurative otitis media, focusing on biofilm formation. The main findings indicated that 21 types of bacteria were identified, predominantly gram-negative bacteria, with the most prevalent being Pseudomonas aeruginosa, followed by *Staphylococcus aureus* and *Proteus mirabilis*. Correlation analysis revealed a significant positive relationship between the biofilm profile and several clinical variables, indicating that the stronger the biofilm profile, the more severe the clinical presentation of the tubotympanic type of CSOM.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

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