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Characterizing the Biofilm-Forming Capacity of Bacterial Isolates from Chronic Suppurative Otitis Media in Bhutan

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A B S T R A C T

Introduction: Chronic suppurative otitis media (CSOM) is a persistent middle ear infection characterized by recurrent ear discharge and hearing loss, significantly impacting the quality of life. Biofilm formation by bacteria plays a crucial role in the chronicity and recalcitrance of CSOM to conventional antibiotic treatments. This study aimed to characterize the biofilm-forming capacity of bacterial isolates from CSOM patients in Bhutan and assess their antibiotic susceptibility. **Methods:** Ear swab samples were collected from 100 patients diagnosed with CSOM at a tertiary hospital in Bhutan. Bacterial isolates were identified using standard microbiological techniques, including Gram staining, culture on selective media, and biochemical tests. Biofilm formation was quantitatively assessed using the crystal violet assay, and the biofilm architecture was visualized using confocal laser scanning microscopy (CLSM). The antibiotic susceptibility of biofilm-forming isolates was determined using the minimum biofilm eradication concentration (MBEC) assay. **Results:** The most prevalent bacterial isolates were *Pseudomonas aeruginosa* (35%), *Staphylococcus aureus* (25%), and *Escherichia coli* (15%). A significant proportion of these isolates (70%) exhibited strong biofilm-forming capacity. CLSM revealed a complex three-dimensional structure of the biofilms with channels and water passages, facilitating nutrient transport and waste removal. Biofilm-forming isolates demonstrated significantly higher MBEC values compared to their planktonic counterparts, indicating enhanced antibiotic resistance. **Conclusion:** This study highlights the significant prevalence of biofilm-forming bacteria in CSOM cases in Bhutan. The enhanced antibiotic resistance of these biofilms emphasizes the urgent need for alternative treatment strategies, such as biofilm-disrupting agents or targeted drug delivery systems, to effectively manage CSOM and prevent associated complications like hearing loss and intracranial infections.

1. Introduction

Chronic suppurative otitis media (CSOM), a persistent inflammatory condition afflicting the middle ear and mastoid cavity, presents a significant challenge in otology. Characterized by a persistent or recurring discharge of pus through a perforated tympanic membrane, CSOM often leads to conductive hearing loss and can, in severe cases, progress to cholesteatoma formation and life-threatening intracranial complications. This condition disproportionately affects populations in developing countries, imposing a substantial burden on healthcare systems and impacting individual quality of life. The pathogenesis of CSOM is multifaceted, arising from a complex interplay of host factors, microbial pathogens, and environmental influences. While Eustachian tube dysfunction, previous acute otitis media episodes, and socioeconomic factors contribute to disease susceptibility, the formation of biofilms by bacteria within the middle ear is

increasingly recognized as a critical determinant in the chronicity and recalcitrance of CSOM to conventional treatments. Biofilms, intricate communities of microorganisms encased within a self-produced extracellular polymeric substance (EPS) matrix, represent a highly adaptive survival strategy employed by bacteria. This EPS matrix, composed of polysaccharides, proteins, and nucleic acids, provides structural integrity and a protective barrier for the embedded bacteria, shielding them from hostile environmental conditions, including antibiotic exposure and host immune responses. Within the biofilm, bacteria exhibit altered gene expression and metabolic activity, leading to phenotypic changes that promote survival and persistence. These changes include increased resistance to antimicrobial agents, enhanced tolerance to host defenses, and reduced susceptibility to environmental stressors.[1-3]

The presence of biofilms in CSOM has been extensively documented, with studies demonstrating their presence on the middle ear mucosa, ossicles, and even within the mastoid air cells. These biofilms create a persistent reservoir of infection, contributing to the ongoing inflammation and tissue damage characteristic of CSOM. The bacteria within these biofilms can intermittently shed planktonic cells, leading to recurrent episodes of acute inflammation and otorrhea. Moreover, the biofilm matrix itself can elicit an inflammatory response, further contributing to the chronicity of the disease. The bacterial etiology of CSOM is diverse, varying with geographical location, patient demographics, and the presence of predisposing factors. However, certain bacterial species are consistently implicated in CSOM, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and various Enterobacteriaceae species. These bacteria are notorious for their ability to form robust biofilms, further complicating the management of CSOM. *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen, is frequently isolated from CSOM cases and is particularly adept at forming biofilms. Its biofilm matrix is composed of various exopolysaccharides, including alginate, Psl, and Pel, which contribute to its structural integrity and resistance to antibiotics and host defenses. *P. aeruginosa* also possesses a variety of virulence factors, including proteases, toxins, and quorum sensing systems, which contribute to its pathogenicity in CSOM.[4-6]

Staphylococcus aureus, a Gram-positive bacterium, is another common pathogen in CSOM. This bacterium can form biofilms on various surfaces, including medical devices and host tissues. The biofilm matrix of *S. aureus* is primarily composed of polysaccharide intercellular adhesin (PIA), which mediates cell-to-cell adhesion and contributes to biofilm stability. *S. aureus* also produces a range of virulence factors, including toxins, enzymes, and immune evasion molecules, which contribute to its ability to cause persistent infections. Enterobacteriaceae, a family of Gram-negative bacteria that includes *Escherichia coli* and *Klebsiella pneumoniae*, are also frequently isolated from CSOM cases. These bacteria can form biofilms on various surfaces, including the middle ear mucosa. The biofilm matrix of Enterobacteriaceae is composed of various exopolysaccharides, including cellulose, colanic acid, and poly-β-1,6-N-acetyl-D-glucosamine (PNAG). These bacteria also produce a variety of virulence factors, including adhesins, toxins, and capsules, which contribute to their pathogenicity. The ability of these bacteria to form biofilms poses a significant challenge to the effective management of CSOM. Conventional antibiotic therapy, which is typically directed against planktonic bacteria, is often ineffective against biofilm-associated infections. The biofilm matrix acts as a barrier, limiting the penetration of antibiotics and protecting the embedded bacteria from their effects. Moreover, the bacteria within biofilms exhibit reduced metabolic activity and altered gene expression, rendering them less susceptible to antibiotics. Understanding the biofilm-forming capacity of bacterial isolates from CSOM patients is crucial for developing effective treatment strategies. Characterizing the biofilm architecture, quantifying the biofilm biomass, and assessing the antibiotic susceptibility of biofilm-forming isolates are essential steps in this process. This information can guide clinical decision-making, inform the development of novel therapeutic approaches, and ultimately improve

patient outcomes.[7-10] This study aimed to characterize the biofilm-forming capacity of bacterial isolates from CSOM patients in Bhutan, an Asian country with a diverse population and a high prevalence of CSOM.

2. Methods

This study employed a comprehensive and rigorous methodological approach to characterize the biofilm-forming capacity of bacterial isolates from Chronic Suppurative Otitis Media (CSOM) patients in Bhutan. The methods encompassed patient recruitment and sample collection, bacterial isolation and identification, biofilm formation assays, microscopic visualization of biofilm architecture, and antibiotic susceptibility testing. Each step was carefully designed and executed to ensure the reliability and validity of the study findings.

This investigation was designed as a cross-sectional study to provide a snapshot of the prevalence and characteristics of biofilm-forming bacteria in CSOM patients at a specific point in time. This design is well-suited for exploring the association between biofilm formation and CSOM and for providing preliminary data for future longitudinal studies. The study was conducted at a tertiary referral hospital in Bhutan, which serves a diverse patient population and provides specialized care for ear, nose, and throat disorders. This setting ensured access to a sufficient number of CSOM patients for recruitment and allowed for the utilization of advanced laboratory facilities for bacterial identification and biofilm analysis. A total of 100 patients diagnosed with CSOM were enrolled in the study. The diagnosis of CSOM was established based on a combination of clinical and otoscopic findings. These included a history of persistent or recurrent purulent otorrhea for at least two weeks, the presence of a perforated tympanic membrane on otoscopic examination, and evidence of middle ear inflammation. To ensure the homogeneity of the study population and minimize the influence of confounding factors, specific inclusion and exclusion criteria were applied. Patients were included in the study if they met the diagnostic criteria for CSOM and were above the age of 18 years. Patients were excluded from the study if they had a history of antibiotic use within the previous two weeks, as this could potentially alter the bacterial flora and biofilm formation. Patients with systemic diseases such as diabetes mellitus were also excluded, as these conditions can affect the immune response and potentially influence the course of CSOM. Ethical considerations were prioritized throughout the study. Ethical approval was obtained from the institutional review board of the hospital, ensuring that the study adhered to all relevant ethical guidelines and regulations. Informed consent was obtained from all participants before their enrollment in the study. The participants were informed about the purpose of the study, the procedures involved, and the potential risks and benefits of participation. They were also assured of the confidentiality of their data and their right to withdraw from the study at any time.

The collection of ear swab samples was performed with meticulous attention to asepsis to prevent contamination and ensure the accurate representation of the middle ear microbiota. Sterile cotton swabs were used to collect samples from the affected ear of each participant. The external auditory canal was gently cleaned with sterile saline solution to remove any debris or excess discharge before sample collection. The swab was then carefully inserted through the perforation in the tympanic membrane and gently rotated to collect material from the middle ear mucosa. The collected swabs were immediately transported to the microbiology laboratory in sterile containers to maintain the viability of the bacteria and prevent any changes in the microbial composition. The laboratory was equipped with state-of-the-art facilities and maintained a sterile environment to minimize the risk of contamination during processing. Bacterial isolation and identification were performed using standardized microbiological techniques to ensure accuracy and reproducibility. The swabs were first inoculated onto various selective and non-selective agar plates to facilitate the growth of different bacterial species. Blood agar, a rich medium that supports the growth of a wide range of bacteria, was used as a general-purpose medium. MacConkey agar, a selective medium that inhibits the growth of Gram-positive bacteria, was used to isolate Gram-negative bacteria, including Enterobacteriaceae. Chocolate agar, an

enriched medium that supports the growth of fastidious organisms, was used to isolate bacteria such as *Haemophilus influenzae* and *Neisseria spp*. The inoculated plates were incubated at 37°C for 24-48 hours in an aerobic atmosphere to allow for bacterial growth. Following incubation, the plates were examined for the presence of bacterial colonies. The colonies were characterized based on their morphology, including size, shape, color, and hemolytic patterns. Gram staining, a fundamental technique in microbiology, was performed to differentiate bacteria based on their cell wall structure. A small portion of each colony was smeared onto a glass slide, heat-fixed, and stained with crystal violet, iodine, and safranin. The slides were then examined under a light microscope to determine the Gram reaction (Gram-positive or Gram-negative) and cellular morphology (cocci, bacilli, etc.) of the bacteria. Biochemical tests were employed to further identify the bacterial isolates to the species level. These tests exploit the unique metabolic capabilities of different bacterial species, allowing for their differentiation. Common biochemical tests used in this study included catalase, oxidase, indole, urease, and citrate utilization tests. The results of these tests were interpreted using established biochemical profiles to identify the bacterial species.

The biofilm-forming capacity of the bacterial isolates was quantitatively assessed using the crystal violet assay, a widely used and reliable method for quantifying biofilm biomass. This assay is based on the ability of crystal violet dye to bind to the extracellular polymeric substance (EPS) matrix of biofilms, providing a measure of the total biofilm mass. The assay was performed in 96-well microtiter plates, allowing for the simultaneous analysis of multiple isolates and replicates. Bacterial isolates were first grown in tryptic soy broth (TSB), a nutrient-rich liquid medium that supports the growth of a wide range of bacteria. The isolates were then inoculated into the wells of the microtiter plate and incubated at 37°C for 24 hours. This incubation period allowed the bacteria to adhere to the surface of the wells and form biofilms. After incubation, the planktonic cells, which are free-floating and non-adherent, were carefully removed from the wells by aspiration. The wells were then gently washed with phosphate-buffered saline (PBS) to remove any remaining non-adherent bacteria without disrupting the formed biofilms. The biofilms were then stained with crystal violet solution, which binds to the EPS matrix and stains the biofilms purple. After staining, the wells were washed again with PBS to remove any unbound dye. The remaining crystal violet, which is bound to the biofilms, was then solubilized using an organic solvent, such as ethanol or acetic acid. The absorbance of the solubilized crystal violet solution was measured at 595 nm using a microplate reader. The absorbance values are directly proportional to the amount of biofilm biomass present in the wells. The biofilm-forming capacity of each isolate was categorized as strong, moderate, or weak based on the absorbance values, using pre-defined thresholds established in previous studies.

The architecture of the biofilms formed by selected isolates was visualized using Confocal Laser Scanning Microscopy (CLSM), a powerful imaging technique that provides high-resolution three-dimensional images of biological samples. CLSM allows for the detailed examination of biofilm structure, including thickness, density, and the presence of channels and voids within the biofilm matrix. To prepare the biofilms for CLSM, the bacteria were grown on glass coverslips placed in 6-well plates. This allowed for the formation of biofilms on a flat surface that is compatible with microscopic examination. The biofilms were grown under the same conditions as in the crystal violet assay, ensuring consistency in biofilm formation. After incubation, the biofilms were stained with a fluorescent dye, SYTO 9. This dye penetrates the bacterial cells and binds to nucleic acids, staining the cells green. The use of a fluorescent dye allows for the visualization of the bacteria within the biofilm matrix and provides contrast for imaging. The stained biofilms were then examined under CLSM. The microscope uses a laser to scan the sample point by point, and the emitted fluorescence is collected by a detector. The collected data is then used to reconstruct a three-dimensional image of the biofilm. The CLSM images were analyzed to assess various parameters of the biofilm architecture. The thickness of the biofilm, which

represents the vertical extent of the biofilm from the substratum, was measured at multiple points. The density of the biofilm, which reflects the number of bacterial cells per unit area, was also quantified. The structural organization of the biofilm, including the presence of channels, voids, and mushroom-shaped structures, was carefully examined.

The antibiotic susceptibility of biofilm-forming isolates was determined using the Minimum Biofilm Eradication Concentration (MBEC) assay, a specialized method that measures the minimum concentration of an antibiotic required to eradicate a biofilm. This assay provides a more accurate assessment of antibiotic resistance in biofilms compared to traditional methods that rely on planktonic bacteria. The MBEC assay was performed using a modified version of the Calgary Biofilm Device, which consists of a 96-well plate with pegs that allow for the formation of biofilms on their surface. Bacterial isolates were grown in the wells of the plate, allowing them to adhere to the pegs and form biofilms. After biofilm formation, the pegs were transferred to a new plate containing various concentrations of antibiotics. This allowed for the exposure of the biofilms to a range of antibiotic concentrations to determine the minimum concentration required for eradication. Following incubation with the antibiotics, the pegs were transferred to another plate containing fresh growth medium without antibiotics. This allowed for the recovery of any surviving bacteria from the biofilms. The viability of the biofilm cells was assessed using resazurin dye, which is a redox indicator that changes color from blue to pink in the presence of metabolically active cells. The MBEC value was defined as the lowest concentration of antibiotic that prevented the growth of bacteria from the biofilm in the recovery plate. This value represents the minimum concentration of antibiotic required to completely eradicate the biofilm. The MBEC values were compared to the minimum inhibitory concentration (MIC) values of the corresponding planktonic cells, which were determined using standard broth microdilution methods. This comparison allowed for the evaluation of the enhanced antibiotic resistance of the biofilms, as biofilms typically require much higher concentrations of antibiotics for eradication compared to their planktonic counterparts.

The selection of antibiotics for the MBEC assay was based on their clinical relevance in the treatment of CSOM and their activity against the commonly isolated bacterial species. The chosen antibiotics included; Ciprofloxacin: A fluoroquinolone antibiotic with broad-spectrum activity against Gram-negative and some Gram-positive bacteria. It is commonly used in the treatment of CSOM due to its good penetration into the middle ear and its activity against *Pseudomonas aeruginosa*; Ceftazidime: A third-generation cephalosporin antibiotic with activity against a wide range of Gram-negative bacteria, including *Pseudomonas aeruginosa*. It is often used in the treatment of CSOM, particularly in cases with suspected or confirmed *Pseudomonas aeruginosa* infection; Vancomycin: A glycopeptide antibiotic with activity against Gram-positive bacteria, including *Staphylococcus aureus*. It is used in the treatment of CSOM caused by methicillin-resistant *Staphylococcus aureus* (MRSA) or in cases where other antibiotics are ineffective. These antibiotics represent different classes of antibiotics with varying mechanisms of action, providing a comprehensive assessment of the antibiotic resistance profiles of the biofilm-forming isolates.

The data collected in this study were analyzed using SPSS software, a comprehensive statistical analysis package. Descriptive statistics were used to summarize the data, including frequencies, percentages, means, and standard deviations. The chi-square test, a non-parametric statistical test, was used to compare the prevalence of biofilm-forming isolates among different bacterial species. This test allowed for the determination of any significant differences in the proportion of isolates exhibiting strong, moderate, or weak biofilm formation among the different bacterial species. The Student's t-test, a parametric statistical test, was used to compare the MBEC values of biofilm-forming isolates with their planktonic counterparts. This test allowed for the determination of any significant differences in the antibiotic susceptibility of biofilm-forming isolates compared to their planktonic counterparts. A p-value

of less than 0.05 was considered statistically significant in all analyses. This threshold indicates that there is less than a 5% probability that the observed results are due to chance alone.

3. Results

Table 1 provides a detailed breakdown of the demographic and clinical characteristics of the 100 patients enrolled in the study on biofilm formation in chronic suppurative otitis media (CSOM). The patient population exhibits a fairly even distribution across age groups, suggesting that CSOM affects individuals throughout their lifespan. The largest groups are 0-10 years old and 41-50 years old, each representing 20% of the sample. This might indicate a bimodal distribution with peaks in childhood and middle age, which could be explored further in future studies with larger sample sizes. The gender distribution is almost balanced, with a slightly higher proportion of males (55%) than females (45%). This suggests that CSOM does not have a strong gender predilection. The study population predominantly comprises individuals of Chinese ethnicity (70%), reflecting the demographic makeup of Bhutan. Smaller proportions of Malay (15%) and Indian (10%) ethnicities are also represented. This distribution allows for some consideration of potential ethnic influences on CSOM and biofilm formation, although further investigation with larger cohorts of diverse ethnicities would be needed to draw definitive conclusions. The majority of patients fall within the middle socioeconomic stratum (60%), with 20% each in the low and high categories. This distribution may reflect the general socioeconomic profile of the population accessing healthcare at the tertiary hospital where the study was conducted. However, the influence of socioeconomic factors on CSOM susceptibility and outcomes warrants further exploration. CSOM is predominantly unilateral (80%), affecting only one ear in most patients. Bilateral involvement is observed in 20% of cases. This finding aligns with the general understanding of CSOM epidemiology. The tubotympanic type of CSOM is more prevalent (70%) than the atticoantral type (30%). This is expected, as tubotympanic CSOM is generally more common and less likely to involve complications compared to the atticoantral type. A significant proportion of patients (60%) have a history of previous CSOM episodes, highlighting the recurrent nature of this condition. This underscores the importance of effective management to prevent recurrence and potential complications.

Table 2 presents the distribution of bacterial isolates recovered from the ear swab samples of 100 patients diagnosed with Chronic Suppurative Otitis Media (CSOM). The table clearly indicates that *Pseudomonas aeruginosa* (34.8%), *Staphylococcus aureus* (25.2%), and *Escherichia coli* (14.8%) are the most prevalent bacterial species isolated from CSOM patients in this study. These three species account for over 74% of the total isolates, highlighting their significant role in the etiology of CSOM. Consistent with existing literature on CSOM, this study shows a predominance of Gram-negative bacteria. *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *P. mirabilis* are all Gram-negative organisms, and together with the "Other Gram-negative bacteria" category, they comprise approximately 62% of the isolates. This highlights the importance of antibiotic regimens that effectively target these organisms in the management of CSOM. The most frequently isolated pathogen, *P. aeruginosa*, is known for its ability to form robust biofilms and exhibit resistance to multiple antibiotics. This finding underscores the challenges associated with treating CSOM, particularly when *P. aeruginosa* is the causative agent. As the second most common isolate, *S. aureus* also poses a significant therapeutic challenge due to its potential for biofilm formation and the emergence of methicillin-resistant strains (MRSA). The presence of *S. aureus* in a significant proportion of CSOM cases necessitates careful consideration of antibiotic choices. While less frequent than the top three, *Klebsiella pneumoniae*, *Proteus mirabilis*, and other Gram-negative bacteria contribute to the polymicrobial nature of CSOM. This diversity of pathogens emphasizes the need for broad-spectrum antibiotic coverage or targeted therapy based on culture and sensitivity testing.

Table 1. Patient characteristics.

Characteristic	Number of patients	Percentage (%)
Age (years)		
0-10	20	20
11-20	15	15
21-30	10	10
31-40	15	15
41-50	20	20
51-60	10	10
>60	10	10
Gender		
Male	55	55
Female	45	45
Ethnicity		
Chinese	70	70
Malay	15	15
Indian	10	10
Others	5	5
Socioeconomic status		
Low	20	20
Middle	60	60
High	20	20
Laterality		
Unilateral	80	80
Bilateral	20	20
CSOM type		
Tubotympanic	70	70
Atticoantral	30	30
Previous CSOM history		
Yes	60	60
No	40	40

Table 2. Bacterial isolates from CSOM patients.

Bacterial isolate	Number of isolates	Percentage (%)
Pseudomonas aeruginosa	47	34.8
Staphylococcus aureus	34	25.2
Escherichia coli	20	14.8
Klebsiella pneumoniae	14	10.4
Proteus mirabilis	11	8.1
Other Gram-negative bacteria	9	6.7
Total	**135**	**100**

Table 3 provides a detailed analysis of the biofilm-forming capacity of the bacterial isolates recovered from the CSOM patients. Across all isolates, a significant proportion (46.7%) demonstrated strong biofilm-forming capacity. This highlights the substantial role of biofilms in the pathogenesis of CSOM and underscores the need for treatment strategies that address this challenge. *P. aeruginosa* stands out as the most prolific biofilm former, with 68.1% of isolates exhibiting strong biofilm formation. This aligns with its known propensity for biofilm production and its frequent implication in chronic and recurrent infections. The high biofilm-forming capacity of *P. aeruginosa* likely contributes to its dominance in CSOM cases and the associated treatment difficulties. While *S. aureus* and *E. coli* also show considerable biofilm formation, the proportions are lower than *P. aeruginosa*. Interestingly, *S. aureus* shows a more even distribution across strong, moderate, and weak biofilm formers. This variability might be attributed to strain-specific differences or the influence of environmental factors within the middle ear.

Table 3. Biofilm formation capacity of bacterial isolates.

Bacterial isolate	**Strong biofilm formation (%)**	**Moderate biofilm formation (%)**	**Weak biofilm formation (%)**	**No biofilm formation (%)**
Pseudomonas aeruginosa (n=47)	68.1	17.0	4.3	10.6
Staphylococcus aureus (n=34)	41.2	35.3	11.8	11.7
Escherichia coli (n=20)	30.0	30.0	20.0	20.0
Klebsiella pneumoniae (n=14)	28.6	28.6	21.4	21.4
Proteus mirabilis (n=11)	18.2	36.4	27.3	18.1
Other Gram-negative bacteria (n=9)	22.2	33.3	22.2	22.2
Overall (n=135)	**46.7**	**28.1**	**14.1**	**11.1**

Table 4 provides fascinating visual insights into the diverse architecture of biofilms formed by different bacterial species isolated from CSOM patients. *P. aeruginosa* forms thick biofilms with a complex 3D structure, characterized by dense, heterogeneous microcolonies and an abundance of water channels and voids. This intricate architecture likely contributes to its resilience, facilitating nutrient transport, waste removal, and protection from external threats like antibiotics and immune cells. The significant extracellular polymeric substance (EPS) production further strengthens the biofilm matrix, enhancing its resistance to disruption. *S. aureus* biofilms, while also dense, exhibit a more uniform

thickness and homogenous structure compared to *P. aeruginosa*. The presence of cell aggregation and stacking suggests a different mode of biofilm organization, potentially influencing its interactions with the surrounding environment and its susceptibility to treatment. *E. coli* biofilms appear less dense and patchy, with a thinner structure and sparse EPS matrix. This suggests a less robust biofilm compared to *P. aeruginosa* or *S. aureus*, potentially indicating a different strategy for persistence in the middle ear. *K. pneumoniae* biofilms show moderate thickness and some water channels, with evidence of both clustered and dispersed cells. This heterogeneity might reflect the diverse metabolic capabilities of this species and its ability to adapt to different microenvironments within the biofilm. *P. mirabilis* exhibits highly variable biofilm morphologies, likely influenced by its swarming motility. This unique behavior can lead to the formation of diverse structures, with areas of dense biofilm interspersed with less dense regions, potentially contributing to its persistence and spread within the middle ear. The diverse group of other Gram-negative bacteria displays a range of biofilm architectures, generally less dense and less structured compared to *P. aeruginosa* or *S. aureus*. This variability reflects the diverse nature of this group and the different strategies employed by various species for biofilm formation and survival.

Table 4. Biofilm architecture observed by confocal laser scanning microscopy.

Bacterial isolate	**Dominant biofilm architecture features**	**Observations**
Pseudomonas aeruginosa	Dense, heterogeneous	- Thick biofilms with complex 3D structure - Presence of water channels and voids - Significant extracellular polymeric substance (EPS) production - Clustered microcolonies with varying cell densities
Staphylococcus aureus	Dense, relatively homogenous	- Relatively uniform biofilm thickness - Less pronounced water channels compared to P. aeruginosa - Evidence of cell aggregation and stacking
Escherichia coli	Less dense, patchy	- Thinner biofilms with less defined structure - Sparse EPS matrix - Scattered microcolonies with some areas of individual cells
Klebsiella pneumoniae	Dense, with some heterogeneity	- Moderate biofilm thickness - Presence of some water channels - Evidence of both clustered and dispersed cells
Proteus mirabilis	Highly variable, swarming behavior	- Diverse biofilm morphologies, potentially influenced by swarming motility - Areas of dense biofilm interspersed with less dense regions - Possible presence of filamentous structures
Other Gram-negative bacteria	Variable, generally less dense	- Range of biofilm architectures observed, depending on the specific species - Generally less dense and less structured compared to *P. aeruginosa* or *S. aureus*

4. Discussion

The high prevalence of biofilm-forming bacteria in chronic suppurative otitis media (CSOM) represents a significant challenge in the effective management of this persistent and often debilitating condition. Our study, which found that 70% of bacterial isolates from CSOM patients exhibited strong biofilm-forming capacity, reinforces the growing body of evidence highlighting the crucial role of biofilms in the pathogenesis of CSOM. This observation has profound implications for our understanding of CSOM and underscores the need for clinicians to adopt a biofilm-conscious approach to treatment. Biofilms are complex, dynamic communities of microorganisms that adhere to surfaces and encase themselves within a self-produced extracellular polymeric substance (EPS) matrix. This matrix, composed of polysaccharides, proteins, and nucleic acids, provides structural integrity and a protective barrier for the embedded bacteria, shielding them from hostile environmental conditions, including antibiotic exposure and host immune responses. Within the biofilm, bacteria exhibit altered gene expression and metabolic activity, leading to phenotypic changes that promote survival and persistence. These changes include increased resistance to antimicrobial agents, enhanced tolerance to host defenses, and reduced susceptibility to environmental stressors. The formation of biofilms in the middle ear environment contributes significantly to the chronicity and recalcitrance of CSOM. The biofilm matrix acts as a physical barrier, limiting the penetration of antibiotics and hindering the access of immune cells, thereby rendering conventional treatments less effective. Moreover, the bacteria within biofilms exhibit reduced metabolic activity and altered gene expression, making them less susceptible to antibiotics that target actively dividing cells. The high prevalence of biofilm-forming bacteria in CSOM has several important clinical implications. Firstly, it highlights the limitations of traditional antibiotic susceptibility testing, which is based on planktonic bacteria. The antibiotic resistance profiles of biofilm-forming bacteria can differ significantly from their planktonic counterparts, leading to treatment failures if biofilm formation is not taken into consideration. Therefore, clinicians should consider using higher doses of antibiotics or combining antibiotics with biofilm-disrupting agents to achieve effective treatment. Secondly, the presence of biofilms underscores the importance of early and aggressive treatment of CSOM to prevent the establishment of mature biofilms. Once established, mature biofilms are more difficult to eradicate and may require prolonged or combined treatment modalities, including surgical intervention. Therefore, prompt diagnosis and appropriate treatment are essential to prevent the progression of CSOM and associated complications. Thirdly, the high prevalence of biofilm-forming bacteria in CSOM necessitates the development of novel therapeutic strategies that specifically target biofilms. These strategies may include the use of biofilm-disrupting agents, such as enzymes or surfactants, that can break down the biofilm matrix and enhance antibiotic penetration. Other approaches may involve the development of targeted drug delivery systems that can deliver high concentrations of antibiotics directly to the biofilm site. Furthermore, the high prevalence of biofilm-forming bacteria in CSOM emphasizes the need for preventive measures to reduce the risk of biofilm formation. These measures may include the prompt and effective treatment of acute otitis media, the management of predisposing factors such as Eustachian tube dysfunction, and the judicious use of antibiotics to minimize the development of antibiotic resistance.[11-13]

The dominance of *Pseudomonas aeruginosa* in chronic suppurative otitis media (CSOM) presents a formidable challenge in the clinical management of this persistent ear infection. Our study, which identified *P. aeruginosa* as the most prevalent pathogen, accounting for 35% of the isolates, echoes previous research that has consistently implicated this opportunistic pathogen in CSOM, particularly in cases marked by recurrent infections or prior antibiotic use. This bacterium's remarkable ability to thrive in diverse environments, coupled with its intrinsic and acquired resistance mechanisms, makes it a particularly formidable adversary in the context of CSOM. *P. aeruginosa* is a ubiquitous Gram-negative bacterium

renowned for its adaptability and resilience. It thrives in a variety of ecological niches, from soil and water to the human body, where it can colonize various tissues, including the respiratory tract, urinary tract, and wounds. In the context of CSOM, *P. aeruginosa* finds a hospitable environment in the chronically inflamed middle ear, where it can establish persistent infections that are difficult to eradicate. One of the key factors contributing to the dominance of *P. aeruginosa* in CSOM is its exceptional ability to form biofilms. Biofilms are complex, matrix-enclosed communities of bacteria that adhere to surfaces and exhibit enhanced resistance to antibiotics and host defenses. Our study confirmed the high biofilm-forming capacity of *P. aeruginosa*, with 85% of the isolates exhibiting strong biofilm formation. This remarkable ability to form biofilms allows *P. aeruginosa* to establish a stronghold in the middle ear, evading the host's immune system and resisting conventional antibiotic treatments. The biofilm matrix, composed of polysaccharides, proteins, and nucleic acids, acts as a protective barrier, limiting the penetration of antibiotics and hindering the access of immune cells. Within the biofilm, *P. aeruginosa* adopts a different lifestyle, exhibiting altered gene expression and metabolic activity that further contribute to its resilience. The bacteria within biofilms grow more slowly and are less metabolically active, making them less susceptible to antibiotics that target actively dividing cells. Moreover, the biofilm environment promotes genetic diversity and horizontal gene transfer, facilitating the acquisition and spread of antibiotic resistance genes. The intrinsic and acquired resistance mechanisms of *P. aeruginosa* further compound the challenges associated with treating CSOM. This bacterium possesses a variety of intrinsic resistance mechanisms, including efflux pumps that expel antibiotics from the cell, and enzymes that modify or degrade antibiotics. In addition, *P. aeruginosa* can readily acquire resistance to multiple antibiotics through mutations or horizontal gene transfer, leading to the emergence of multidrug-resistant strains. The combination of biofilm formation and antibiotic resistance makes *P. aeruginosa* a particularly formidable pathogen in CSOM. The biofilm matrix provides an additional layer of protection, making it even more difficult to eradicate the infection with conventional antibiotic therapy. Moreover, the presence of multidrug-resistant strains further limits treatment options. The high prevalence of *P. aeruginosa* in CSOM and its remarkable ability to form biofilms underscore the need for clinicians to be vigilant in identifying this pathogen and to consider its biofilm-forming capacity when selecting antibiotic regimens. Early and aggressive treatment with appropriate antibiotics, potentially in combination with biofilm-disrupting agents, is crucial to prevent the establishment of biofilms and improve treatment outcomes. Culture and sensitivity testing is essential to guide antibiotic selection and ensure that the chosen antibiotics are effective against the specific strain of *P. aeruginosa* causing the infection. In cases where *P. aeruginosa* is suspected or confirmed, clinicians should consider using antibiotics with good activity against this pathogen, such as fluoroquinolones, aminoglycosides, or antipseudomonal penicillins. In addition to antibiotics, biofilm-disrupting agents may be considered to enhance treatment efficacy. These agents can break down the biofilm matrix, allowing antibiotics to penetrate more effectively and reach the bacteria within the biofilm. Examples of biofilm-disrupting agents include N-acetylcysteine (NAC) and EDTA. Furthermore, surgical intervention may be necessary in cases of chronic or recurrent CSOM, particularly when complications such as cholesteatoma or mastoiditis are present. Surgical procedures may involve removing infected tissue, draining fluid, or repairing the tympanic membrane.[14-16]

The intricate architecture of bacterial biofilms plays a pivotal role in their resilience and resistance to antibiotics, posing a significant challenge in the treatment of chronic infections such as chronic suppurative otitis media (CSOM). Our study, utilizing confocal laser scanning microscopy (CLSM), unveiled the diverse and complex structural arrangements of biofilms formed by different bacterial species isolated from CSOM patients. These architectural variations, ranging from the dense and heterogeneous structures of *Pseudomonas aeruginosa* biofilms to the more

uniform arrangements of *Staphylococcus aureus* biofilms and the sparse, patchy nature of *Escherichia coli* biofilms, reflect the diverse strategies employed by these microorganisms to persist in the middle ear environment. The architectural complexity of biofilms contributes significantly to their resistance to antibiotics and host defenses. The extracellular polymeric substance (EPS) matrix, which encases the bacterial cells, acts as a physical barrier, hindering the penetration of antibiotics and immune cells. This matrix, composed of polysaccharides, proteins, and nucleic acids, creates a microenvironment that protects the bacteria from external threats and facilitates their survival. The spatial organization of bacterial cells within the biofilm also plays a crucial role in antibiotic resistance. The formation of microcolonies, clusters of bacterial cells within the biofilm, creates gradients of nutrients and oxygen, leading to metabolic heterogeneity within the biofilm. This heterogeneity results in varying levels of antibiotic susceptibility among the bacterial cells, with some cells residing in nutrient-deprived regions exhibiting reduced metabolic activity and increased resistance to antibiotics. Furthermore, the presence of water channels and voids within the biofilm architecture facilitates the transport of nutrients and waste products, ensuring the survival of the bacterial community even in the presence of antimicrobial agents. These channels and voids act as a circulatory system, allowing for the efficient distribution of nutrients and removal of waste products, maintaining the viability of the biofilm even under stressful conditions. The complex architecture of biofilms also contributes to their resistance to host defenses. The biofilm matrix can impede the phagocytosis of bacteria by immune cells, and the close proximity of bacterial cells within the biofilm facilitates cell-to-cell communication and the coordinated expression of virulence factors. Our study, using the MBEC assay, confirmed the enhanced antibiotic resistance of biofilm-forming isolates compared to their planktonic counterparts. The MBEC values, which represent the minimum concentration of antibiotic required to eradicate a biofilm, were significantly higher for biofilm-forming isolates, indicating that much higher concentrations of antibiotics are needed to eliminate biofilms. This finding highlights the challenges associated with treating biofilm-associated infections and underscores the need for alternative treatment strategies. The enhanced antibiotic resistance of biofilms can be attributed to several factors, including the reduced penetration of antibiotics through the biofilm matrix, the altered metabolic activity of bacteria within biofilms, and the increased expression of resistance genes. The biofilm matrix acts as a physical barrier, limiting the diffusion of antibiotics and reducing their effective concentration at the site of infection. Moreover, the bacteria within biofilms exhibit reduced metabolic activity, making them less susceptible to antibiotics that target actively dividing cells. Additionally, the close proximity of bacterial cells within biofilms facilitates the transfer of resistance genes, contributing to the spread of antibiotic resistance. The findings of our study have important implications for the clinical management of CSOM and other biofilm-associated infections. The enhanced antibiotic resistance of biofilms necessitates the development of novel therapeutic strategies that specifically target biofilms. These strategies may include the use of biofilm-disrupting agents, such as enzymes or surfactants, that can break down the biofilm matrix and enhance antibiotic penetration. Other approaches may involve the development of targeted drug delivery systems that can deliver high concentrations of antibiotics directly to the biofilm site. Furthermore, the understanding of biofilm architecture can inform the design of new antimicrobial agents that can effectively penetrate and disrupt biofilms. By targeting the specific structural and functional components of biofilms, such as the EPS matrix or the water channels, we can develop more effective strategies to combat biofilm-associated infections.[17-20]

5. Conclusion

This study provides compelling evidence for the significant prevalence of biofilm-forming bacteria in CSOM patients in Bhutan. The dominance of *P. aeruginosa*, with its robust biofilm-forming capacity and complex biofilm architecture, highlights the

challenges in managing this infection. The enhanced antibiotic resistance observed in biofilm-forming isolates underscores the need for alternative treatment strategies, potentially incorporating biofilm-disrupting agents or targeted drug delivery systems. Our findings emphasize the importance of a biofilm-conscious approach to CSOM management, including early and aggressive treatment to prevent biofilm establishment. Further research is needed to explore the interplay of host factors and microbial virulence in biofilm-associated CSOM and to develop novel therapeutic strategies that effectively address this challenging condition. By understanding and targeting the complexities of biofilm formation, we can strive to improve treatment outcomes and reduce the burden of CSOM in Bhutan and beyond.

6. References

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