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"To Study the Molecular Characterization of Metallo-Beta Lactamase Gene bla_{IMP-1} in Imipenem Resistant *Pseudomonas aeruginosa* isolates from Patients of Chronic Suppurative Otitis Media"

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ABSTRACT

Introduction: A chronic inflammation of the middle ear and mastoid cavity that lasts more than two weeks is known as chronic suppurative otitis media (CSOM). A common organism that causes CSOM is *pseudomonas aeruginosa*. Although carbapenems are among the best medicines for treating Pseudomonas infections, the development of metallo-ß-lactamases strains is frequently linked to carbapenem resistance. Finding strains that produce MBLs can help ensure that patients receive the best care possible to stop the development of resistance. Finding the imipenem-resistant *Pseudomonas aeruginosa* carrying the metallo-ß-lactamase (MBL) geneblaIMP-1 is the primary goal of the research.

Aim and Objective: To study the Molecular Characterization of Metallo-Beta Lactamase Gene bla_{IMP-1} in Imipenem Resistant *Pseudomonas aeruginosa* isolates from Patients of Chronic Suppurative Otitis Media.

Material and Methods: This was a cross sectional study carried out in the Department of Microbiology and ENT Department for a period of 1 year i.e, May 2023 to May 2024. A total of

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200 patients clinically suspected cases for CSOM was studied. Swabs taken from discharging ears were sent for Gram's staining, culture and antibiotic sensitivity test as per the latest CLSI guidelines 2023. The DNA was extracted by using Qiagen DNA Extraction kit and bla_{IMP-1} gene for *Pseudomonas aeruginosa* isolates was detected by conventional PCR.

Results: In the present study the number of cases clinically diagnosed of having CSOM were 200, out of which 70 (35%) was found to be culture positive for CSOM infection. Males were 44 (62.8%) as compared to that of female 26 (37.1%), the age group of 0-10 years followed by 11-20 years were being affected the most and the least number of cases was seen in the age group above 51 years. The side of the ear affected was almost in equal distribution, with the left ear being (51.4%) and the right ear being (40%) while (8.5%) were bilateral. In our study it was observed that the maximum number of cases was found in Gram negative isolates (98.5%) as compared to

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the Gram positive isolates (14.2%). It was also observed that 62 isolates (88.5%) samples showed growth of single isolates while 8 (11.4%) were mixed isolates.
Pseudomonas aeruginosa being the most common isolate with 42.8% followed by *Klebsiella sp*. with 21.4% and among gram positive isolates *Staphylococcus aureus* was 11.4% and *Streptococcus pneumonia* (2.8%) being the least observed.

The sensitivity observed in *P. aeruginosa* for Colistin was (97.1%), Piperacillin-tazobactam (78.5%), Amikacin (82.8%), and cefepime (78.5%) were found to be the most effective Antibiotics. The resistance to ciprofloxacin was (56.6%), Levofloxacin (50%), Piperacillin(46.6%), Gentamicin(37.1%), Imipenem (31.4%), Tobramycin(31.4%), Ceftazidime (31.4%) and Gentamycin (37.1%). The bla_{IMP-1} gene was detected in 14 (20%) of the isolates of *Pseudomonas aeruginosa*

Conclusion: *Pseudomonas aeruginosa* was the most frequently isolated strain in the current investigation, and the most effective antibiotics were cefepime, amikacin, piperacillin-tazobactam, and colonistin, ciprofloxacin and levofloxacin were the least effective. The Kanpur region (%) is seeing an increase in *P. aeruginosa* isolate resistance to imipenem as a result of MBL enzymes. Understanding the CSOM etiological agents and their antibiogram is crucial for effective treatment and prevention of antimicrobial resistance as well as clinical consequences.

Keywords: CSOM, bla_{IMP-1}, Imipenem, DNA, PCR

INTRODUCTION

An inflammation of the middle ear cleft with or without an intact tympanic membrane is known as otitis media. It is well recognised as one of the most prevalent paediatric infections and a major contributor to the prescription of antibiotics in the developed world. Hippocrates first mentioned it around 450 B.C., and it's still one of the most puzzling children medical issues that's commonly noticed today, as well as the main contributor to hearing loss [1].

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In a developing nation like India, particularly among those with lower socioeconomic standing, it is a prevalent health issue. In India, chronic suppurative otitis media is also among the most prevalent ear conditions. It is the leading cause of deafness in India and requires a significant amount of otolaryngologists' clinical and surgical work. Depressingly, it is not uncommon in rural India for a benign upper respiratory infection (URI) to proceed to acute otitis media with perforation and recurring/persistent infection (CSOM), which can lead to hearing loss, even though it is very avoidable [2,3].

Nearly 90% of CSOM is mostly seen in younger children less than 2 years of age but its occurrence may also be seen in adults [4,5]. The vulnerability of CSOM in relation to aetiopathogenesis is due to the involvement of multiple factors such as demographic, genetic, environmental and other health related factors like infections, allergy, asthma, eustachian tube dysfunction, cleft palate, and adenoid hypertrophy etc [6-8]. The presence of fluid in middle ear leads to long term morbidity with varying degrees of hearing loss in children and adults [9-11].

CSOM differs from chronic serous otitis media in that chronic serous otitis media may be defined as a middle ear effusion without perforation that is reported to persist for more than 1-3 months. Generally, microbiological culture of the ear discharge simplicates *Pseudomonas aeruginosa*, *Proteus* spp and *Staphylococcus* as the prevalent causative organism [12].

Pseudomonas aeruginosa, is one of the most common organisms to cause CSOM. Among the beta lactams, carbapenems are considered as the potent drug of choice for serious treatment of gram-negative bacteria infections. The most effective antibiotics that can be used against *Pseudomonas aeruginosa* are ß- lactam antibiotics in which imipenem as a carbapenem is considered as the most appropriate antibiotic to be used against the mentioned organisms.

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Worldwide, the prevalence of gram negative bacteria with multi-drug resistance profiles is now recognized. Carbapenem resistance occurs because of the production of carbepenemases as there is decrease in antibiotics absorption. Carbapenemases acquires resistance belongs to Ambler

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molecular classes A, B and D. Metallo–beta-lactamases (MBL) enzymes are the most significant carbapenemases [13].

Nowadays the emergence of antibiotic resistance strains is one of the challenges in treating patients, such as MBLs producing *Pseudomonas aeruginosa* [14].
The VIM, IMP and SPM types are the most clinically significant carbapenemases which is encoded by *bla*IMP, *bla*VIM and *bla*SPM genes [15].

MBLs in Carbapenem-resistant Pseudomonas aeruginosa can be detected by different phenotypic methods and these methods are based on the ability of metal chelators to inhibit the activity of MBLs such as EDTA and thiol-based compounds. 9 These include Combined Disk Test (CDT) 9 using EDTA with imipenem (IPM), Modified Hodge test (MHT), MBL Epsilometer test (E-test) and EDTA disk potentiation test . A PCR detection assay is considered as a gold standard method for the detection of MBL producers. Because of the increasing rate of resistance to the carbapenems, the treatment of infections produced by MBLs producing P. aeruginosa is becoming critical [16].

Therefore, the present study was undertaken to study the Molecular Characterization of Metallo-Beta Lactamase Genebla_{IMP-1} in Imipenem Resistant *Pseudomonas aeruginosa* isolates from Patients of Chronic Suppurative Otitis Media at a Tertiary care hospital.

MATERIAL AND METHODS

This was a hospital based cross sectional study carried out in the Department of Microbiology and ENT Department at a tertiary care centre, over a period of one year from May 2023 to May 2024. Ethical clearance was duly obtained from the Institute Ethical Committee for conducting the study. A total of 200 patients was included in our study.

Inclusion Criteria

1. All adult patients who consented to participate in the study were included

2. Patients of both sexes.

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3. Patients with complaints of CSOM.

Exclusion Criteria

1. Patients who did not consent to be the part of study.

2. Patients with any other medical problem

Specimen collection and sample processing:

The sample was collected using Pus swab from the external auditory canal and introduced into Amies transport medium bottle and sent for laboratory analysis.The sample was processed to primary gram stain for pus cells and inoculated into Blood agar (Oxoid, UK), and MacConkey agar (Oxoid, UK) and incubated aerobically at 37 °C for 24–48 h.

Screening, Isolation and Identification of organisms

Microscopy and colony characteristics—such as haemolysis on blood agar, changes in the physical appearance of the differential media, and colony morphology—were used to identify the pathogens. Biochemical testing for gram negative isolated bacteria included oxidase, triple sugar iron (TSI), sulphur indole and motility (SIM), urease synthesis, and citrate utilisation [17]. Grame positive isolates were evaluated for catalase and coagulase assays.

Antimicrobial susceptibility testing

Antibiotic susceptibility test of isolated bacteria pathogens was performed using modified Kirby Bauer disc diffusion method according to theclinical and Laboratory Standard Institute (CLSI) guidelines[17].

A colony suspension with concentration equivalent to 0.5 McFarland solution was prepared for each identified isolate and inoculated into Mueller–Hinton-Agar (Oxoid, UK). Appropriate Selected Antibiotic discs were placed onto the media and incubated at 37 °C for 24 h.

Gram positive isolates were tested against Ampicillin (10 µg), Amoxicillin/clavulanate (20/10 µg), Ceftriaxone (30 µg), Gentamycin (10 µg), Ciprofloxacin (5 µg), Trimethoprim/sulfamethoxazole (1.25/23.75 µg), Chloramphenicol (30 µg), Amikacin (17 µg) and Cephalexin (18 µg), Cefoxitin (30µg).

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Gram negative organisms were tested sensitivity to amikacin (AMK, 30 μg), gentamicin (GM, 10 μg), tobramycin (TOB, 10 μg), ceftazidime (CAZ, 30 μg), cefepime (CFP, 50 μg), piperacillin (PIP, 100 μg), PIP/tazobactam (PTZ, 100/10 μg), imipenem (IMP, 10 μg), ciprofloxacin (CIP, 5 μg), and levofloxacin (LFX, 5 μg) by modified Kirby Bauer disc diffusion method using Mueller Hinton agar (MHA) medium.

A suspension of the isolated colonies of each test strain equivalent to a 0.5 McFarland's standard was prepared in sterile normal saline. Briefly, a suspension of each strain was made so that the turbidity was equal to 0.5 McFarland standards and then plated as a lawn culture onto MHA. Antibiotic discs were placed and plates were incubated at 37°C for 18–24 h. Results were interpreted in accordance with CLSI guidelines [17] *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as control strains. Reference stains used for quality control were *Staphylococcus aureus* (American Type Culture Collection; ATCC 25922 and ATCC 29213), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC2785) [17].

Genotypic Detection of bla_{IMP-1} gene in *P. aeruginosa*

The DNA was extracted from *P. aeruginosa* using the Qiagen DNA Extraction Kit as per manufactures guidelines. The DNA was isolated using the Qiagen DNA Extraction Kit as per the manufactures guidelines. The DNA was eluted in 60 μl elution buffer and preserve at -20 °C till PCR analysis. For amplification of the target gene, PCR was carried out in a 50 μL reaction mixture. The primers were purchased from "**Saha gene"** and was reconstituted with sterile double distilled water based on the manufacturer's instruction.

MOLECULAR ANALYSIS

Polymerase Chain Reaction (PCR): After the DNA Extraction, the PCR was done for the gene detection. Polymerase chain reaction (PCR) was carried out for detection of bla_{IMP}, gene on a thermal cycler (Eppendorf, Germany) .The primer pair sequences used in this study and the PCR conditions is described in the below Table no. 1. The DNA extraction was performed and the electrophoresis unit was run where 2% agarose gel was prepared with ethidium bromide. The

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bromophenonol blue dye was used for loading our DNA product which was then visualized in the gel documentation system.

Positive controls used in this test were SPM-1 producing *P. aeruginosa* 16 strain (provided by Prof. Patrick Nordmann), bla_{IMP-1} from *Seratia marcesens* (sequenced by Bioneer company), and bal_{VIM}. *P. aeruginosa* ATCC 27853 was used as a negative control [12].

Figure No. 1: The *bla*$_{IMP}$ primer from the Saha gene

Gene	**Primer sequence**	**Length (bp)**	**Reference**
bla_{IMP-1}	Forward 5'-TGAGCAAGTTATCTGTATTC 3′	740	[12]
	Reverse 5'- TTAGTTGCTTGGTTTTGATG 3		

Table no. 1: The Primer sequence used for the detection of bla_{IMP-1} gene

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PCR Condition					
Primer name	**Denaturing**	**Anneal**	**Extension**	**Cycles**	**Size(bp)**
bla_{IMP-1}	94°C, 60 s	57°C, 60 s	72°C, 2 min	35	740

Table No. 2: The Nucleotide sequences of primers used for detection of metalo-beta lactamase gene [12]

Gel electrophoresis : The Agarose Gel Electrophoresis was performed in order to identify the Purified PCR Product which was previously identified by its amplified DNA fragments. The resulting PCR product was subjected to 1 % agarose gel electrophoresis and visualized by Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories Inc., Hercules, CA, USA). A 1 kb DNA Ladder (Thermo Fisher Scientific ™, Waltham, MA, USA) was used as the marker to evaluate the PCR product of the sample [18].

RESULTS

In the present study the number of ears clinically diagnosed of having CSOM were 200, out of which 70 were found to be positive for CSOM infection with the prevalence rate of 35%. The ratio of Male 44 (62.8%) were found to be more as compared to that of Female 26 (37.1%), which is illustrated in the Table No. 3.

Table 3 : Genderwise Distribution of chronic suppurative otitis media patients

S.N.	**Gender**	**Isolates N=70**	**Percentage (%)**
1.	**Male**	44	62.8%
2.	**Female**	26	37.1%

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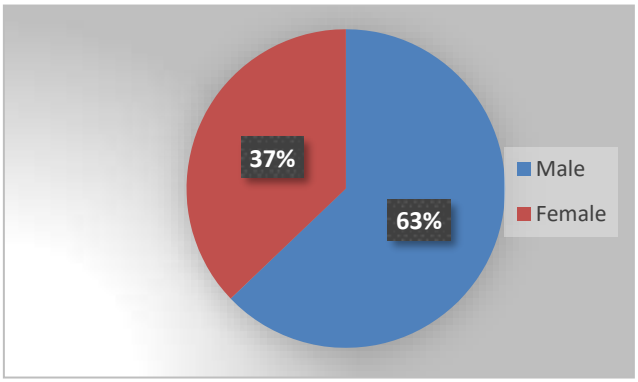

Graph No. 1 Genderwise Distribution of chronic suppurative otitis media patients

The maximum number of cases reported was observed in the age group of 0-10 years followed by 11-20 years of age and the least number of cases was seen in the age group above 51 years of age [Table No. 4].

Table-4 Age wise distribution of the CSOM culture positive isolates

S.N.	Age group (Years)	Male N=44	Female N=26	Percentage (%)
1.	**0-10**	**18**	**13**	**44.2%**
2.	**11-20**	**13**	**5**	**25.7%**
3.	21-30	7	1	11.4%
4.	31-40	1	2	4.2%
5.	41-50	2	2	5.7%
6.	51-60	1	1	2.8%
7.	61-70	2	-	2.8%

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8.	≤ 80	-	2	2.8%

Table 5: Bilateral distribution of chronic suppurative otitis media culture positive patients

S.N.	Gender	Isolates N=70	Percentage (%)
1.	**Left**	36	51.4%
2.	**Right**	28	40%
3.	**Bilateral**	6	8.5%
4.	**Total**	70	100%

The side of the ear affected was almost in equal distribution, with the left ear being (51.4%) and the right ear being (40%) while (8.5%) were bilateral [Table 5].

In our study it was observed that the maximum number of cases was found in Gram negative isolates (98.5%) as compared to the Gram positive isolates (14.2%). It was also observed that 62 isolates (88.5%) samples showed growth of single isolates while 8 (11.4%) were mixed isolates.

Table 6: Distribution of bacterial species associated with chronic suppurative otitis media patients

Bacterial Isolates	No. of Isolates N= 70	Percentage (%)
Staphylococcus aureus	8	11.4%
Streptococcus pneumoniae	2	2.8%
Gram negative bacteria		
Pseudomonas aeruginosa	30	42.8%

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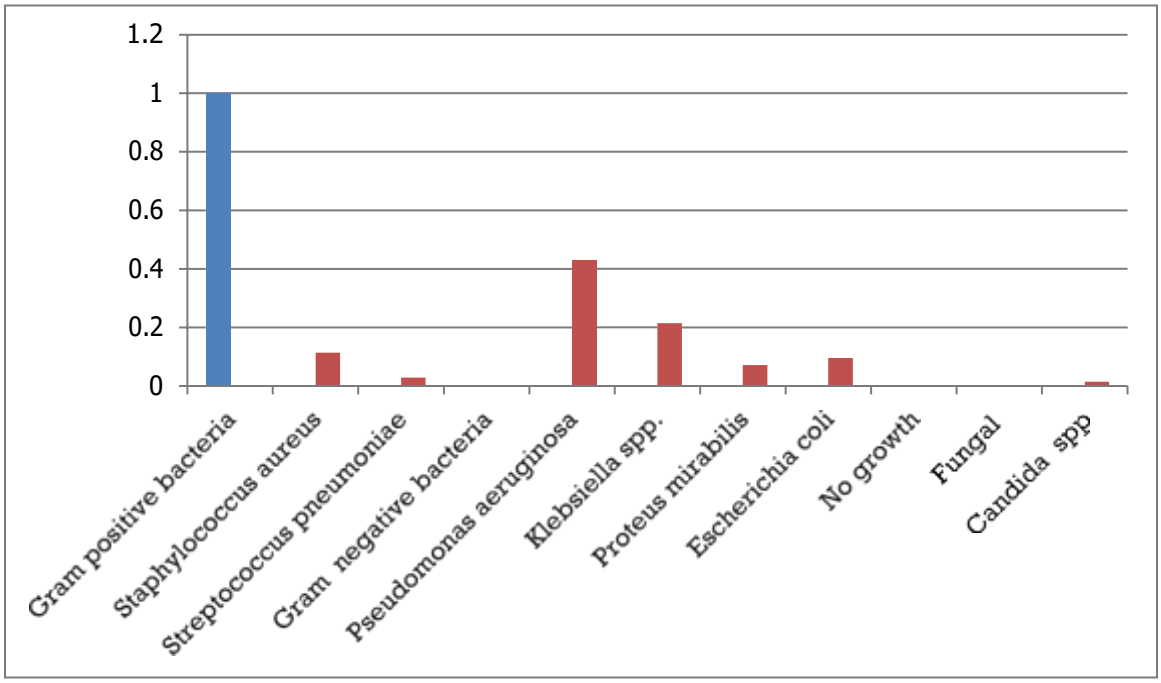
Klebsiella spp.	15	21.4%
Proteus mirabilis	5	7.1%
Escherichia coli	9	12.8%
No growth	130	
Fungal		
Candida spp	1	1.4%



The maximum number of isolates of CSOM was found for *P. aeruginosa* (42.8%) had the highest prevalence followed by *Klebsiella* spp 21.4% and among gram positive isolates *Staphylococcus aureus* was 11.4%.

The sensitivity observed in *P. aeruginosa* for Colistin was (97.1%), Piperacillin-tazobactam (78.5%), Amikacin (82.8%), and cefepime (78.5%) were found to be the most effective Antibiotics.

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The resistance to ciprofloxacin was (56.6%), Levofloxacin (50%), Piperacillin(46.6%), Gentamicin(37.1%), Imipenem (31.4%), Tobramycin(31.4%), Ceftazidime (31.4%) and Gentamycin (37.1%). The bla_{IMP-1} gene was detected in 14 (20%) of the isolates of *Pseudomonas aeruginosa.*

S. aureus showed a 100% sensitivity to Cefoxitin, Vancomycin , Teicoplanin and Linezolid.
S. pneumoniae showed 100% sensitivity to gentamicin, netilmicin, levofloxacin, and ofloxacin.

P. mirabilis showed a sensitivity of 80% sensitivity to levofloxacin and 81.4 % to ofloxacin, respectively, followed by ceftazidime and gentamicin 64.2%, ceftriaxone 58.5%.
E. coli showed a sensitivity of 90% and 92.8% to gentamicinand levofloxacin, respectively, followed by ofloxacin 91.4%,ceftazidime 78.5%, ceftriaxone 78.5%, netilmicin 78.5%, and tetracycline 68.5%.
Klebsiella species showed a sensitivity of 100% to levofloxacin and ofloxacin, respectively, followed by netilmicin, ceftazidime , ceftriaxone with 94.2% , tetracycline and gentamicin with 74.2 %.

The DNA Extraction was performed by the Qiagen DNA kit and the DNA was isolated from the samples.

Fig. 2 The DNA extraction from *P. aeruginosa* isolates

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Fig. 3 The amplified bla$_{IMP}$ gene in *P. aeruginosa* ; the amplified DNA band size was obtained 740bp, , Lane 1 and L 3 is the sample positive for bla$_{IMP;}$ L2 corresponding to 100bp DNA ladder ; L4 corresponds to the Negative control and L5 corresponds to the Positive control

DISCUSSION

Chronic suppurative otitis media (CSOM) is one of the most common childhood infectious diseases worldwide and is a leading cause of hearing impairment in resource-limited settings. It is less frequently seen in resource-abundant settings. It is characterized by chronic drainage from the middle ear associated with tympanic membrane (TM) perforation. CSOM is usually preceded by an episode of acute otitis media (AOM).

The most effective antibiotics that can be used against *Pseudomonas aeruginosa* are ß-lactam antibiotics in which imipenem as a carbapenem is considered as the most appropriate antibiotic to be used against the mentioned organisms. Carbapenem resistance occurs because of decrease in antibiotics absorption due to lack of an outer membrane porin, as oprD, exclusion from the cell by efflux pump, decrease in outer membrane permeability and production of MBL [4]. According to some recent reports, infection with metallo-beta-lactamase producing *P. aeruginosa* strains has increased mortality. Nowadays the emergence of antibiotic resistance strains is one of the challenges in treating patients, such as MBLs producing *Pseudomonas aeruginosa* [12, 13].

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In the present study the number of ears clinically diagnosed of having CSOMs was 200, out of which 70 (35%) was found to be positive for CSOM infection. Our study was in support with the study performed by Deepthi Maringanti et al.,[19] where, the ear discharge swabs were sent for Culture and Sensitivity in which only 106 patients out of 180, showed culture positives.

The ratio of Male 44 (62.8%) were found to be more as compared to that of Female 26 (37.1%) in our study. This finding was similar to the study by Mohammed Jamiu Kazeem [20] where 198 (52.1%) patients were male while 182 (47.9%) were female. Other studies by Okesola and Fasina [21] and Akingbade*et al*.,[22] was also in support with our study but in contrast with the study by Shrestha *et al*.,[23]. The maximum number of cases reported was observed in the age group of 0-10 years followed by 11-20 years of age and the least number of cases was seen in the age group above 51 years of age. This study was in support with the study performed by the other author [20] where maximum number of cases was reported in the age group of 10 years age with the fact that CSOM is predominantly a childhood disease, particularly the under 10 and least was observed above the age group of 50 years. This may be because of the week immune system in the young age and also because eustachian tubes are wider, shorter, and straighter compared to that of the adult.

The side of the ear affected was almost in equal distribution, with the left ear being (51.4%) and the right ear being (40%) while (8.5%) were bilateral. This study was in support with the study by Mohammed Jamiu Kazeem [20] where the distribution pattern of the right and the left ear was in and the equal while bilateral was 3.4%.

In our study it was observed that the maximum number of cases was found in Gram negative isolates as compared to the Gram positive isolates and only 1.4% with Fungal isolates. It was also observed that 88.5% samples showed growth of single isolates while 11.4% were mixed isolates. This was in accordance with the other study [20].

This current study showed that *P. aeruginosa* (42.8%) has the highest prevalence of the isolated organism. This correlates with the studies performed by other authors where the incidence of *P. aeruginosa* as the most commonly isolated organism in CSOM ranging from 21%-52.94% [24]. Another study by Loy *et al*. (33.3%) [55] and Mansoor*et al*. (40%) [26] also stated the rate of

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P. aeruginosa was more with no fungal isolate, but in Contrast with the study by Adoga et al. where Klebsiella species (40%) as the predominant organism [27].

In our study *Klebsiella* spp. was the second most common isolate followed by *Staphylococcus aureus* with 11.4 % , *Escherichia coli* (12.8%) , *Proteus mirabilis* (7.1%) and *Streptococcus pneumonia* (2.8%) being the least observed. This correlate with the study by the Nwankwo and Salisu [28].

Furthermore, this study showed that the commonly available antibiotics such as ciprofloxacin, and levofloxacin were generally ineffective against *P. aeruginosa*, species, which is the most isolate in our study. This was in support with the reports of Nwabuisi and Ologe [29]. Moreover, the ineffectiveness may be due to indiscriminate use of antibiotics, resulting in the emergence of resistant strains.

Piperacillin –tazobactam, Imipenem, Cefepeme and Amikacin though highly sensitive, are considered as reserve drugs in CSOM cases which are not responding to ciprofloxacin and Levofloxacin. The knowledge of resistance mechanisms in Pseudomonas is an important issue for antimicrobial treatment [30]. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most predominant pathogens that cause CSOM. Although the pathogenesis of AOM is well studied, very limited research is available in relation to CSOM [31,32]. With the emergence of antibiotic resistance as well as the ototoxicity of antibiotics and the potential risks of surgery, there is an urgent need to develop effective therapeutic strategies against CSOM [33].

Although still the sensitivity is declining so there is a need to quickly check the menace of inappropriate treatment of CSOM by quacks and intensify campaign against self-medication.

CONCLUSION

Like other chronic illnesses, CSOM can have a negative impact on a person's quality of life and employment. The most common isolate in our study was Pseudomonas aeruginosa, and the most effective antibiotics were found to be cefepime, amikacin, piperacillin-tazobactam, and colonistin; ciprofloxacin and levofloxacin were the least effective.

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As a result, since antibiotic resistance is growing, CSOM patients should have an aural discharge culture, and data on antibiotic susceptibility should be used to formulate medications that will help stop the emergence and spread of organisms that are resistant to antibiotics.

Therefore, the identification of genetic and prognostic markers will help in predicting CSOM-susceptible individuals and possibly even novel therapeutic strategies. Understanding the molecular mechanisms leading to CSOM will provide avenues to design novel treatment modalities against the disease and consequent hearing loss.

Declarations:

Conflicts of interest: There is no any conflict of interest associated with this study

Consent to participate: We have consent to participate.

Consent for publication: We have consent for the publication of this paper.

Authors' contributions: All the authors equally contributed the work.

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