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Molecular characterization of Extended Spectrum Beta lactamases producing bacteria causing Chronic Suppurative Otitis Media in South India

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ABSTRACT

Introduction: Chronic suppurative otitis media (CSOM) is one of the most common middle ear infections leading to extra and intracranial complications if not diagnosed promptly. Early identification and detection of the etiological agents and antibiotic susceptibility patterns assist in preventing complications. **Methods:** Two hundred twelve ear swabs were collected using sterile cotton swabs. Direct gram staining was done and then inoculated into blood, MacConkey, and Nutrient agar. Bacterial isolates were identified using conventional methods. According to CLSI guidelines, Antibiotic susceptibility testing was performed by Kirby-Bauer disc diffusion method. Minimum inhibitory concentration (MIC) was performed by the agar dilution method. Extended-spectrum beta-lactamases producing bacteria were detected by the phenotypic confirmatory test and then corroborated by uniplex PCR. **Results:** Out of 212 samples, 157 samples (74.06%) were culture-positive for bacteria. The isolated bacteria included *Pseudomonas aeruginosa* (46.24%), *Staphylococcus aureus* (26.59%), *Klebsiella pneumoniae* (14.45%), coagulase-negative *Staphylococcus aureus* (5.20%), *Proteus mirabilis* (4.05%), *Enterococcus faecalis* (2.89%), and *Escherichia coli* (0.58%). The *P. aeruginosa* isolates showed 96.25% and 95% susceptibility to amikacin and ofloxacin, respectively. All Gram-negative bacilli isolates were 100% sensitivite to imipenem. Ten (30.30%) isolates were ESBL producers with the *CTX-M-14* gene detected in most of them. **Conclusion:** Our study found that *P. aeruginosa* was the most common isolated pathogen bacteria. Knowledge of CSOM causing bacteria and their susceptibility to antibiotics would help choose an appropriate treatment, thereby preventing antibiotic resistance and complications in these cases.

INTRODUCTION

Chronic suppurative otitis media (CSOM) is one of the major causes of preventable hearing loss, particularly in developing nations [1]. This medical condition results from poorly managed acute otitis media in early childhood, leading to recurrent episodes of chronic discharging ears. In 1996, the WHO/CIBA Foundation workshop defined CSOM as a stage of disease resulting from a chronic infection of the middle ear cleft, i.e., eustachian tube, middle ear, and mastoid, with a non-intact tympanic membrane (e.g., perforation or tympanostomy tube) and discharge (otorrhoea) for at least two weeks or more [2].

Around 90% of the disease burden is in countries like Africa, Southeast Asia, and Western Pacific regions, and some ethnic minorities occur in the Pacific Rim. Among the South-East Asian countries, India, with 7.8%, had the highest CSOM prevalence. This persistent and destructive disease results in irreversible sequelae leading to severe intra- and extracranial complications [3]. Conductive (acquired) hearing loss is a worldwide medical condition, especially in children living in developing countries. CSOM infections can lead to prolonged hearing loss in both children and adults, which affects the developmental impairment of linguistic, behavioral, motor, and social skills and the performance at school or the workplace [4]. Typically, viral infections of the upper respiratory tract precede the disease, but soon the conditions become favorable in the middle ear for pyogenic organisms invasion [3]. Over the past few years, various studies have isolated *Pseudomonas* spp. from 48%-98% of patients diagnosed with CSOM followed by *Staphylococcus aureus* [5]. CSOM has received significant attention due to its increasing incidence, chronic nature, and ototoxicity resulting from the application of topical and systemic

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antibiotics and emerging bacterial resistance [4]. Inappropriate use of medications results in antibiotic resistance, leading to treatment failure and persistent discharge in the ears. The introduction of sophisticated synthetic antibiotics has led to changes in the microbiological flora, emphasizing the significance of reappraisal of the present-day flora in CSOM [6].

This study investigated the bacterial profile, antimicrobial susceptibility pattern, and molecular pattern of extended-spectrum beta-lactamases (ESBL) producing bacteria causing CSOM.

MATERIAL AND METHODS

This cross-sectional study was conducted on 212 clinically diagnosed CSOM patients referring to ENT OPD, Department of Otorhinolaryngology, Government Kilpauk Medical College, Chennai for one and a half years from January 2013 to August 2014. The CSOM patients belonged to various age groups, both sexes, and were not on systemic or topical antibiotic treatment for at least one week before sample collection. We excluded from the study the patients with nonbacterial-related CSOM, other middle ear infections, and a discharge for less than three weeks.

Sample collection. After obtaining informed content from patients, samples from ear discharge were collected under aseptic conditions using two sterile cotton swabs and immediately sent to the Department of Microbiology, Government Kilpauk Medical College, Chennai [7].

Gram staining and culture. One swab was used for a direct gram staining to examine the presence of pus cells, epithelial cells, and bacteria. The second swab was inoculated onto Nutrient agar, 5% sheep blood agar, and Mac Conkey agar and incubated at 37 ºC for 24 h. Bacteria species were identified based on standard microbiological methods. The isolates were assessed based on clinical history, presence of pus cells in direct gram stain, and pure growth on culture. In case of growing any contaminants or commensals like diphtheroids, the organisms were not considered pathogenic.

Antibiotic Susceptibility Testing. The antibiotic susceptibility testing of the isolates to antimicrobial agents was performed using the Kirby-Bauer disk diffusion method according to Clinical & Laboratory Standards Institute (CLSI) recommendations [8,9]. Antibiotic disks (µg/disk) purchased from HiMedia included amoxicillin (20), cefoxitin (30), cefalexin(30), ceftazidime (30), cefotaxime (30), gentamicin (10), amikacin (30), tobramycin (10), amoxicillin-clavulanic acid (20/10), erythromycin (15), ciprofloxacin (5), ofloxacin(5), imipenem (10), kanamycin (30), trimethoprim/sulfamethoxazole (1.25 /23.75), tetracycline (30), doxycycline(30), piperacillin-tazobactam(100/10), vancomycin(30), and linezolid (30).

Detection of ESBL. Screening for ESBL production was performed using cefotaxime, ceftazidime, and cefpodoxime antibiotic disc [10]. Isolates with zone diameters less than 27mm for cefotaxime and less than 22mm for ceftazidime and cefpodoxime were further confirmed for ESBL production by phenotypic confirmatory test [11]. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 were included in the assay as controls.

Phenotypic Confirmatory test or Disc potentiation test. This test was done for all Enterobacteriaceae isolates against ceftazidime (30 µg) antibiotic discs with and without clavulanic acid (10 µg). The discs were placed on a Mueller-Hinton agar plate inoculated with bacterial suspension equivalent to 0.5 McFarland standards. Following overnight incubation at 37°C, if the zone diameter of ceftazidime plus clavulanic acid was ≥ 5 mm compared with ceftazidime alone, the isolate was assumed positive for ESBL production **(Fig. 2).**

MIC determination by agar dilution method. Freshly prepared and autoclaved Mueller Hinton agar was allowed to cool in a 50 ºC water bath. Preparation of serial dilution of 3rd generation cephalosporin (ceftazidime, cefotaxime) was prepared in sterile distilled water, with final concentrations ranging from 2µg to 2048µg/ml of agar. The antibiotics were added to the medium at 50 ºC, mixed well, and poured onto sterile Petri plates. A culture without the antibiotic was used as a control plate for each series of tests.

Detection of ESBL by PCR. DNA was extracted from the bacteria using a commercial kit (PureFast Bacterial Genomic DNA purification kit, Helini Biomolecules, Chennai). All ten *Klebsiella pneumoniae* isolates confirmed as ESBL producers were checked for *blaCTX-M-14, blaTEM,* and *blaSHV-12* genes by a uniplex PCR. The primers were obtained from Helini Biomolecules, Chennai (Table 1). The 50 µl reactions contained 25 µl of 2x master mix, 5 µl extracted DNA, 5µl of forward and reverse primers (3 pmol/µl) and nuclease-free water to the final volume (Helini Biomolecules, Chennai, India). The 2x master mix included 2 units of *Taq* DNA polymerase, 10X *Taq* reaction buffer, 2 mM $MgCl_2$, 10mM dNTPs, 1µl and Polymerase Chain Reaction additives. The amplification program included an initial denaturation at 95ºC for 3 min followed by 35 cycles each of denaturation at 95 ºC for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 30 sec and final extension at 72 °C for 5 min. The PCR samples were loaded alongside 10µl of a 100bp DNA ladder (Helini Biomolecules, Chennai, India) in 2% agarose gels after mixing with gel loading dye and then visualized under a UV Transilluminator.

Statistical analysis. The data for demography, distribution of isolates, and antimicrobial susceptibility tests were tabulated and analyzed. Data comparison was achieved via an analysis of Pearson's Chi-square test.

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Table. 1. The Primers used for detecting ESBL genes, *bla CTX-M*, *bla TEM*, and *bla SHV*.

Target genes	Primers	Size	Reference
bla $_{\text{CTM- M-14}}$	5'-TTATGCGCAGACGAGTGCGGTG-3'	120bp	[12]
	5'-TCACCGCGATAAAGCACCTGCG-3'		
bla $_{\text{SHV-12}}$	5'-CGCCGCCATTACCATGAGCGAT-3'	276bp	[12]
	5'-ACCCGATCGTCCACCATCCACT-3'		
bla $_{\text{TEM}}$	5'-CCAAACGACGAGCGTGACACCA-3'	148bp	[12]
	5'-AGCGCAGAAGTGGTCCTGCAAC-3'		

Table 2. Antibiotic susceptibility of Gram-negative bacteria identified in this study (n=113).

Antibiotics	*P.aeruginosa* (n=80) n (%)	*K. pneumoniae* (n=25) n (%)	*P. mirabilis* (n=7) n (%)
Amoxycillin	NA	7 (28.00)	2 (28.57)
Amoxycillin-clavulinic acid	63 (78.75)	17 (68)	6 (85.71)
Cefoxitin	72 (90)	21 (84)	7 (100)
Cephalexin	52 (65)	13 (52)	4 (57.14)
Cefotaxime	74 (92.50)	15 (60)	7 (100)
Ceftazidime	74 (92.50)	15 (60)	7 (100)
Amikacin	77 (96.25)	20 (80)	6 (85.71)
Gentamicin	59 (73.75)	14 (56)	3 (42.85)
Ciprofloxacin	58 (72.50)	12 (48)	3 (42.85)
Ofloxacin	76 (95)	18 (72)	6 (85.71)
Piperacillin- Tazobactam	78 (97.50)	25 (100)	7 (100)
Imipenem	80 (100)	25 (100)	7 (100)

NA, not applicable; according to CLSI guidelines, amoxicillin was not tested against *P. aeruginosa*

RESULTS

Demographic Characteristics of the study. The incidence of CSOM was higher within the age group of 21-30 years compared to other age groups, and males (52%) were more affected than females (48%).

Gram staining and culture positivity. Of 212 specimens, 153 (72.17%) showed bacteria by direct smear and culture. Four (1.89%) were direct smear-negative and culture-positive, while 55 (25.94%) were direct smear- and culture-negative. None of the specimens was direct smear-positive and culture negative. The culture was positive for 157 (74.06%) swabs. The number of sterile pus swabs was 55 (25.94%). Of 157 culture-positive swabs, 141 (89.80%) showed a single monomicrobial infection, and 16 (10.19%) polymicrobial infections.

Distribution of organisms. *P. aeruginosa* (46.24%) was the most common isolated organism among Gram-negative bacilli, followed by *K. pneumoniae* (14.45%). *S. aureus* (26.59%) was the common Gram-positive cocci detected. Other isolated organisms were *Proteus mirabilis*, *E. coli*, coagulase-negative *Staphylococcus* (CONS), and *Enterococcus faecalis* (Fig. 1).

Antibiotic sensitivity of bacterial isolates. *P. aeruginosa* showed 100% sensitivity to imipenem. All Gram-negative bacilli isolates were 100% sensitive to imipenem (Table. 2). All the Gram-positive cocci were 100% sensitive to vancomycin and linezolid.

Detection of ESBL. Out of 113 Gram-negative isolates, 10 (40%) *K. pneumoniae* isolates and 6 (7.5%) *P. aeruginosa* isolates showed resistance to both cefotaxime and ceftazidime. *P. mirabilis* and *E. coli* showed 100% sensitivity to both cephalosporins. All ten *K. pneumoniae* were further subjected to phenotypic confirmatory test and MIC determination. The phenotypic confirmatory method identified all ten K. pneumoniae isolates as ESBL producers (Fig. 2).

MIC Determination. The minimum inhibitory concentration of isolates to ceftazidime for the ESBL producing organism in the study was between 0.5 (µg/ml) of agar to 128 (µg/ml) of agar in the presence of 4µg/ml clavulanic acid in the agar showing a reduction in ≥3 doubling dilution.

Detection of ESBL by PCR. All ten *K. pneumoniae* isolates identified as ESBL producers by the phenotypic confirmatory test were confirmed by PCR for ESBL genes, including *blaCTX-M-14*, *blaTEM*, and *bla SHV-12*. Of ten *K. pneumoniae* isolates, 3 (30%) were positive for *bla CTX-M-14* (Fig. 3), 4 (40%) for *bla SHV-12* (Fig. 4), and 3 (30%) for *bla TEM* gene (Fig. 5).

DISCUSSION

CSOM is the most well-known disease encountered in otolaryngology practices in developing countries like India. It is known for its dreadful complications if not diagnosed and treated promptly. Such complications range from persistent otorrhoea, mastoiditis, labyrinthitis, and facial nerve paralysis to more serious intracranial abscesses or thromboses. Early bacteriological diagnosis in cases will assure accurate and appropriate therapy.

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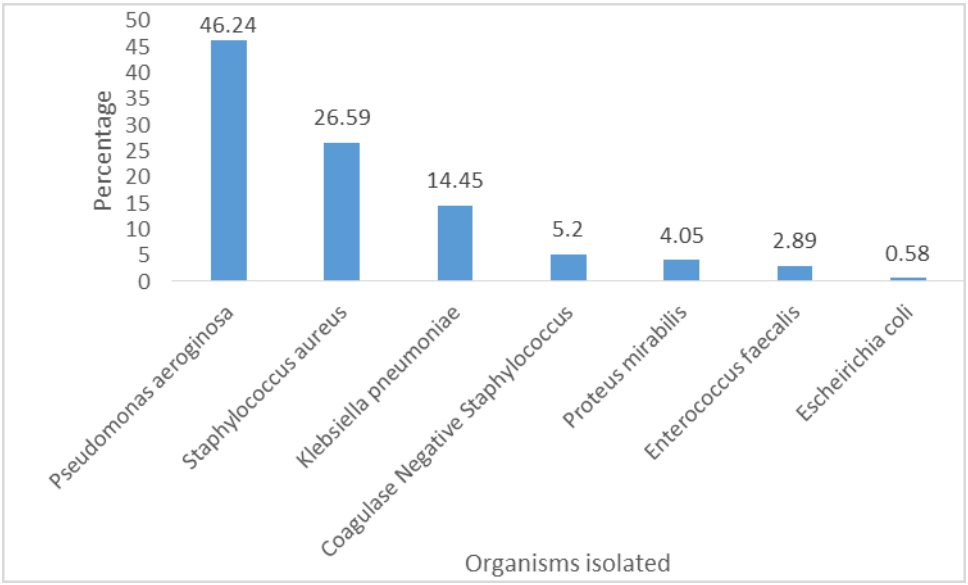


Fig. 1. Distribution of Gram-positive and Gram-negative organisms isolated from the CSOM patients.



Fig. 2. Phenotypic confirmation test (disc potentiation test) for detecting ESBL producer isolates.

In the present study, the incidence of CSOM was higher within the age group of 21-30 years, concordant with two similar studies in South India [13, 14], which showed 22.34% and 30% in the age group of 21-30 years. The reason for delayed presentation could be the patients' ignorance to approach the health services at the early stages of the disease.

In our study, males were more affected than females, similar to previous reports from India [15, 3]. A higher incidence in males may be due to their increased outdoor activity. The majority of the previous studies showed male preponderance similar to our study, whereas reports from Singapore [16], Nepal [17], and India. [18, 19] showed female preponderance.

The culture positivity rate was 74.06%, and 25.94% of swabs did not result in bacterial growth, corresponding to similar studies from India [20, 21] and Nigeria [7].

A high percentage of negative cultures in the present study could be due to the following reasons: 1) CSOM patients might have been referred to our clinic after being diagnosed and partially treated by local doctors, 2) infections with anaerobic bacteria, *Mycoplasma*, and *Chlamydia* which require specific culture media. In our cases, no association between two bacteria species was observed, similar to the report by Kumar H *et al.* [14].

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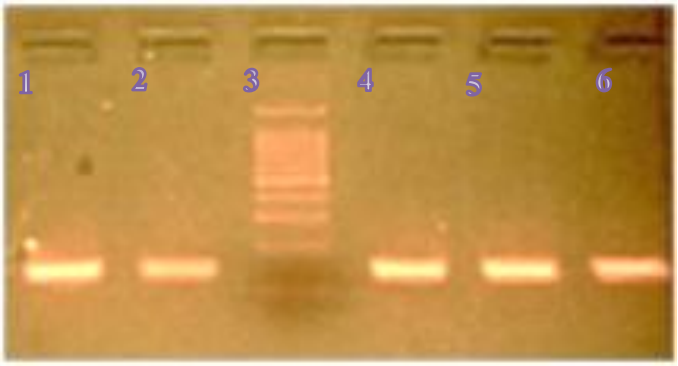


Fig. 3. PCR detection of *blaCTX-M-14* gene (120bp); Lane 3- Ladder (100bp), Lanes 1, 2, 4, 5, and 6, clinical samples.

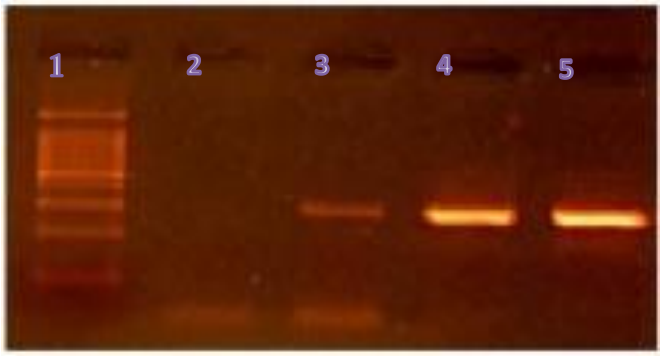


Fig. 4. PCR detection of *blaSHV-12* gene (276 bp); Lane 1, 100 bp ladder; lane 2, negative control; lane 3, positive control; lanes 4 and 5, clinical specimens.

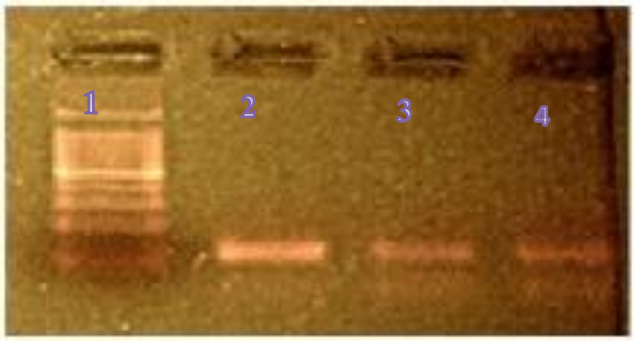


Fig. 5. PCR for detection of *blaTEM* gene (148 bp); Lane 1, a 100bp ladder. Lanes 2, 3, and 4 clinical samples.

Among cultures, 147 (89.37%) were monomicrobial, and 17 (10.69%) polymicrobial. This finding corroborates well with the previous studies from India [6, 3, 22, 23]. In the present study, the predominant isolated bacteria were *P. aeruginosa* (46.10%) followed by *S. aureus* (26.59%), similar to previous studies from India [15,13,4,3,24,22, 25,26] and Nigeria [7], which also reported *P. aeruginosa* as the most common organism in CSOM cases. The occurrence of *P. aeruginosa* as the dominant organism can be due to this opportunistic organism's ability to grow in the moist external auditory meatus of CSOM patients and outgrow other organisms due to its minimal nutrition requirement.

The other most common organism in our study were *S. aureus* (26.59%), followed by *K. pneumoniae* (14.45%), CONS (5.20%), *P. mirabilis* (4.05%), *Enterococcus* spp (2.89%), and *E. coli* (00.58%). Unlike the present study, similar studies in India have reported *S. aureus* as the most common pathogen in CSOM patients [16, 27-29], which might be due to various geographical reasons.

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Although *CONS* are generally considered commensal, their association in some cases can be attributed to the decreased resistance in the middle ear due to invasion by other organisms. Under these circumstances, they assume a pathogenic role either singly or more often alongside other organisms.

In the present study, *P. aeruginosa* showed maximum sensitivity to amikacin (96.25%), followed by ofloxacin (95%), similar to reports from various parts of India. [3,6,28,30]. In our study, among *K. pneumoniae* isolates, 80% and 72% were susceptible to amikacin and ofloxacin, respectively, whereas they showed 56% susceptibility to gentamicin, 60% to ceftazidime, and cefotaxime. Among *P. mirabilis* isolates, 85.71% were sensitive to amikacin and ofloxacin. All Gram-negative isolates were 100% sensitive to imipenem and piperacillin-tazobactam except *P. aeruginosa,* which showed 97.50% sensitivity to piperacillin-tazobactam. *K. pneumoniae* and *P. mirabilis* showed less than 30% sensitivity to amoxycillin.

Many studies on the etiology of CSOM and sensitivity patterns in India and other countries are available, but only a few have reported the prevalence of ESBL in India. In the present study, 40% were ESBL producers, similar to the other Indian studies [14, 21, 31]**.**

P. aeruginosa and *S. aureus* were the most common organisms isolated in our study. *P. aeruginosa* was sensitive to piperacillin-tazobactam, ofloxacin and amikacin. *S. aureus* was sensitive to vancomycin. Therefore, studying the microbial flora in CSOM and their antibiotic sensitivity pattern is essential for the clinicians to start empirical treatment, thereby minimizing the complications and preventing the emergence of resistant strains.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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