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Detection and Genomic Characterization of Carbapenemase producing Pseudomonas aeruginosaisolates in Chronic SuppurativeOtitis Media(CSOM)

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ABSTRACT

Introduction: *Chronic Suppurative Otitis Media is a common infection in the middle ear and an important cause ofhearing impairment in developing countries.*

Aims and Objectives: *To determine the Carbapenemase producing Multi drug resistant (MDR) Pseudomonas aeruginosa isolates in CSOM.*

Materials and Methods: *140 ear swabs were collected from 130 patients with CSOM. The isolates were identified and tested for antimicrobial susceptibility.Pseudomonas isolates with MIC >32 were selected for Carbapenemase production.* ***Results***: *The Predominant isolate was Pseudomonas aeruginosa36%.Among 24 MDR Pseudomonas isolates 29.1% and 37.5%were confirmed as MBL producer by phenotypic Modified Hodge test &combined disk test respectively and 13 were positive for OXA genes&all were negative for IMP & VIM genes by molecular method.* ***Conclusion***: *The study concludes that Ceftazidime resistance should be considered as an indicator for detection of resistant enzymes pattern in antimicrobial susceptibility tests*

KEYWORDS :CSOM, MDR, Carbapenemase

Introduction

Chronic suppurative otitis media is a common infection in the middle ear. It iswell known for its persistence and recurrence in spite of treatment. CSOM is defined as chronic inflammation of the middle ear and mastoid cavity, with recurrent ear discharge through the perforated tympanic membrane[1].

It is an important cause of mild to moderate hearing impairment among children and young people particularly in developing countries. In India over all prevalence rate is 46 per thousand individuals in rural areas and 16 per thousand individuals in urban population[2].

In microbiological aspect, *Pseudomonas aeruginosa* is the most common among GNB which causes CSOM next to hospital infections and Staphylococcus aureus is a common organism among GPC. Infections caused by *Pseudomonas aeruginosa* are often severe and life threatening and are difficult to treat because it has limited susceptibility to antimicrobial agents. In developing countries like India there is rapidly increasing resistance for antimicrobial therapy due to indiscriminate, irrational use of antibiotics and poor follow up of the patients. These factors will lead to persistent low grade infection and changes in the microbiology of the disease[3].

In recent years the development of drug resistance by *Pseudomonas aeruginosa*is mainly through inducing bacterial enzymes like Betalactamases, Extended Spectrum Betalactamases and Carbapenemases causing great concern to ENT surgeons[4]. *Pseudomonas aeruginosa* resist Betalactam antibiotics by producing Beta-lactamases that can express resistance to penicillin, third generation Cephalosporins and Carbapenems[5]. According to Amblers classification Carbapenemases which include Metallo beta lactamases (class B) and Oxacillinases (class D)resistant *Pseudomonas aeruginosa* are more prevalent and gives emerging infections recently. The Carbapenemases observed in *Pseudomonas aeruginosa* are either plasmid or Chromosome mediated[6]. They often continue to spread the resistance pattern to other gram negative organisms like Enterobacteriaceae group of organisms through vertical gene transfer. The precipitating factor responsible for this mechanism is inappropriate dose and duration of antibiotics. Hence the evaluation of antimicrobial susceptibility pattern of *Pseudomonas aeruginosa* and implementation of judicious use of antibiotics in CSOM is mandatory to prevent the spread of resistant pathogens[7].

In view of above, this study was undertaken to determine the frequency of MDR *Pseudomonas aeruginosa* isolates in patients of active CSOM and theirgenomiccharacterization in our community.

Aims and Objectives

To determine the prevalence of MDR *Pseudomonas aeruginosa* in CSOM

To detect the Carbapenemase producing *Pseudomonas aeruginosa* and to demonstrate the genomic pattern of MDR *Pseudomonas aeruginosa* isolates by PCR.

Materials and Methods: A prospective study was conducted in the department of Microbiology, Thanjavur Medical College Hospital, Thanjavur, during the period from August 2009 to July 2010 after Ethical committee approval. 140 ear swabs were collected from 130 patients with CSOM attending ENT op after obtaining informed consent. For collection of samples the following inclusion and exclusion criteria were considered[7,8].

Inclusion criteria

All age groups and genders.

- Patients with chronic or recurrent ear discharge for more than 3 months.
- Patients having perforated TM with ear discharge.

Exclusion criteria

Acute otitis media

Antibiotic therapy before 5days of sample collection (systemic or topical)

Clinical signs suggestive of unsafe ear i.e. Cholesteatoma

Under above criteria two samples were collected from each patient. Single use minitip culture swabs were used to harvest the middle ear flora through the perforated tympanic membrane withoutcontaminating with external auditory canal flora. After collection, the swabs were transported to the lab immediately and processed as per standard guidelines. The first swabused for gram stain and the second swab was inoculated into Nutrient agar, MacConkey agar and 5% sheep Blood agar & incubated at 37°C for 24 – 48 hours.After incubation the bacterial isolates were identified and speciated by standard protocol. Antimicrobial susceptibility test was done for all bacterial isolates as per CLSI guidelines by Kirby-Bauer Disk Diffusion method on MullerHintonagar[9,10]. The panel of antimicrobial disc like Ampicillin, Amox/clav, Piperacillin, Piperacillin / Tazobactum, Gentamicin, Amikacin, Chloramphenicol, Ciprofloxacin, Ofloxacin, Cephalexin, Ceftriaxone, Cefotaxime, Cefepime, Aztreonam, Imipenem and Meropenem were used for all bacterial isolates and for GNB Ceftazidime

disc was used along with above antibiotic discs. MIC for Ceftazidime was determined by an Antimicrobial Gradient strip (Hicomb MIC test) for all Pseudomonas isolates as per standard guidelines. Out of 54 isolates, 24 were selected for Carbapenemase detection (with MIC >32µg/ml and resistant to 3-4 group of drugs) in spite of the susceptibility to Carbapenems. Carbapenemases include Metallo Betalactamases (MBL) and Oxacillinase enzymes. MBL were detected by phenotypic tests by Modified Hodge test and Combined disk test as per CLSI guidelines[11,12].

Modified Hodge Test

In MHT, a lawn culture of 1:10 dilution of 0.5 McFarland's standard E coli ATCC 25922 was done on a Muller Hinton Agar plate. A 10 µg Imipenem disc was placed in the centre of the plate. Then, Meropenem resistant *P. aeruginosa* (test strains) were streaked from the edge of the disk to the periphery of the plate in four different directions. After overnight incubation, the plates were observed for the presence of a 'clover-leaf' shaped zone of inhibition and the plates with such zones were interpreted as modified Hodge test positive[13,14].(Fig:1)

Fig:1 Modified Hodge test

Fig:2Combined disk test

Combined disk test

0.5 M EDTA solution was prepared by dissolving 186.1g of disodium EDTA.2H2O in 1000 ml of distilled water and its pH was adjusted to 8.0 by using NaOH & sterilized by autoclaving. The test organisms were inoculated on MH agar plate and two 30mcg Meropenem (MRP) were placed at centre to centre with distance of 20mm& 10µl 0f 0.5M EDTA (750µg) solution was added to one of the MRP disc & incubated at 37°C for 16-18 hours.After incubation the increase in inhibition zone with MRP-EDTA disc by more than 7mm than MRP alone was considered as MBL positive[13,14]. (Fig:2)

There are no standard phenotypic tests for Oxacillinase detection, the isolates taken for molecular study directly.

Molecular Methods

In our study 24 MDR *Pseudomonas aeruginosa* isolates were taken for confirmation of Carbapenemases production by molecular study. Multiplex Polymerase chain reaction (PCR) method was used to identify the IMP and VIM gene for MBL & OXA gene for Oxacillinase. Bacterial DNA template was prepared as follows; the pure organisms (3-5 colonies) were inoculated into 10 ml of Nutrient broth (Himedia) and incubated for 24 hours at 37°C. Then the broth was centrifuged at 10,000 rpm for 5 minutes. After centrifugation the supernatant was decanted, the bacterial pellet was taken for next step. The pellet was purified further with DNA purification kit. The kit used in this procedure was **PureFast®** Bacterial Genomic DNA purification kit (from HELINI Biomolecules Chennai, India). DNA purification kit includes PCR Master Mix, Agarose gel electrophoresis consumables and Primers. **PCR Master Mix:** It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and PCR additives. **Agarose gel electrophoresis:** Agarose, 50X TAE buffer, 6X gel loading buffer and Ethidium bromide **Procedure:** Genomic DNA extraction from bacterial pellet using HELINI PureFast Genomic DNA purification kit. The following **Primer Sequences were used;**

- OXA23-F-AGTATTGGGGCTTGTGCT
- OXA23-R-AACTTCCGTGCCTATTTG
- OXA58-F-ATGCAAAGTGAATTGCAACG
- OXA58-R-CCCCAGCCACTTTTAGCATA
- IMP-DIA-F-GGAATAGAGTGGCTTAATTCTC
- IMP-DIA-R-GTGATGCGTCGCCAAYTTCACT
- VIM-DIA-F-CAGATTGCCGATGGTGTTTGG
- VIM-DIA-R-AGGTGGGCCATTCAGCCACA

The amplicons were found after Agarose gel electrophoresis. Genomic sizes of the amplicons (PCR Products)were 453bp &233bp for OXA23 and OXA58 respectively and compared with control. No amplicons were found for IMP and IMP gene. Datas were analysed by descriptive statistics study[15].

Results and Discussion:

In our study a total number of 140 specimens were collected from 130 CSOM patients (with 10 bilateral CSOM). Among the total population, 71 male (54.6%) and 59 female (43%) were affected by CSOM. Out of the 140 specimens, 137 (97.8%) & 3 (2.1%) were showed significant and insignificant growth respectively. Out of total samples, 136 (97.1%) bacterial and 6(4%) were fungal. Out of total cultures, the predominant isolate was *Pseudomonas aeruginosa*36% followed by *Staphylococcus auerus*26%, *Proteus species* 11.3%, *Klebsiellaspp*7.3%, CONS 4.6%, *Escherichia coli* 3.3%, *Citrobacterspp*(1.3%), *Enterobacterspp*0.6% and *Candida spp*4% [Table-1]. Regarding epidemiological analysis, out of 130 patients, 54.6% were male and43% werefemale. In age wise, paediatric age group 26.1% showed more incidence followed by middle age group 20.7%. Among *Pseudomonas aeruginosa* isolates from CSOM, female 57.4% are more affected than male42.5%.

Table – 1: Distribution of pathogens in CSOM

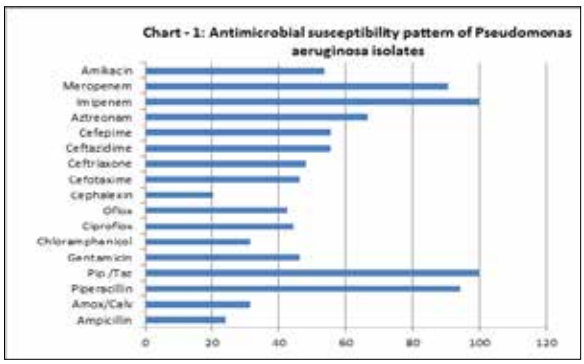
S.No	Name of the organism	Number	%
1.	Pseudomonas aeruginosa	54	36
2.	Staphylococcus aureus	39	26
3.	Proteus mirabilis	11	7.3
4.	Proteus vulgaris	6	4
5.	Klebsiella pneumoniae	9	6
6.	Klebsiella oxytoca	2	1.3
7.	CONS	7	4.6
8.	Escherichia coli	5	3.3
9.	Citrobacter species	2	1.3
10.	Enterobacter species	1	0.6
11.	Candida species	6	4

ur study showed *Pseudomonas aeruginosa* was the prevalent organism 36% in CSOM. This is supported by Indudharan et al 27.2%[4], Loy *et al*33.3%[16], and also in Lee *et al* study 25.8%[14].

The antibiogram of *Pseudomonas aeruginosa* was showed highest sensitivity against Imipenem100% Piperacillin / Tazobactum 100%, Piperacillin 94.4%, Meropenem 90.7%. The other sensitive drugs are Aztreonam 66.6%, Ceftazidime 55.5%, Cefepime 55.5%, Amikacin53.7%, Ceftriaxone 48.1%, Ceftriaxone 48.1%, Cefotaxime46.2%, Gentamicin 46.2%, Ciprofloxacin 44.4%, Ofloxacin42.5%, and less sensitive to Ampicillin 24%, Amox / Clav31.4%, Chloramphenicol 31.4%, Cephalexin 20.3%, among 54 isolates [Chart-1].

In contrast Mansoor*et al*[8]showed highest sensitivity Amikacin96% of isolates, followed by ceftazidime 89%, ciprofloxacin 85%, Gentamicin 81%, Imipenem76%, Aztreonam42% and ceftriaxone 21%. In Nikakhlagh*et al*[17] study the most effective antibiotic against *Pseudomonas* was ofloxacin. The higher rate of sensitivity against Quinolones and Amino glycosides than our study is due to variation in the of drug preference for treatment

In our study 24(44.4%) *Pseudomonas* isolates were identified as MDR isolates. Similarly Bhalerao*et al*[18]study showed 67.5% were MDR isolates. Our study showed higher incidence of MDR *Pseudomonas* in young adults 37.5% followed by paediatric age group 20.8%. In contrast Deeba*et al* study[19] showed higher incidence of MDR *Pseudomonas* isolates in old age group 46.6%.



Among 24 MDR *Pseudomonas* isolates 7 (29.1%) were confirmed as MBL producer by phenotypic Modified Hodge test. This is higher than Attal *et al* 11.4 % study[20]. Among 24 isolates, 9(37.5%) were positive for Combined disk test. Similarly Clare Franklin *et al*[21]study 84 isolates out of 134and Deeba *et al* 33(11.6%)[19] were positive by Combined Disk test.

Multiplex PCR was done for all 24 MDR *Pseudomonas* isolates, to detect the genotypes of Carbapenemases particularly for IMP, VIM and OXA genes. The PCR result showed that all isolates were negative for IMP and VIM genes (Fig:4). Similar report seen in Manoharan *etal* study among 48 tested isolates all were negative for IMP and 15 were positive for VIM gene[22]. Similarly Bhalerao *et al* study15 were positive for VIM and all were negative for IMP gene out of 43 isolates[18]. In our study the negative results for IMP and VIM genes due to the isolates may possessed with other MBL genes like SPM, SIM and GIM.Our study showed 13 were positive for OXA genes(Fig:3) out of 24 isolates. Similarly by Frank Daniel *etal*[23]study 2, Didier Hocquet*etal*[24] study 5 and Jing-Jou*et al*[2]2 isolates were positive for OXA gene among 43, 140 & 26 Ceftazidime resistant *Pseudomonas* respectively.

Fig :3 PCR Gel Document showing bands for OXA Genes

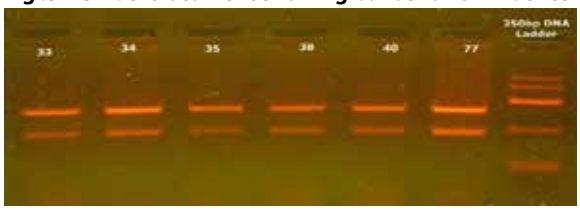
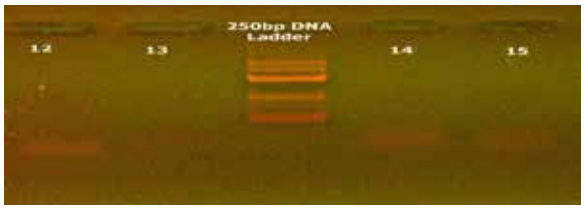


Fig: 4 PCR Gel Document negative MBL gene



CONCLUSION

Chronic suppurative otitis media is a disease of childhood and young adults predominantly with ear discharge as an early and frequent symptom along with deafness. Untreated cases of CSOM may result into extra cranial and intracranial complications. Hence the treatment needs to be instituted early and effectively to avoid the complications. Our study showed that *Pseudomonas aeruginosa*is the most prevalent isolate in CSOM and showed higher degree of resistance towards third generation Cephalosporins. The study revealed that though the *Pseudomonas* isolates are susceptible to Carbapenems in vitro, the Ceftazidime resistance should be considered as an indicator for detection of in vivo resistance to Carbapenems since there are **Hidden genes** present in the organisms now a days.

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