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RESEARCH ARTICLE

A STUDY ON THE METALLO-BETA-LACTAMASE PRODUCING *PSEUDOMONAS* AERUGINOSA ISOLATED FROM CASES OF SINUSITIS

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ABSTRACT

About 15 strains of *Pseudomonas aeruginosa* isolated from cases of sinusitis were used in this study. Drug susceptibility pattern and molecular mechanisms of resistance were evaluated. Drug susceptibility was studied for the following antimicrobial agents. The antibiotics used were aminoglycosides - amikacin and gentamicin, the quinolones – ciprofloxacin and ofloxacin, the third generation cephalosporins - cefotaxime, the cefoperazone - sulbactam, cefpodoxime and other agents piperacillin and imipenem. Sensitivity to amikacin and gentamicin were observed in 11(73.3%) strains and resistance was observed in 4(26.6%) strains. It was observed that 4(26.6%) out of 15 strains were resistant to ciprofloxacin and 6(40%) were resistant to ofloxacin. To the cephalosporins, i.e to cefotaxime, cefoperazone - sulbactam and to cefpodoxime, 14(93.3%), 10(66.6%) and 12(80%) strains were resistant respectively. To piperacillin and imipenem, 14(93%) out of 15 strains were resistant. Plasmid was present in 67% of the *Pseudomonas* strains examined. PCR for IMP 1 was negative in all the strains tested. However, 9(60%) out of 15 strains were positive for VIM 1.

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INTRODUCTION

Dissemination of Gram-negative bacteria producing metallo-β-lactamases (MβLs) is of great concern. A variety of acquired MβLs, including IMP, VIM, SPM, GIM, and SIM have been described in species of clinical importance (Queenan and Bush, 2007). MβLs exhibit wide hydrolysis spectra affecting virtually all β-lactams including carbapenems. Moreover, MβL-encoding genes are commonly carried by multi-resistant integrons. MβLs, mainly of the VIM and IMP types, have been widely diffused among non-fermenters such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* and enterobacteria (mainly *Klebsiella pneumoniae*) (Walsh *et al*., 2005; Queenan and Bush, 2007). The early and accurate detection of MβL producers would facilitate administration of the appropriate therapy and the implementation of infection control measures, especially in high prevalence settings. Novel detection approaches utilizing molecular methods have been proposed for the timely detection of MβL producers (Cornaglia *et al*., 2007; Miriagou *et al*., 2010; Walsh *et al*., 2005). Currently, polymerase chain reaction (PCR) is considered as a sensitive and reliable method for detection of MBLs (Behera *et al*., 2008). This study was conducted to investigate the prevalence of MBL-producing *P. aeruginosa* and to detect MBL-encoding genes (VIM-1 and IMP) among imipenem-nonsusceptible (fully resistant or intermediate) isolates. In order to see if blaVIM-1 and IMP could be located on a non-transferable plasmid, DNA plasmid extraction was also performed.

MATERIALS AND METHODS

Clinical samples

About 200 non-repetitive clinical samples collected during the period, June 2011 to January 2014, from the patients attending the Diagnostic Nasal Endoscopy section of the Out Patient, (OP) Department of Ear, nose and throat (ENT), General Hospital, Chennai, were used in the study. Endoscopic pus samples were collected from the middle meatus region, in sterile transport medium after obtaining due permission from the concerned health authorities. Samples collected were immediately transported to the laboratory and processed.

Bacterial identification

The clinical samples were cultured in appropriate media. *Pseudomonas* species were isolated and identified by performing standard biochemical tests (Balows, *et al*., 1991).

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The organisms that were identified as *Pseudomonas* species were stored in Brain Heart Infusion agar in air tight vials for further study.

Antimicrobial susceptibility test

Susceptibility to anti-pseudomonal antimicrobials (Table 1) was performed by disk-diffusion method, (Bauer, *et al*. 1966) according to Clinical and Laboratory Standards Institute (CLSI, 2010) guidelines. The antibiotics employed were Gentamicin (10 µg) , oflaxacin (5µg), piperacillin (100 µg), ceftazidime (30 µg), cefoperazone-sulbactam (30 µg), cefotaxime (30 µg), amikacin (30 µg), ciprofloxacin (5 µg) and imipenem (10 µg) obtained from Hi-Media Laboratories, BD Diagnostics Pvt. Ltd, India. The MIC of ceftazidime was determined by MIC strip test method. The MIC strip test method was performed according to manufacturer's instructions.

MBL screening

The MIC of ceftazidime and the activity of metallo beta lactamase were determined by MIC strip test method (Walsh, *et al*., 2002) Radianz Biotechnologies, Perungudi, Chennai . The MIC strip test method was performed according to manufacturer's instructions. Screening for MBL production was done in ceftazidime resistant isolates by Ceftazidime (CDM)-EDTA combined disc test. The CDM-EDTA combined disk test was performed as described by Yong *et al*., (2002). Test organisms were inoculated on to plates with Mueller Hinton agar as recommended by the CLSI (2010). The strip was designed in such a way so that one half of the strip contains Ceftazidime (CDM)-EDTA and the other half contains Ceftazidime (CDM) only. The final concentrations of Ceftazidime and EDTA used in the strip corresponded to 256 µg/ml. The inhibition zones of the Ceftazidime and Ceftazidime -EDTA disks were compared after 16 to 18 hours of incubation at 37°C. In the MIC strip test, if the increase in inhibition zone with the Ceftazidime and EDTA disc is more than 3 twofold dilutions than the ceftazidime disc alone, it is considered as MBL positive.

Plasmid DNA extraction

Extraction of plasmid DNA was done for all the 15 strains. Plasmid DNA extraction from bacterial cell pellets was performed by alkali lysis method. Plasmid DNA obtained was electrophoresed through 1% agarose gel with ethidium bromide. Plasmid bands were visualized under UV light.

PCR

About 15 isolates of *Pseudomonas aeruginosa*, exhibiting phenotypic traits compatible with MβL production as described in a recent consensus paper (Cornaglia, *et al*., 2007), were examined for carriage of blaVIM1 by PCR assays. This was described previously using oligonucleotides blaVIM1A-F 5′TCT ACA TGA CCG CGT CTG TC--3′and blaVIM1B-R5′-TGT GCT TTG ACA ACG TTC GC-3′ (Dallenne, *et al*., 2010) which yielded a product of 748bp. Amplification of the target gene was carried out using bacterial cell lysate as the source of template DNA. *Pseudomonas aeruginosa* cells which was grown overnight at 37°C, on Luria Bertani agar (LB Agar) were picked up, inoculated into LB broth and kept for overnight incubation in the shaker incubator.

The bacterial cells were pelleted by centrifugation at 3000 rpm for 10 minutes. The cell pellets obtained were washed with Tris EDTA buffer and were resuspended in 200µl of Tris EDTA buffer and boiled for 10 minutes. Cell debris was removed by centrifugation at 10,000 rpm for 10 minutes and the supernatant containing the template DNA was used for PCR assay. The amplification was performed in a thermocycler (Eppendorf Gradient Cycler). Amplification was carried out in 0.2 ml tubes. Two µl of the primer mix, 12.5 µl of the master nucleotide mix, 5 µl of the sample supernatant were added in each reaction. The cycling conditions were: initial denaturation step at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and elongation at 72 °C for I.5 min. After a final elongation step of 5 min at 72°C, the amplification products were obtained. The PCR products were electrophoresed through 1.5% agarose gel with ethidium bromide, to resolve the amplified products and they were visualized under UV light.

RESULTS AND DISCUSSION

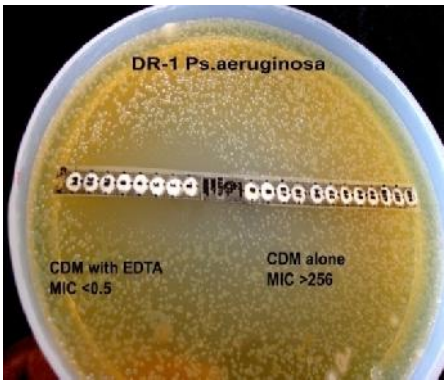
About 15 strains of *Pseudomonas aeruginosa* obtained from sinusitis patients were used in this study. Antibiotic resistance profiles of the isolates using disk diffusion tests demonstrated that the isolates were most resistant to cefotaxime, piperacillin and imipenem 14 (93%), and least resistant to ciprofloxacin (4 isolates, 27%) (Table- 1). Results of antibiotic susceptibility test (Table - 1) indicated that 80% of isolates were multidrug resistant (MDR) (resistant to more than 3 antibiotics from different classes) The MIC of CDM-EDTA and CDM was observed to be lesser than 0.5 and greater than 256µg/ml respectively (Fig-1). When tested phenotypically for the presence of MβL gene by E-strip test method, 9 strains were found to contain the enzyme metallobetalactamase. These results correlated with the presence of VIM1 gene by PCR. IMP1 gene was absent in all the 15 strains. It was seen that both the methods, CDM-EDTA combined disk test and PCR were equally effective for MBL screening. Plasmids were present in 10 strains and absent in 5 strains (Fig- 2).

Table 1 Antimicrobial susceptibility testing results of *P. aeruginosa* isolates

Antibiotic	Resistant no.(%)	Intermediate no.(%)	Sensitive no. (%)
Amikacin (10 µg)	4 (26.6%)	0 (0%)	11 (73.3%)
Cefotaxime (30µg)	14 (93%)	0 (0%)	1(6.6%)
Cefoperazonesulbactam (30µg)	10(66.6%)	4(26.6%)	1(6.6%)
Cefpodoxime(30µg)	12 (80%)	0 (0%)	3(20%)
Ciprofloxacin (30 µg)	4 (26.6%)	2 (13.3%)	9 (60%)
Gentamicin(10 µg)	4(26.6%)	3 (20%)	11 (73.3%)
Imipenem(10µg)	14 (93%)	0(0%)	1 (6.6%)
Ofloxacin (30 µg)	6 (40%)	1 (6.6%)	9 (60%)
Piperacillin(100 µg)	14 (93.3%)	0 (0%)	1(6.6%)

MBLs have been identified from clinical isolates worldwide, with an increasing frequency over the past few years and strains producing these enzymes have been responsible for prolonged nosocomial outbreaks that were accompanied by serious infections, as reported by Senda *et al*. (1996) In our

study about 93 % of *P. aeruginosa* strains showed resistance to Imipenem of which about 60% were detected as MBL producers which is much higher than that obtained in a study conducted by Navneeth *et al* .,(2002) who reported 12% of MBL mediated imipenem resistance in *P. aeruginosa*. However the incidence of *P. aeruginosa* has been reported to be 10-50% from various clinical specimens across the country (Taneja *et al*., 2003).



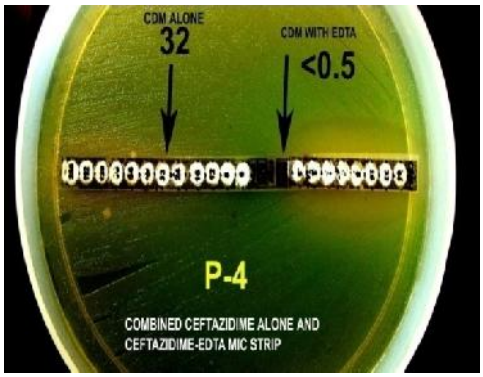


Fig 1 MIC strip test against *Ps.aeruginosa*

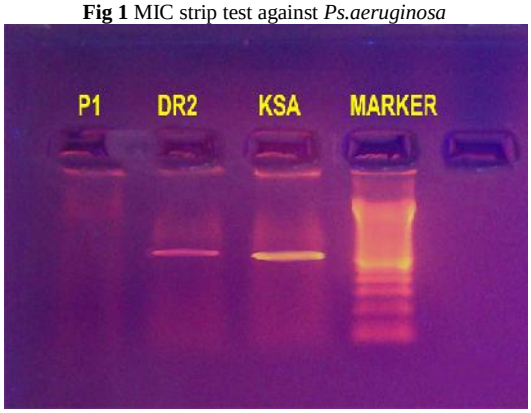


Fig 2 Amplified product of VIM 1

A study conducted by Jesudason *et al*. reported 42% MBL production by *Pseudomonas* (Jesudason MV). Sarkar *et al*, 54.54% of MBL production by *Pseudomonas* has been recorded (Sarkar *et al*., 2006). In a study carried out by Hasan (2005) in a tertiary care hospital in India, MBL production in Imipenem resistant isolates of *Acinetobacter* was 96.6% which is high as compared to our study which showed 93% MBL production in Imipenem resistant *Pseudomonas*. A total of 9 VIM-positive microorganisms were identified in 15 clinical samples confirming the high prevalence of VIM producers in this setting. IMP-producing microorganisms were not found in this collection. This was consistent with findings of previous surveys indicating the lack of IMP-positive bacteria in Greek hospitals (Vatopoulos, 2008). The emergence and rapid dissemination worldwide of carbapenem resistance due to the NDM-1-encoding gene should be seriously monitored in order to avoid any risk of pandemia (Kouda *et al*., 2009)

CONCLUSION

Our study showed the presence of MBL positive *P. aeruginosa* in the study region that emphasizes the necessity of screening all imipenem-nonsusceptible isolates for MBL production and implementation of infection control programs to avoid spreading of drug resistance. Timely and reliable detection of pathogens producing MβLs conferring resistance to virtually all β-lactams including carbapenems is of major clinical importance. Direct detection of MβL genes may also circumvent the problems of the phenotypic detection of carbapenemase producing microorganisms especially those exhibiting low carbapenem MICs.

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