Research Article

Study of Metallo β- lactamase producing *Pseudomonas aeruginosa* in diabetic foot infections

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Abstract

Introduction: *Pseudomonas aeruginosa* , gram negative aerobic rod, is an opportunistic and worrisome nosocomial pathogen. Metallo β- lactamase (MBL) mediated resistance to carbapenems is an emerging threat. Foot ulcers in diabetics are prone to polymicrobial infections. Infection with such multidrug resistant organisms increases morbidity and mortality in these patients. **Materials and methods**: Present study was undertaken to find out metallo β- lactamase producing *Pseudomonas aeruginosa* in diabetic foot infections. The study period was October 2009 – August 2011. Samples from the wounds of 121 Type II diabetic patients with foot infection were studied. *P. aeruginosa* isolates were identified by conventional methods. Antibiotic susceptibility testing of these isolates was done by Kirby- Bauer Disk Diffusion method. The isolates showing resistance to imipenem were subjected to EDTA- Disk Synergy test (EDS) and Imipenem – EDTA Combined Disk method for metallo β- lactamase detection. Minimum Inhibitory Concentration (MIC) of imipenem and imipenem- EDTA was determined in these isolates by agar dilution method. **Results**: Total 139 isolates were obtained from the samples. Out of which 22 isolates were of *Pseudomonas aeruginosa* (15.82%). Metallo β- lactamase production was seen in 12 isolates (54.54%) by both the tests. **Conclusion**: It is concluded that prevalence of metallo β- lactamase producing *Pseudomonas* aeruginosa in diabetic foot infection was 54.54%. Continuous surveillance of such resistant strains will help the clinician to facilitate the development of effective strategies to combat growing problem of resistance.

Key words - Diabetic foot infection(DFI), EDTA- Disk Synergy test (EDS), Metallo β- lactamase (MBL), Minimum Inhibitory Concentration (MIC)

Introduction

Pseudomonas aeruginosa is a classic opportunistic pathogen with innate resistance to many antibiotics and disinfectants [1]. It is a notable cause of nosocomial infections of respiratory & urinary tracts, wounds, blood stream and even central nervous system. For immunocompromised patients, such infections are often severe & frequently life threatening [2].

Infections caused by *Pseudomonas aeruginosa* are particularly problematic because the organism is inherently resistant to many drug classes and is able to acquire resistance to all effective antimicrobial drugs [3]. Its general resistance is due to combination of factors- 1) It is intrinsically resistant to antimicrobial agents due to low permeability of cell wall. 2) It has genetic capacity to express wide repertoire of resistance mechanisms. 3) It can become resistant through mutation in chromosomal genes which regulate resistant genes.4) It can acquire additional resistance genes from other organisms via plasmids, transposons and bacteriophages [4].

Pseudomonas aeruginosa produces enzymes which are responsible for widespread β- lactam resistance. Four molecular classes of β- lactamases are known, A-D according to Ambler classification. Class B β- lactamases include carbapenemases that hydrolyze most of β- lactams including carbapenems [5]. Based on molecular studies , 2 types of carbapenem hydrolyzing enzymes have been described : 1) Serine enzymes (possessing serine moiety at the active site) 2)Metallo β- lactamases. Characteristics of metallo β- lactamases are – 1) They require zinc for their catalytic activity. 2) metallo β- lactamase hydrolyzes all β- lactam antibiotics including carbapenems with the exception of aztreonam (monobactam). 3) MBL producing strains are not susceptible to serine β- lactamase inhibitors.(clavulanate)[6].

Diabetic foot ulceration and infections are a major medical, social, economic problem and leading cause of morbidity and mortality especially in developing countries like India[7].*Pseudomonas aeruginosa* may cause severe tissue damage in diabetics and more difficult to treat than infections in non-diabetics[8]. Proper

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management of these Diabetic foot infections (DFI) require appropriate antibiotic selection based on culture and antimicrobial susceptibility results. So the present study was done in Department of Microbiology, Dr. D.Y. Patil Hospital &Research Institute, Kadamwadi road, Kolhapur to find prevalence of metallo β- lactamase producing *Pseudomonas aeruginosa* isolated from diabetic foot infections.

Materials and Methods

The present study was done in Dr D Y Patil Hospital & Research Institute, Kolhapur. Study period was October 2009 to August 2011. Total of 121 type II diabetic patients with clinically infected foot ulcers attending as inpatient and outpatient department in the hospital were studied. Informed consent was taken from all the patients. Ethics committee clearance was obtained.

Collection of sample – Samples such as pus, debrided material, scrapings from the base of ulcer from the wounds of diabetic foot patients were collected in sterile container with all aseptic precautions and transported immediately to laboratory.

Processing of sample – Preliminary gram staining was done. The samples were inoculated on Nutrient agar, Blood agar, Mac-Conkey agar & Cetrimide agar plates. Plates were incubated at 35^{o}C aerobically.

Isolates of *Pseudomonas aeruginosa* were identified on the basis of colony characteristics & pigment production. Colony smears were made and gram staining was done. Motility was determined by hanging drop preparation. Confirmation was done by standard biochemical reactions like Oxidase test, Catalase test, Nitrate reduction test, Methyl red test, Indol production, Voges-proskauer test, Citrate utilization, Urease test, Triple Sugar Iron test, Arginine dehydrolase test and growth at 42^{o}C[1,2].Antibiotic susceptibility testing of these isolates was done by Kirby-Bauer Disk Diffusion method. Results were recorded as per CLSI guidelines [9]. Antibiotics used were Cefotaxime (30µg), Ceftazidime(30 µg), Cefepime(30µg), Ciprofloxacin(5µg), Gentamicin(10µg), Amikacin(30µg), Imipenem(10µg), Piperacillin(100 µg), Piperacillin – tazobactam(100/10 µg), Aztreonam(30 µg).

Isolates showing resistance to Imipenem were tested for metallo β-lactamase production. These isolates were subjected to EDTA Disk Synergy test (EDS) and Imipenem –EDTA Combined Disk method.

1) **EDTA Disk Synergy test (EDS)** [10]- Test organism (turbidity adjusted to 0.5 Mc Farland standard) was inoculated on Mueller Hinton Agar (MHA) plate. An Imipenem disk (10 µg) was placed 20 mm centre to centre from blank filter paper disk (Whatmann filter paper no.2)containing 10µl of 0.5M EDTA solution. Interpretation- Enhancement of zone of inhibition in the area between Imipenem & EDTA disk in comparison with zone of inhibition on the far side of IPM was interpreted as positive.

2) **Imipenem –EDTA Combined Disk method** [11]- The test organism was inoculated in sterile peptone broth &turbidity adjusted to 0.5 Mc Farland std. Lawn culture of test strain was done on dried Mueller Hinton agar plate. After drying two 10 µg Imipenem disks were applied firmly on the surface of the agar plate, at a distance of 20mm centre to centre on the plate. To one of the disk 10µl of 0.5M EDTA solution was added &plates were incubated for 16-18 hrs aerobically at 35^{o}C.

Interpretation- Zone diameters of two IPM disks were compared. Difference in the zone of inhibition between two disks more than or equal to 7mm was considered as positive result. **(**In both the above tests *Pseudomonas aeruginosa* ATCC27853 was used as negative control)

Isolates which were positive for MBL production by above two tests were subjected to determination of Minimum Inhibitory Concentration (MIC) of Imipenem by agar dilution method [12].MIC of IMP-EDTA was determined. One ml EDTA solution of 0.5M was added to 1ml Imipenem solution spanning similar concentration as done for MIC Imipenem. Each 2ml of IMP-EDTA solution was added to 18 ml molten Mueller Hinton agar& poured on plates. They were allowed to set. A fixed inoculum of test strains was spot inoculated on these plates. Reading was taken after 18-24 hrs incubation. The highest dilution that inhibits the growth of the organism was taken as MIC [12].

Data was collected from Dr. D.Y. Patil Hospital & Research Institute, Kolhapur. Data analysis is done by using MS-Excel computer language (Data analysis tool park option). Commercially available antibiotic disks and EDTA powder manufactured by Hi- Media Laboratories Pvt .Limited, Mumbai were used.

Results

Total 121 pus samples from wounds in the diabetic patients with foot infection were studied. Out of these 8 samples were cultures negative. Total number of isolates obtained were 139. Out of which 80 isolates were of gram negative bacilli (57.55%) & remaining 59 isolates were of gram positive cocci (GPC 42.44%). Total of 22 strains *P.aeruginosa* were isolated (15.82%)

Table1: Resistance pattern of *P.aeruginosa* isolated from pus samples obtained from wounds in Diabetic foot infections

Antibiotic	**No. of isolates resistant (n=22)**	**Percentage**
Ceftazidime	17	77.27%
Cefotaxime	18	81.81%
Cefepime	12	54.54%
Gentamicin	12	54.54%
Amikacin	11	50%
Aztreonam	14	63.63%
Piperacillin	19	86.36%
Piperacillin-tazobactam	18	81.81%
Ciprofloxacin	15	68.18%
Imipenem	15	68.18%

Resistance to imipenem showed by strains of *P. aeruginosa* was 68.18%. Least resistance was shown to Amikacin 50%.

Table 2: Percentage of MBL producing *P.aeruginosa* isolated from pus samples obtained from wounds in Diabetic foot infections (n = 22)

Name of the test	**No. of isolates positive for MBL production**	**Percentage of MBL positive isolates**
EDTA Disk Synergy Test(EDS)	12	54.54%
Imipenem-EDTA combined Disk Method	12	54.54%

In our study, we used two methods for detection of MBL production- 1) EDTA Disk Synergy Test & 2) IMP-EDTA combined disk method. By using both the methods MBL production was seen in 12 isolates (54.54%).

All MBL producing *P.aeruginosa* strains were subjected to determination of Minimum Inhibitory Concentration (MIC) of Imipenem by agar dilution method. MIC of all MBL producing isolates ranged from 16 µg /ml to 256 µg/ml. 16 to 128 fold reductions in MIC of IMP-EDTA combination was observed in our study.

Table 3: Minimum Inhibitory Concentration (MIC) of Imipenem in MBL producing *P.aeruginosa* strains (n=12)

MIC values (µg /ml)	**No. of isolates**	**Percentage of isolates**
1	0	0
2	0	0
4	0	0
8	0	0
16	2	16.66%
32	2	16.66%
64	3	25%
128	2	16.66%
256	2	16.66%
512	1	8.33%

Discussion

Diabetes mellitus has reached epidemic properties worldwide as we enter the new millennium. Increase in

both prevalence & incidence of type II diabetes occurred globally. Diabetic foot lesions are one of the most serious causes of morbidity among diabetic people and require long hospital stay. Due to frequent hospitalization, these patients suffer from infection with multidrug resistant (MDR) organisms which leads to serious complications.

Pseudomonas aeruginosa is most frequent nosocomial pathogen. Acquired drug resistance is common in nosocomial isolates of *Pseudomonas spp.* So the present study was conducted to find out metallo β-lactamase producing *Pseudomonas aeruginosa* in diabetic foot infections. Total 121 diabetic patients with foot infection were studied.107 patients were from IPD & 14 were from OPD. Age of the patients ranged between 36-76 years (mean age 50years). 87 patients were male & 34 were female. Out of 121 pus samples 8 samples were culture negative (6.61%)

Total no. of organisms isolated were 139.Out of these 80 were GNB (57.55%) & remaining 59 were gram positive cocci (42.44%). Isolates of *P. aeruginosa* obtained from these samples were 22 (15.82%). A bacteriological study of DFI in Tertiary Care Hospital of Dhaka city by Smir Paul et al reported isolation of *P. aeruginosa* in DFI as 26.7%[13]. Another study of MBL producing *P. aeruginosa* in DFI by Murugan et al revealed isolation of 18.91% of *P. aeruginosa* from patients of diabetic foot infection[8]. Ravishekhar Gadepalli et al (2006), reported isolation of *P. aeruginosa* from patients of DFU as 9.8%[7]. Another Microbiological study of DFI by Sivaraman Umadevi et al in 2011, reported 17% *P. aeruginosa* in DFU[14].

In the present study, antibiotic susceptibility testing of *P. aeruginosa* isolates showed resistance to antibiotics as follows- Piperacillin 86.36%, Piperacillin- tazobactam 81.81%,Amikacin 50%,Ceftazidime 77.27%,Gentamicin 54.54% and Imipenem 68.18%. In 2010,S.Murugan et al, in his study found almost all *P. aeruginosa* isolates were 100% resistant to Piperacillin, Cefepime, Cefotaxime, Aztreonam & 71.4% resistance to Imipenem,42.8% resistance to Amikacin & gentamicin[8]. Samir Paul et al in his study found resistance pattern of *P. aeruginosa* in DFI as follows – ceftazidime 66.7%, ciprofloxacin 66.7%, gentamicin 55.6%, amikacin 61.1% [13]. He also reported that only Imipenem was most effective against GNB.

In this study both the tests used to detect MBL production i.e. EDTA Disk Synergy Test (EDS) & Imipenem- EDTA Combined Disk Method showed equal no. of MBL producing *P. aeruginosa* strains (12/22) 54.54% .Varaiya et al (2008) reported 60% MBL producers in DFU[15]. Murugan et al,in 2010 reported 70% isolates of *P.aeruginosa* as MBL producers by Double Disk Synergy Test (DDST) in DFI[8]. There are reports of MBL producing *P. aeruginosa* in countries like Singapore [16] and Korea [17].

In present study, MIC of IMP & IMP-EDTA was detected by agar dilution method. MIC of all MBL producing isolates ranged from 16µg/ml to 256µg/ml. 16 - 128 fold reduction in MIC was observed when IMP-EDTA combination was used. Similar results were reported by Hemlatha et al, MIC values of MBL producing *P.aeruginosa* 8-128µg/ml & MIC reduction of 8- 128 fold with IMP-EDTA combination method [12].

Metallo β-lactamases (MBL) require divalent cations of zinc as a cofactor for enzyme activity and have potent hydrolyzing activity not only against carbapenem but also against other β-lactam antibiotics[18]. The genes responsible (IMP & VIM) for MBL production can be chromosomally or plasmid mediated & poses a threat of spread of resistance by gene transfer among gram negative bacilli[19].

Infections caused by resistant strains are a matter of concern in many hospitals worldwide, since they are associated with three fold higher rate of mortality, nine fold higher rate of secondary bacteremia ,two fold increase in length of hospital stay and considerable increase in health care cost [20].

P.aeruginosa has now clearly emerged as a major nosocomial pathogen in immunocompromised & debilitated patients. It has always been considered as difficult target to antimicrobial therapy.

Conclusion

Foot ulceration & infection is most frequent & serious complication of diabetes mellitus. These patients need repeated hospitalization so also they suffer from polymicrobial infections. *Pseudomonas aeruginosa* has one of the broadest ranges of infectivity. Present study documents prevalence of metallo β-lactamase producing *P.aeruginosa* in diabetic foot infections as 54.54%.Infection with such multidrug resistant organism will increase in morbidity & mortality in these patients. So regular reporting of such MBL producing *P.aeruginosa* will help the clinician to start the proper antibiotic the earliest.

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