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Prevalence and Antimicrobial Susceptibility Pattern of Metallo β Lactamase Producing *Pseudomonas aeruginosa* in Diabetic Foot Infection

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Abstract: *Pseudomonas aeruginosa* is an invasive organism that frequently causes severe tissue damage in diabetic foot ulcers. A major problem in *P. aeruginosa* infection may be that this pathogen exhibits a high degree of resistance to a broad spectrum of antibiotics. Here we isolated and characterized *P. aeruginosa* population from a diabetic foot ulcers and their resistant pattern. It is a retrospective study, which comprised of 128 diabetic foot ulcer patients. All the pus samples were subjected to Gram staining, bacterial culture and the antibiotic sensitivity to 16 different antibiotics as per the standard procedures. Metallo beta lactamase producing *P. aeruginosa* strains have been reported to be an important cause of nosocomial infections. There is not enough data from Coimbatore, South India regarding their prevalence among the diabetic foot infection patients. The present study was undertaken over a period of one year from June 2007 to May 2008 to study their prevalence and their antimicrobial susceptibility pattern according to CLSI guidelines. 14 isolates were obtained from diabetic foot ulcer patients. These isolates were subjected to susceptibility testing to antipseudomonal drugs as per CLSI guidelines. They further screened for MBL production by Modified Hodge test and Double Disk Synergy test. Of the 14 (18.9%) isolates of *P. aeruginosa,* 14 (100%) and 7 (71.4%) isolates were found to be resistant to meropenem and imipenem, respectively and 7 (50%) were found to be MBL producers.

Key words: Metallo beta lactamase · *Pseudomonas aeruginosa* · Diabetic foot infection

INTRODUCTION

Diabetes is a disease of complications popularly known as Iceberg disease [1] and it is a chronic disorder which affects large segment of population [2]. Diabetes is estimated to touch 73.5 million by 2025 in India as a consequence of longer life expectancy, sedentary life style and changing dietary patterns [3]. As many as 25 % of diabetic individuals are expected to develop severe foot problems at some point in their life time, that often ends with amputation [4]. Diabetic foot infections are more severe and more difficult to treat than infections in non-diabetics. *Pseudomonas aeruginosa* may cause severe tissue damage in diabetic and should never be disregarded as insignificant in diabetic foot ulcers. The consequence of considering the bacteria as contaminants or commensals may result in sepsis and amputation [5].

P. aeruginosa exhibits resistance to a variety of antimicrobials including beta lactams. Carbapenems are often used as antibiotics for treatment of infections caused by β-lactam resistant *P. aeruginosa* [6]. The carbapenems available for use in India are imipenem and meropenem [7]. However, a group of carbapenem hydrolyzing beta lactamases known as metallo β lactamases (MBLs), belonging to Ambler class B [8] have been reported in several countries particularly in clinical isolates of *P. aeruginosa* [9]. Pitout *et al.* [10] reported the presence of IMP-non susceptible. *P. aeruginosa* in clinical isolates and showed that 46% of the strains were MBL positive. MBL producing *P. aeruginosa* strains are resistant to most broad-spectrum β lactams, aminoglycosides and fluoroquinolones, the traditional antipseudomonal antimicrobials [11]. Resistance to carbapenem is due to decreased outer membrane

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permeability, increased efflux systems, alteration of penicillin binding proteins and carbapenem hydrolyzing enzyme carbapenemase [12].

Metalo β lactamases (MBL) requires divalent cations of zinc as cofactors for enzyme activity and have potent hydrolyzing activity not only against carbapenem, but also against other β-lactam antibiotics [13]. The genes responsible (IMP and VIM) for MBL production can be chromosomally or plasmid mediated and poses a threat of spread of resistance by gene transfer among the Gram-negative bacilli [12]. The appearance of MBL genes and their spread among bacterial pathogens is a matter of concern with regard to the future of antimicrobial chemotherapy [14]. The detection of MBL producing Gram- negative bacilli, especially *P. aeruginosa* is vital for the optimal treatment of patient's particularly for ill and hospitalized patients [15]. The rapid detection of MBL-positive Gram- negative bacilli is necessary to aid infection control and also to prevent their dissemination [16].

The Modified Hodge test uses *Escherichia coli* ATCC 25922 and 10 μg imipenem disc instead of *Staphylococcus aureus* ATCC 25923 and a 10 μg penicillin disc, respectively [17, 18]. The double disk synergy test has been modified using EDTA instead of a β lactamase inhibitor. Modified Hodge and EDTA disk synergy tests detect and differentiate carbapenemase and metallo β lactamase production from other β-lactamases [18]. A simple reliable test to detect carbapenemase and metallo β lactamase production is useful particular in situations where carbapenem and β-lactams are commonly used in the therapeutic regimen [15]. There is not much information available on MBL producing isolates form *diabetes mellitus* patients with foot ulcers in Coimbatore, South India. We therefore undertook this study to evaluate the usefulness of these tests for the detection of MBL producer *P. aeruginosa* isolated from diabetes patients with foot ulcers of tertiary care hospitals in Coimbatore, South India.

MATERIALS AND METHODS

In the present study, a total of 128 pus samples were collected for *P.aeruginosa* screening from diabetic patients with foot ulcers and they were obtained from reputed diabetic clinics and tertiary care hospitals in and around Coimbatore, South India. "Soft tissue infections" includes foot wound and limb threatening infection specimens received during June 2007 to May 2008 were included in the analysis. Specimens were obtained using aseptic techniques to avoid contamination and were promptly transported to the laboratory in a sterile swab in an ice-cold condition.

Antimicrobial susceptibility test was performed by disc diffusion technique according to Clinical and Laboratory Standards Institute guidelines [19]. The following antibiotic discs (drug concentration in ig) were used: Amikacin (30 μg), Ampicillin (30μg), Aztreonam (30μg), Carbenicillin (30μg), Cefepime (30μg), Cefotaxime (30 μg), Gentamicin (30 μg), Imipenem (10 μg), Levofloxacin (30μg), Meropenem (10 μg), Norfloxacin (30μg), Ofloxacin (30μg), Piperacillin (10μg), Polymyxin-B (10μg), Ticarcillin (30μg), Tobramycin (30μg).

Imipenem resistant strains were checked for production of metallo β-lactamase by the modified Hodge test and EDTA disc synergy test. Modified Hodge test was carried out on Mueller Hinton agar and the plate was inoculated using a cotton swab dipped in an overnight culture suspension of *E. coli* ATCC 25922 (0.5 McFarland's standard). After brief drying, 10 μg of imipenem disc was placed at the centre of the plate and test strains were streaked from the edge of the disc to the periphery of the plate in four different directions. After overnight incubation, the plates were observed for the presence of 'cloverleaf shaped' zone of inhibition. The plates with such zones were interpreted as modified Hodge test positive.

For the EDTA disk diffusion synergy test, an overnight broth culture of the test strain (0.5 McFarland's standard) was used to inoculate a plate of Mueller Hinton agar. After drying, 10 μg of imipenem disc and a blank filter paper disk (6 mm in diameter, Whatmann filter paper no.2) were placed 10 mm apart from edge to edge, 10 ml of 0.5 M EDTA solution was then applied to the blank disc, which resulted in approximately 1.5 mg/disc. After overnight incubation, the presence of an enlarged zone of inhibition was interpreted as EDTA synergy positive.

RESULTS AND DISCUSSION

Of the total 128 diabetic patients suffering from foot infection taken for the study, 94 (73.4%) were males and 34 (26.5%) were females and the male to female ratio being 3:1. The age of the patients ranged between 40 to 60 years. Among these 10 (7.18%) patients have underwent amputation.

Sof the 128 specimens of diabetic foot infections, 74 (57.81%) specimens showed culture positive and the rest 40 (31.25%) were negative. Among the isolates, the aerobic Gram-negative *Pseudomonas species* were found

Table 1: Resistance pattern of *P. aeruginosa* from diabetic foot ulcers

Antimicrobial agents	No of Resistant (14) Strains (%)
Amikacin (Ak)	6 (42.8)
Ampicillin (Am)	14 (100)
Aztreonam (Ao)	14 (100)
Carbenicillin (Cb)	14 (100)
Cefepime (Cpm)	14 (100)
Cefotaxime (Ce)	14 (100)
Gentamicin(G)	6 (42.8)
Imipenem (I)	10 (71.4)
Levofloxacin (Le)	8 (57.1)
Meropenem (M)	14 (100)
Norfloxacin (Nx)	2 (14.3)
Ofloxacin (Of)	12 (85.7)
Polymyxin (Pb)	14 (100)
Piperacillin (Pc)	14 (100)
Ticarcillin (Ti)	14 (100)
Tobramycin (Tb)	10 (71.4)

to be 60 (81.08%). From the 74 positive specimens of diabetic foot infection, *Pseudomonads* isolates includes *P. aeruginosa* 14 (18.91%) and non-pigmented *Pseudomonas species* 60 (81.08%), respectively. The occurrence of non-pigmented *Pseudomonas* was higher than the pigmented *P. aeruginosa*. [20, 21] reported 16.80 and 29.8% of *P. aeruginosa* in Chennai. This emphasizes the high prevalence of aerobic Gram- negative pathogens in the diabetic soft tissue infections in Southern India. In their retrospective study on diabetic foot ulcers in tertiary care hospital in New Delhi [22] revealed that *P. aeruginosa* (12.0%) is becoming predominant in diabetic foot ulcer patients. *P. aeruginosa* is a ubiquitous versatile environmental microorganism that is the leading cause of opportunistic human infections [23]. Similarly, Sharma *et al.* [24] reported that *P. aeruginosa* were the most common causes of diabetic foot infections.

The drug resistance patterns (Table 1) of *P. aeruginosa* isolated from diabetic foot ulcer patients were found to be as follows: almost all of the 14 *P. aeruginosa* strains screened showed 100% resistant to piperacillin, meropenem, cefepime, cefotaxime, aztreonam, ampicillin, polymyxin-B, carbenicillin and ticarcillin, 71.4% resistant to imipenem and tobramycin, 42.8% resistant to amikacin and gentamicin, 57.1% resistant to levofloxacin, 85.7% resistant to ofloxacin. The percentage of intermediate resistance observed against gentamycin and norfloxacin were found to be 45% (not shown in the table). No single antibiotic showed 100% sensitivity to all *P. aeruginosa* clinical isolates. Resistance was least with norfloxacin (14.3%) followed by gentamicin and amikacin (42.8%).

In the present study, 10 (71.4%) strains were resistant to imipenem by disc diffusion test. Both the modified Hodge and DDST detected equal number of *P. aeruginosa* strains isolated from diabetes patients with foot ulcers (50%) and (70%) as carbapenemase and metallo β lactamase producers. Varaiya *et al.* [25] have reported 60% MBL producer from diabetes patients. The modified Hodge test is a simple method for screening for metallo β lactamase production but occasional false positive have been reported in literature [6]. But, in the present study, false positive results have not been observed. Moreover, it has been stated that these can be avoided by adding 10 µl of 50 mM Zinc Sulphate (100µg/disc) to the imipenem disc or by incorporating a final concentration of 70µg/ml into Mueller Hinton agar [6]. However, this has also not been carried out in this study

There are reports on MBL production in *P. aeruginosa* from various countries like Brazil [26], Korea [27], Singapore [28] and France [29]. Metallo β-lactamases was first reported as a zinc dependent enzyme in *Bacillus cereus* in mid 1960s [30]. A few decades' later imipenem hydrolyzing metalloenzymes were found in *Aeromonas hydrophila* [31] and *Bacteroides fragilis* [32].

MBL production among the isolates of *P. aeruginosa* is a significant problem in diabetes patients with foot ulcers. With increasing isolation of ESBL producing isolates in the foot ulcers of diabetes patients necessitates the use of carbapenems; the problem of MBL producing is also increasing. The development of simple screening tests, designed to detect acquired MBL production will be a crucial step towards large scale monitoring of these emerging resistant determinants. *P. aeruginosa* is a pathogen associated with numerous nosocomial infections in immunocompromised patients [33]. Carbapenems are the drugs of choice for multidrug resistant *P. aeruginosa* and ESBL producing organisms. However, resistance to carbapenems due to reduced uptake of drug leads to imipenem/meropenem resistant isolates [34]. In various studies across the world, varying resistance (4-60%) has been seen towards imipenem and meropenem [35, 36]. We found 100 and 71.4 percent resistance to imipenem amd meropenem. In 2002 from India, Navneeth *et al.* [37] was the first to report MBL production in *P. aeruginosa* to be 12 per cent. Since then, the incidence of MBL production in *P. aeruginosa* has been reported to be 10-30 per cent from various clinical specimens across the country.

Table 2: Multi- Drug resistance pattern among the clinical isolates of *P. aeruginosa*

No of drugs resistant	No of isolates(n=14)	Resistance (%)
≥ 10	14	100
≥ 11	12	85.7
≥ 12	12	85.7
≥ 13	6	42.8
≥ 14	4	28.6
≥ 15	4	28.6
≥ 16	0	0

From the above Table 2, it is found that about 85.7% of the isolates were found to be resistant from 10 to 12 antibiotics, which implies that an alternative choice of antibiotic is the need of the hour to combat *P. aeruginosa* infection associated with diabetic foot ulcer. Shankar *et al.* [21] reported the antibiotic resistance pattern exhibited by *P. aeruginosa* against cefotaxime, cefeperazome and gentamicin to be in the order of 63.6, 54.5 and 50%, respectively. In the same study intermediate resistance was also observed against imipenem (13.6%), amikacin (9%) and piperacillin (9%).

The clinical isolates are more resistant to antimicrobial agents, which may be due to the fact that clinical strains had been submitted to the selective action of both antibiotics and disinfectants and as expected they exhibited resistant to imipenem, piperacillin, cefotaxime, cefoperazone, ciprofloxacin and gentamicin indicating the emergence of multi-drug resistance strains.

With increasing numbers of diabetic population worldwide and with principal importance to the world's diabetic capital (India), there is a significant rise in prevalence of foot infections. Studies have been done on the microflora of diabetic foot infections involving both aerobic and anaerobic bacteria, but very few reports are available on the specific prevalence of metalllo β lactamase producer *Pseudomonas* in them. India has the largest number of diabetic individuals with appreciable poor socioeconomic conditions; the study on this intrinsically resistant organism in diabetic foot infections assumes greater significance. Contrary to previous studies, our study has shown the incidence of *P. aeruginosa* in a significant number (18.91%) of diabetic foot ulcers. Antibiogram also reveals that piperacillin, amikacin and imipenem retain the high level of antipseudomonal activity and gentamicin, cefoperazone and cefotaxime with least activity. Amikacin, piperacillin and imipenem were found to be better drug choices for patients in this part of the region when compared to ciprofloxaicin, gentamicin and other third-generation cephalosporins.

The emergence of carbapenamase producing strains represents a serious therapeutic and epidemiological problem which can be circumvented only by the early detection and control of such multidrug resistant pathogens in diabetic foot ulcer patients. The rapid detection of carbapenemases and metallo-β-lactamase producing isolates must be followed up on a routine basis in the laboratory, since the number of metallo-β-lactamase producing isolates are increasing and are posing a problem for the clinicians during the treatment of diabetic patients with foot ulcers. The routine detection of MBLs among *P. aeruginosa* isolated from diabetic foot ulcers will ensure optimal patient care and the timely introduction of appropriate infection control procedures.

To conclude, the present study detected a high level of resistance in *P. aeruginosa* isolated from diabetic foot ulcers to most antibiotics tested. Moreover, the study indicates the urgent need of action to prevent spread of MBL producing *P. aeruginosa* with reference to diabetic foot ulcers by developing reliable and rapid detection methods. So, that it will enable the clinical microbiologist to start detecting the MBL's accurately and routinely in diagnostic laboratories.

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