Original article :

Bacteriological profile and antibiotic susceptibility pattern in diabetic ulcers with specialreference to metallo-beta- lactamase production

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ABSTRACT

Diabetes is one of the most common complaints identified in India. Diabetic foot problems like ulcerations, infections and gangrene, are the general reasons of hospitalization in diabetic patients. Foot ulcerations occur as a result of trauma (often trivial) in the presence of neuropathy or peripheral vascular disease with infections occurring as a secondary phenomenon following disruption of the protective epidermis. The treatment of diabetic foot infections, ulcer care etc. are expensive and place an incredible burden on the health care system. The main objectives of the present study are to isolate and identify the microorganisms causing diabetic foot ulcers, their antibiotic susceptibility pattern and detection of metallo-beta-lactamase (MBL) production. One hundred fifty pus samples over the age groups 0-90 years were collected from the deep wounds and processed microbiologically using standard methods for culture and sensitivity. One hundred eleven samples showed microbial growth and 39 samples were culture sterile.*Pseudomonas aeruginosa* was the predominant microorganism 29 (19.3%), followed by *Klebsiella pneumoniae* 9(6%). Both the organisms showed high susceptibility to piperacillin.Twenty one isolates of *Pseudomonas aeruginosa* showed carbapenem resistance (37%) and screening for MBL production by EDTA disc potentiation test showed, 38 isolates (66.6%) were MBL positive. The double disc synergy test revealed 47 (82.4%) was positive for MBL production among 57 *P.aeruginosa* isolates. The study revealed double disc synergy test is more sensitive than EDTA disc potentiation test for the detection of MBL of *P.aeruginosa*.The microorganisms causing diabetic foot ulcers are usually multidrug resistant; hence screening of multidrug resistant bacteria and the detection of carbapenamase production in diabetic foot ulcer patients is a key step to formulate effective treatment strategies for these patients.

Key words: Diabetic foot ulcer, Microorganisms, Antibiotic susceptibility, MBL production.

INTRODUCTION:

Diabetes mellitus is a metabolic disorder that primarily affects microvascular circulation. In the extremities microvascular disease due to “Sugar Coated Capillaries” limits the blood supply to the superficial and deep structures (Vishnu datta, 2014). Pressure due to ill-fitting shoes or trauma further compromises the local blood supply at the microvascular level, predisposing the patient to infections. The infections may involve skin, soft tissues, bone or and other tissues. Most Diabetic foot infections occur in the setting of good dorsalis pedis pulses, this finding indicates that the primary problem in diabetic foot infections is microvascular compromise (Damir, 2011). Impaired microvascular circulation hinders white cell migration into the area of infection and limits the ability of antibiotics to reach the site of infection in an effective concentration. Diabetic neuropathy

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may be encountered in conjunction with vasculopathy (Russell, 2014). This may allow for incidental trauma that goes unrecognized like blisters, penetration of foreign body etc.Diabetes mellitus is recognized as an epidemic in the Asian sub-continent affecting nearly 25 million in India alone (Rewale*et al*., 2014). Diabetic foot ulcers are estimated to affect 15% of all Diabetics during their life time and precede almost 85% of all foot amputations. The causative pathogens are varied and can range from aerobic organisms such as *Staphylococcus aureus*, β-hemolytic *Streptococci*, *Enterococci*, Coagulase positive *Staphylococci*, *Proteus mirabilis*, *Proteus vulgaris*, *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp., *Pseudomonas* spp., carry special role for causing continuous and extensive tissue destruction with the poor blood circulation of the foot (Bowler et al., 2001). A high frequency of anaerobic infections has also been noted.However, bacteria have proven to be much more innovative and adoptive than we imagined. And have developed resistance to antibiotics at an ever increasing pace. Antibiotic resistance problems are detected as they emerge and actions are taken immediately to curtail them.Carbapenem group of antibiotics play a vital role in the management of hospital acquired infections because of the broad spectrum activity and stability to hydrolysis by most of the β- lactamases, including extended spectrum β-lactamases (ESBLs) (Nicolau, 2008). Nosocomial outbreaks of carbapenem resistant *Pseudomonas aeruginosa* and Acinetobacter spp due to Metallo-β-lactamase (MBLs) production have been reported from different regions (Crespo *et al*., 2004). The emergence of these MBLs in gram negative bacilli is becoming a therapeutic challenge as these enzymes possess high hydrolytic activity that leads to degradation of higher generation Cephalosporins. Moreover, the treatment alternatives are unavailable or expensive or toxic with poor outcome. Plasmid mediated MBL genes spread rapidly to other species of gram-negative bacilli;therefore rapid detection of Metallo-β-lactamases production is necessary to modify therapy and to initiate effective control to prevent their dissemination (Crespo *et al*., 2004). Using the right antibiotic in an infectious situation as determined by antibiotic sensitivity testing can prevent unnecessary development of multiple drug resistance. In the present investigation, various bacterial isolates have been isolated from patients with diabetic foot ulcers, studying their antibiotic susceptibility pattern specifically in relation to *Pseudomonas aeruginosa* and detection of metallo-beta-lactamase production in *Pseudomonas aeruginosa* are the key objectives of the study.

MATERIALS AND METHODS:

The present study was undertaken in the Department of Microbiology, GIMSR, Visakhapatnam, over a period of one year-six months from March 2015 to September 2016. One-hundred-fifty diabetic patients with foot ulcer attending the Medicine and Surgery outpatient and inpatient departments were included in the study.Based on Meggit Wagner's classification, diabetic foot ulcers were gradedin the present study. Two pus swabs were collected from each patient and kept in a sterile glass bottle and were subjected for smear preparation and culture.The heat fixed smears were subjected to Gram's staining initially for the identification of microorganisms. Later the pus samples were inoculated onto blood agar and MacConkey agar plates and incubated overnight at 37^{o}C in an incubator. After 24h the plates were examined for colony morphology and different biochemical reactions like Indole, Methylred, Voges-Proskauer, Citrate utilization tests (IMViC), Triple sugar iron agar test (TSI) and detection of urease, catalase, coagulase, oxidase production by the microorganisms are used as the confirmatory tests (Collee, 2006).

Antibiotic susceptibility testing: Antibiotic susceptibility testing was done by Kirby-Bauer disc diffusion method. The isolates were grown in peptone water by incubating at 37^{o}C for two hours or till the turbidity

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matched that 0.5 McFarland Nephlometer tube. The inoculum was streaked onto the Mueller Hinton agar plate with the help of sterile swabs and commercially available antibiotic discs (Hi media) were placed on the surface and plates were incubated at 37^{o}C in an incubator overnight. Next day the zones of inhibition were measured and interpreted according to CLSI guidelines. The antibiotics used were Imipenem (10 µg), ciprofloxacin (5 µg), Amoxyclav (20/10 µg), Amikacin (30 µg), Piperacillin (100 µg), Cefotaxime (30µg), Ceftazidime (30µg) and Cefuroxime (30µg) (Collee, 2006).

Detection of Metallo-β-Lactamase production:*Pseudomonas aeruginosa* isolates which are susceptible or resistant to imipenem were tested for MBL production by EDTA-Disc potentiation test and Double disc synergy test (Uma Choudhary*et al*., 2008).

EDTA-Disc potentiation test: Test organisms were inoculated onto Mueller-Hinton agar plates. A blank filter paper (whatmann no.2) was placed and the following discs ceftazidime(30µg), cefotaxime(30µg), cefuroxime(30µg), Ceftriaxone(30µg) were placed 25mm center to center from blank disc. 10µl of 0.5M EDTA solution was added to the blank disc and the plate was incubated overnight at 37^{o}C.Enhancement of the zone of inhibition in the area between EDTA disc and anyone of the four cephalosporin discs, in comparison with the zone of inhibition on the far side of the drug was interpreted as positive result (Uma Choudhary*et al*., 2008).

Imipenem-EDTA Double disc synergy test (DDST): Test organisms were inoculated onto Mueller Hinton agar plates as recommended by CLSI guidelines. An imipenem disc (10µg) was placed 20mm center to center from a blank disc contianing10µl of 0.5M EDTA (750µg). Enhancement of zone of inhibition in the area between imipenem and EDTA disc in comparison with the zone of inhibition on the far side of the drug was interpreted as positive. (Lee *et al*., 2003, Agarwal and Lodhi, 2008).

RESULTS:

Total of 150 Diabetic patients with foot ulcers were included in the present study. According to Meggits Wagner's classification, diabetic foot ulcers were given grades from 0-5. Majority of the patients in the present study were reported in grade-1 (92%) followed by grade-4 (8.7%) (Table 1).

Table 1: Meggit Wagner's classification of diabetic foot ulcers

Grade (Meggit Wagner's classification)	**Lesion**	**Number of Patients**	**Percentage (%)**
0	No open lesion may have deformity or cellulitis.	0	0
1	Superficial Diabetic ulcer (partial or full thickness)	138	92%
2	Ulcer extension to ligament, tendon, joint capsule or deep fascia without abscess or osteomyelitis.	0	0
3	Deep ulcer with abscess, osteomyelitis or joint sepsis.	0	0
4	Gangrene localized to portion of forefoot or heel.	12	8%

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5	Extensive gangrenous involvement of the entire foot.	0	0
	Total:	150	100%

Age and sex wise distribution shows male predominance i.e. 110 (73.3%) males and females 40 (26.7%) in the age group of 30-80 years with the p ≥0.05 (Table 2). While studying the duration of diabetes in 150 patients as on the demographic variable, predominant duration ranging from 6-10 years i.e. 64 (42.5%) followed by 11-15 years i.e. 40 (26.6%), 0-5 years (26 17.4%), 16-20 years 20(13.4%) with the p ≥0.05.

Table 2: Age and Sex wise distribution of cases

Age in Years	**Male**	**Percentage (%)**	**Female**	**Percentage (%)**
0-10	0	0	0	0
11-20	0	0	0	0
21-30	0	0	0	0
31-40	1	0.6%	0	0
41-50	5	3.3%	4	2.6%
51-60	43	28.6%	19	12.6%
61-70	56	37.3%	17	11.3%
71-80	5	3.3%	0	0
81-90	0	0	0	0
Total	**110**	**73.3%**	**40**	**26.7%**

Duration of Diabetic ulcer is shown in Figure 1. Predominant duration is 21 to 30 days where 63 (42%) of patients were present and least cases were reported in 91-120 days 5 (3.4%). Out of 150 samples pure isolates were reported in 61 samples (40.6%), polymicrobial isolates in 50 samples (33.3) and 39 samples (26%) were cultures sterile. Among the pure isolates, 29 samples (19.3%) showed the growth of *P.aeruginosa* followed by *K.pneumoniae* 9 (6%), *E.coli* 4 (7%), *S.aureus* 12 (21%), and coagulase negative staphylococci 7 (12.2%). Analysis of polymicrobial isolates showed that, predominant mixture was *P.aerugonisa*+ *Proteus* spp., + *Klebsiella* spp., 12(24%) followed in order *Staphylococcus aureus* + *Proteus* spp., 9(15.7%), *E.coli* + *Proteus* spp., 8(14%), *Pseudomonas aeruginosa* + *Staphylococci* 6(10.5%), *Klebsiella pneumoniae* + *Proteus*spp., 4 (6.2%),*Klebsiella pneumoniae* + *Pseudomonas aeruginosa* 4 (7%), CONS + *P.aeruginosa* 4 (7%) and *Pseudomonas aeruginosa* + *Proteus* spp., 3(5.2%).

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Figure 1:Duration of diabetic ulcer

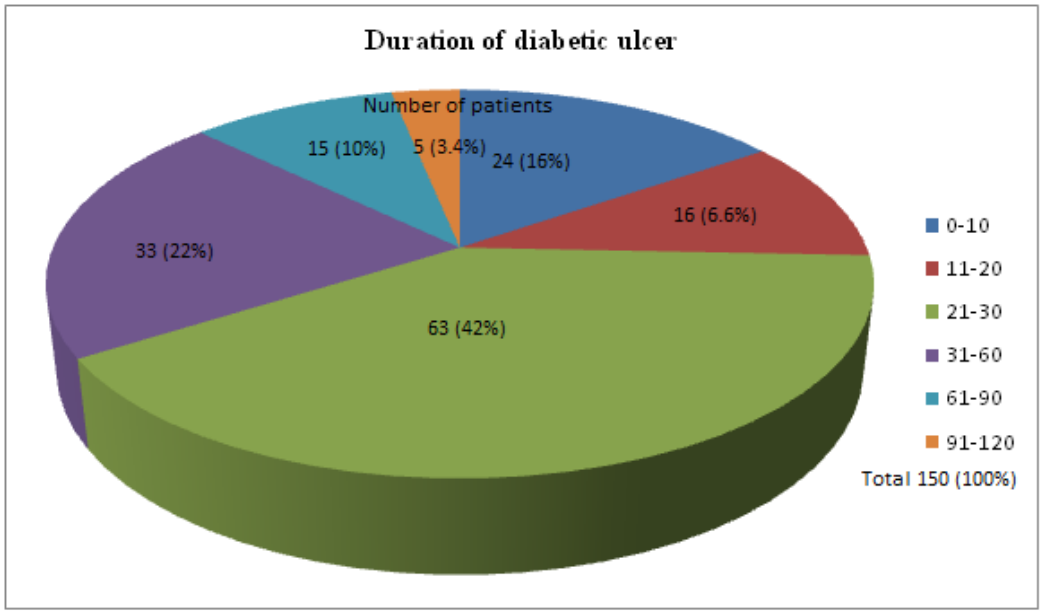

Table 3 showed the antibiotic susceptibility pattern of bacterial isolates. *P. aeruginosa* was highly susceptible to piperacillin 34 (59.6%), Klebsiellaspp showed maximum susceptibility to piperacillin 24 (85.7%), Proteus spp., was highly susceptible to imipenem 26 (86.6%), E.coli sensitive to most of the drugs imipenem, amoxyclav, Amikacin, Cefotaxime, ceftazidime 4 (80%), *Staphylococcus aureus* highly sensitive to Cefotaxime 21 (77.7), CONS showed high sensitivity to Ciprofloxacin and amoxyclav 11(100%). The results also revealed that, 21 isolates of *Pseudomonas aeruginosa* showed carbapenem resistance (37%) and 36 showed sensitivity (63.1%).Screening for MBL productionby EDTA disc potentiation test showed, among 57 isolates of *P.aeruginosa* 38 (66.6%) were MBL positive and 19(33.3%) were MBL negative. Both imipenem resistant strains and sensitive strains were screened for MBL production. Whereasdouble disc synergy test revealed 47 (82.4%) were positive and 10(17.5%) were negative for MBL production among 57 *P.aeruginosa*isolates. Table 4 showed the comparison of MBL detection by EDTA disc potentiation test and double disc synergy test. EDTA detected 38(66.6%) and Double disc synergy test detected 47(82.4%) of 57 isolates. It indicates double test synergy test is more sensitive than EDTA disc potentiation test for the detection of MBL of *Pseudomonasaeruginosa*.

Table 3: Antibiotic susceptibility pattern of clinical bacterial isolates

Micro organisms	**Imipenem (10µg)**	**Ciproflox acin (5 µg)**	**Piperacillin (100µg)**	**Amoxycl av (20/10 µg)**	**Amikacin (30 µg)**	**Cefotaxime (30 µg)**	**Ceftazidime (30 µg)**	**Cefuroxime (30 µg)**

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P.aeruginosa	21 (36.8%)	32 (56.1%)	34 (59.6%)	27 (47.3%)	18 (31.5%)	16 (25%)	12 (25%)	21 (36.8%)
K.pneumoniae	23 (82%)	19 (67.5%)	24 (85.7%)	10 (35.7%)	16 (57.1%)	13 (46.4%)	13 (46.4%)	9 (36.5%)
P.mirabilis	26 (86.6%)	20 (66.6%)	20 (66.6%)	19 (63.3%)	19 (63.3%)	15 (50%)	13 (46.3%)	11 (36.6%)
E. coli	4 (80%)	2 (40%)	3 (60%)	4 (80%)	4 (80%)	4 (80%)	4 (80%)	1 (20%)
S.aureus	20 (74%)	20 (74%)	13 (45.5%)	19 (70.3%)	21 (77.7%)	21 (77.7%)	19 (70.3%)	9 (33.3%)
CONS	8 (72.7%)	11 (100%)	10 (90.9%)	11 (100%)	2 (18%)	10 (90.9%)	6 (54.5%)	4 (36.3%)

Table 4: Comparison of Metallo-beta-lactamase Detection by Two methods

Double Disc Synergy Test	**Positive**	**Negative**	**Percentage (%)**	**EDTA Disc Potentiation Test**	**Positive**	**Negative**	**Percentage (%)**
57	47	10	82.4%	57	38	19	66.6%

DISCUSSION:

Diabetes mellitus is a chronic disorder which causes serious morbidity and mortality due to microvascular and macrovascular complications. As the age of maturity onset diabetes is 45 and above, the majority of cases are in that age group. Mean age group of diabetes was 60 years. Male to female ratio is 2.8:1. Male dominance is only due to fact that more number of patients were admitted in surgical wards with complications of diabetes.Alavi*et al*., (2007) reported that foot ulcers are the significant complications of diabetes and most common cause of non-traumatic lower extremity amputation in the world. *Pseudomonas* species, *Enterobacter* species, *Proteus* species carry a special role and responsible for continuing and extensive tissue destruction with poor blood circulation of foot. Our study showed high number of monomicrobial isolates 61 (40.6%) and low number of polymicrobial isolates 50 (33.3%) however in contrast to the study of Alavi*et al.,* (2007), reported less number of monomicrobial isolates 10 (31.2%) and more number of polymicrobial 16 (50%) isolates. Ranjini et al. (2015) also reported more number of polymicrobial isolates 56.73% along with anaerobic organisms like Bacteroides and Peptococcus.Zubair*et al.* (2015) isolated the predominant bacteria *Staphylococcus aureus* 27.7% followed by *Klebsiella pneumoniae* 19.7% which coincides the current study reported majority of the

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isolates were *Pseudomonas aeruginosa* 29 (19.3%) in pure culture and 28 (18.6%) in mixed culture, making a total of 57 (51.35%) *Pseudomonas aeruginosa* isolates.The other organisms were *Staphylococcus aureus* 12 (8%) in pure and 15 (10%) in mixed infections, also *Klebsiella pneumoniae* 9 (6%) in pure and 19 (12%) in mixed infections.The results of the present investigation revealed that, *Pseudomonas aeruginosa* showed highest sensitivity to piperacillin 34(59.6%), *Klebsiella pneumoniae* was sensitive to piperacillin 24(85.7%), *Staphylococcus aureus* was sensitive to amikacin 21(77.7%).*Pseudomonas aeruginosa* was resistant to imipenem 36(63.1%) which was similar to the study of Sarkar *et al.,* (2000), Bisiklis*et al.* (2005) showed 34 isolates 77.2% showed imipenem resistance. *Pseudomonas aeruginosa* strains resistant to carbapenem group of antibiotics were further tested for MBL production. In contrast, Lavilla(2005)had reported majority of *P.aeruginosa*strains showed imipenem resistance 96.3% and meropenem 84%. There was carbapenem sensitivity by 21 (37%) of the 57 isolates of *Pseudomonas aeruginosa* and 36(63.1%) resistant to imipenem in the current study. Varaiya*et al*., 2008 reported 26% strains were resistant to imipenem.Attal *et al*., 2010 reported 12% resistance to imipenem.Solanki*et al*., 2015 reported 67% resistance to imipenem.Our study was in accordance to study of Mehndiratta*et al*., 2001.There were 38 (66.6%) positive by EDTA disc potentiation test and 19 (33.3%) were negative by this test.There were 47 (82.4%) positives and 10 (17.5%) were negatives by Double disc synergy test for MBL detection of *Pseudomonas aeruginosa*. The positivity rate was increased by 16.4% for MBL detection by Double disc synergy test. Kaur*et al*., 2013 reported that, Double disc synergy test was the most accurate phenotypic test to detect Metallo-β-lactamase production.In our study Double disc synergy test is noted to be more superior and sensitive test as compared to EDTA disc potentiation test in detection of MBL. The test is simple and economical, so that it can be used as a routine diagnostic work especially for the organisms found to be multi drug resistance.

CONCLUSION:

The study was aimed to isolate the bacterial flora of Diabetic foot ulcers from patients with diabetes attending the Surgical and Medical O.P and I.P departments of GIMSR Hospital, Visakhapatnam and to assess their invitro susceptibility to commonly used antibacterial agents. The incidence of Diabetic foot ulcers is more in the maturity onset Diabetics, also when long standing more than 10 years duration. The infective organisms isolated were in pure as well as polymicrobial growths. Among the polymicrobial infections *P. aeruginosa*, Proteus spp., and Klebsiella spp., were common. *P. aeruginosa* was dominant organism. It is also resistant tocarbapenems. All isolates of *P.aeruginosa* was tested for MBL production by EDTA disc potentiation test which was positive 66.6% and Double disc synergy test which was positive by 82.4%. Double disc synergy test is economical, cheap & can be done routinely in every Microbiological Laboratory. The study highlights the prevalence of multiple antibiotic resistant bacteria in foot infections. Increasing isolation of ESBL producing organisms like *Escherichia coli*, Klebsiellaspp, and *P.aeruginosa* is necessitating the use of carbapenems. The inadvertent use of carbapenems in hospital setup has increased the MBL producing *P.aeruginosa*. This study documents the need to institute correct antibiotics. By detecting MBL producers we can prevent the spread of MBL by using Double disc synergy test as screening test is essential. Rapid dissemination of carbapenem resistance is worrisome and calls for implementation of surveillance studies and judicious selection of antibiotics in clinical practice.

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