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Original article

Detection of Multidrug Resistant Gram Negative Bacilli in Type II Diabetic Foot Infections

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ABSTRACT

Diabetes is a major health challenge not only in the developed countries but also in the developing nation like India .Diabetic patients usually suffer from foot ulceration leading to polymicrobial infections. Frequent infections with drug resistant organisms will not respond to penicillin & cephalosporins .Therefore study was conducted in the Dept. of Microbiology, Dr. D.Y.Patil Hospital &Research centre, Kadamwadi ,Kolhapur to find out multidrug resistance in gram negative bacilli mediated through Extended Spectrum β-lactamase (ESBL) & AmpC β-lactamase in type II diabetic foot infections during period Jan 2010 to June 2011.Total 100 clinical samples from wounds in 100 diabetic foot patients were studied. Total no. of isolates found were 108.Out of these 63 were gram negative bacilli (GNB) (58.3%) & 45 were gram positive cocci (GPC)(41.66%).GNB isolates were processed further for study. Antibiotic sensitivity testing was done by Kirby Bauer disk diffusion method .Results were recorded as per Clinical Laboratory Standard Institute – CLSI guidelines. ESBL production was confirmed by Double Disk Synergy Test (DDST) & Minimum Inhibitory Concentration (MIC).Organisms showing resistance to β-lactamase inhibitors like clavulanic acid were screened for AmpC β-lactamase production by Disk Antagonism Test (DAT) & confirmed by AmpC Disk Test. Out of 63 GNB isolates 21 were ESBL producers (33.3%) & AmpC β-lactamase was found in 13 isolates (20.63%).All ESBL producing strains were sensitive to β-lactamase inhibitors like clavulanic acid. All AmpC producers were resistant to β-lactamase inhibitors but were sensitive to Imipenem.

KEYWORDS:

Diabetic foot ulcer (DFU),Multidrug resistance(MDR),Extended spectrum β lactamase(ESBL),AmpC β-lactamase

INTRODUCTION

Diabetic foot lesions are one of the most serious causes of morbidity among diabetic people which require long hospital stay & repeated hospitalization .Resistance to β-lactam antibiotics like penicillin & cephalosporins through production of Extended spectrum β-lactamase(ESBL) & AmpC β-lactamase enzymes by gram negative bacilli is increasing worldwide. Third generation (3GC) cephalosporins were thought to be resistant to hydrolysis by β-lactamase but in mid 1980's a new type of β-lactamase was produced which could hydrolyze them called as ESBL.[1]ESBL can be classified on the basis of their primary structure into 4

molecular classes A-D. Class A & C are most common .They have serine residue at their active site.[1,2] ESBL are plasmid mediated & as a result of enzymes TEM1(Temoneira),TEM2,SHV1(sulph-hydryl variables).[2]ESBL producing organisms show resistance to 3GC,monobactam such as Aztreonam but not to Cephamycins or Carbapenems. ESBLs are inhibited by β-lactamase inhibitors like clavulanic acid, tazobactam, sulbactam .AmpC producers show resistance to β-lactamase inhibitors like clavulanic acid as well as Cefoxitin. AmpC class of enzymes is encoded by chromosomes & also have been carried by plasmid.[3]They show sensitivity to Carbapenems.

Diabetic patients have life time risk of developing foot ulcerations as high as25%. [4]Uncontrolled infection is a major cause of necrosis & hospitalization in these patients. Multidrug resistant organisms (mediated through ESBL & AmpC β-lactamase) will not respond to penicillins & cephalosporins. Use of appropriate antibiotics in the treatment of diabetic foot will reduce morbidity.

MATERIALS AND METHODS

Study was conducted in Dr. D.Y. Patil Hospital, Research Center, Kadamwadi, Kolhapur (period Jan.2010 to July 2011).Clearance from ethics committee was obtained. Informed consent was obtained from all the patients.

Collections of sample – Various samples like pus, discharge, debrided material were collected from wound in100 type II diabetic foot patients aseptically in sterile container. Gram staining was done. Microscopic characters of these organisms were studied. Isolation of organisms from the said samples was carried out by using suitable media like Nutrient agar , Blood agar, McConkey agar & biochemical tests .Motility was observed by hanging drop preparation. Isolated GNB were selected for further study.

All GNB isolates were tested for antibiotics routinely used by Kirby-Bauer Disk Diffusion method. Results were recorded as per CLSI norms.[5] Antibiotics used were Amikacin (30µg),Gatifloxacin (5µg),Aztreonam (30µg),Ceftazidime (30µg),Ceftriaxone (30µg),Cefotaxime (30µg)Piperacillin-tazobactam (100/10µg),Ceftazidime-clavulanic acid(20/10µg),Imipenem (10µg),Cephoxitin (30µg).

Screening for ESBL producer – Isolates showing resistance or decreased sensitivity to any one of the 3rd Generation Cephalosporins (3GC--Ceftazidime, Cefotaxime, Ceftriaxone) were considered as probable ESBL producers.

Double Disk Synergy Test (DDST) – Probable ESBL producing organisms were subjected to DDST for confirmation. A lawn culture of test strain (0.5 Mc Farland std.) was done on Mueller-Hinton Agar (MHA) plate. Antibiotic disks of Ceftazidime & Ceftazidime-clavulanic acid were placed 15 mm apart. Plates were incubated at 37 ^{0}C aerobically. Accentuated zone of inhibition towards combination disk was taken as positive test.[6]

Hi-Comb MIC Test – Minimum Inhibitory Concentration (MIC) was detected using Hi-Comb MIC strips of Ceftazidime for ESBL positive isolates. (*E.coli* ATCC 25922 was used as negative control).

Screening for AmpC β-lactamase producers – The strains which show decreased susceptibility to 3rd generation cephalosporins(3GC) as well as resistance to β –lactamase inhibitors like clavulanic acid were taken as positive for screening test. Disk Antagonism Test (DAT) [7] – Lawn culture of test strain isolate (0.5 Mc Farland std.) was done on Mueller Hinton Agar plate. Disks of Ceftazidime (30µg) & Cefoxitin (30µg) were placed 15 mm apart. Plates were incubated overnight at 37^{o}C. Isolate showing blunting of zone of inhibition of Ceftazidime adjacent to Cefoxitin disk was considered as presumptive AmpC producer.

AmpC Disk Test [7,8] – Confirmation of AmpC β-lactamase producer is done by AmpC disk test. A lawn culture of Std. strain *E.coli* ATCC 25922 was done on Mueller-Hinton Agar plate. Sterile filter paper disks (6mm) were moistened with sterile distilled water (20µl).Several colonies of test organism were inoculated on filter paper .A Cefoxitin (30µg) disk was placed on inoculated media. Above mentioned inoculated filter paper disk was placed beside Cefoxitin disk (almost

touching).Plate was incubated overnight at 37^{o} C. Results were interpreted as **a)** Positive – If flattening or indentation of zone of inhibition of Cefoxitin in the vicinity of test disk. **b)** Negative – Undistorted zone.

Data was collected from D. Y. Patil Medical College, Hospital & Research Institute, Kolhapur .Data analysis is done by using MS-Excel computer language. (Data analysis tool park option.) Antibiotic disks & MIC strips manufactured by High Media,Mumbai were used.

RESULTS

Total 100 pus samples were studied. 4 samples were culture negative. Total 108 organisms were isolated. Out of these 63 were GNB(58.3%) &45were GPC(41.66%).The gram negative bacilli isolated were *E.coli* 20 (31.74%), *K.pneumoniae* 8(12.69%), *K.oxytoca* 4 (6.34%), *C.freundii* 7 (11.11%), *C.koseri* 4 (6.34%), *Proteus mirabilis* 5 (7.93%), *Proteus vulgaris* 3(4.76%), *Pseudomonas aeruginosa* 12 (19.04%).

Out of 63 GNB isolates, 35 were found positive by ESBL screening test (55.6%).All 35 probable ESBL producers were subjected to confirmatory test (DDST & MIC).Out of 35 isolates 21 were confirmed as ESBL producers (33.3%).Remaining 14 isolates out of 35 were negative by confirmatory test .Reason for this was as follows – 1)10 isolates were found resistant to combination disk (ceftazidime & clavulanic acid).So they were further tested for AmpC β –lactamase production & found positive for AmpC β-lactamase production.2)Four isolates of *Ps.aeruginosa* were found sensitive to Aztreonam,3GC,Amikacin & Gatifloxacin. ESBL production was highest in *Citrobacter spp*(63.63%),followed by *E.coli* (50%).In the present study MIC of Ceftazidime ranges from 16µg/ml to 256µg/ml in ESBL positive strains .

ESBL producing organisms showed resistance to 3GC,Aztreonam,Gatifloxacin.All ESBL producers were sensitive to Imipenem. Most of them were sensitive to Ceftazidime-clavulanic acid ,Piperacillin-tazobactam & Amikacin.

Disk Antagonism Test was used for screening AmpC β-lactamase production .Out of 63 GNB isolates 16 were positive by screening test (25.39%).All these isolates showed resistance to any one of the 3GC & lack of inhibition by β-lactamase inhibitors. These 16 probable AmpC producers were subjected to AmpC Disk Test for confirmation Out of these 13 isolates were confirmed as AmpC producers (20.63%).

Most of AmpC producers were resistant to 3GC,Cefoxitin,Amoxy-clav. Piperacillin tazobactam&Aztreonam.All AmpC producers showed sensitivity to Imipenem(100%).

Table 1: Organisms found in pus samples studied.

Type of bacteria	Number of isolates	%
Gram – ve bacilli	63	58.3
Gram + ve cocci	45	41.66
Total No. of isolates	108	

Table 2: Gram negative bacilli isolated from Diabetic Foot Ulcer

Name of bacteria	Number	%
Escherichia coli	20	31.74
Klebsiella pneumoniae	8	12.69
Klebsiella oxytoca	4	6.34
Citrobacter freundii	7	11.11
Citrobacter koseri	4	6.34
Proteus mirabilis	5	7.93
Proteus vulgaris	3	4.76
Pseudomonas aeruginosa	12	19.04

Table 3: Gram negative isolates positive for ESBL production by confirmatory test

Name of Bacteria	Number	%
Escherichia coli (n =20)	10	50
Klebsiella pneumoniae (n=8)	1	12.5
Klebsiella oxytoca (n=4)	0	00
Citrobacter freundii (n=7)	4	57.14
Citrobacter koseri (n=4)	3	75
Proteus mirabilis (n=5)	1	20
Proteus vulgaris (n=3)	1	33.3
Pseudomonas aeruginosa (n=12)	1	8.33
Total	21	33.3

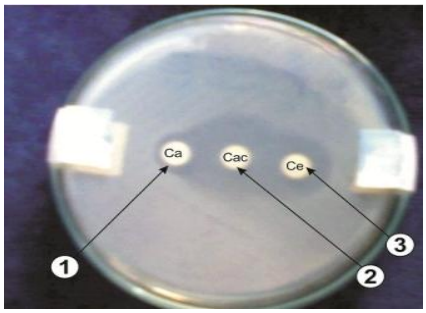
Table 4: Detection of Minimum Inhibitory Concentration (MIC) of Ceftazidime in ESBL positive Strains

MIC of Ceftazidime (µg / ml)	**Number of strains**				
	E.coli **N=10**	***Klebsiella*** **spp. N= 1**	***Citrobacter*** **spp. N=7**	***Proteus*** **spp. N= 2**	***Pseudomonas*** **Spp. N= 1**
2	-	-	-	-	-
4	-	-	-	-	-
8	-	-	-	-	-
16	-	-	-	-	1
32	2	-	2	1	-
64	4	-	4	-	-
128	3	1	1	1	-
256	1	-	-	-	-

Table 5: Gram negative isolates positive for AmpC production by Confirmatory test:

Name of Bacteria	**No.**	**%**
Escherichia coli (n =20)	4	20
Klebsiella pneumoniae (n=8)	2	25
Klebsiella oxytoca (n=4)	2	50
Citrobacter freundii (n=7)	2	28.57
Citrobacter koseri (n=4)	1	25
Proteus mirabilis (n=5)	1	20
Proteus vulgaris (n=3)	1	33.3
Pseudomonas aeruginosa (n=12)	0	0
Total	13	20.63

Fig 1: Double Disk Synergy Test- Enhanced zone of inhibition around central disk indicates positive ESBL producer.



1. Disk of Ceftazidime.
2. Disk of Ceftazidime and Clavulanic acid.
3. Disk of Cefotaxime.

Fig 2 : MIC by Hi-Comb Test- Increased MIC of Ceftazidime indicates ESBL producer.

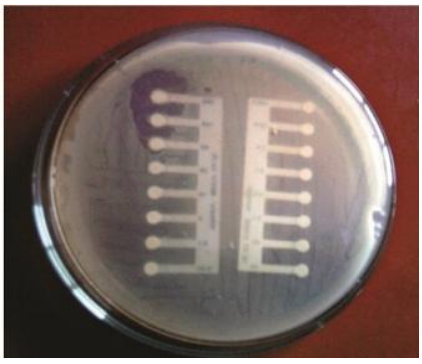
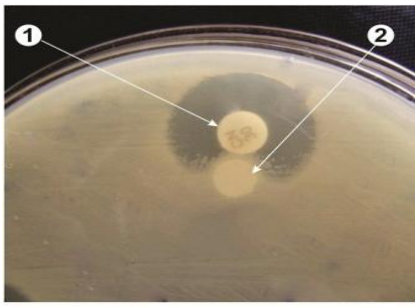
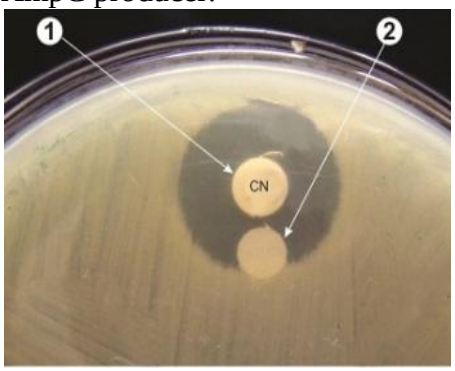


Fig 3a : AmpC Disk Test- Indentation of zone of inhibition around Cefoxitin disk near disk 2 indicates AmpC producer.



1. Disk of Cefoxitin.
2. Disk containing Test organism.

Fig3b : AmpC Disk Test- No distortion of zone of inhibition around Cefoxitin disk indicates non-AmpC producer.



1.Disk of Cefoxitin
2. Disk containing Teat organism

DISCUSSION

In modern medical practice, Extended Spectrum β-lactamase producing strains pose one of the greatest challenges to the clinicians, resulting in limitation of therapeutic options. In this study 100 type II diabetic patients with foot infection were studied. 92 patients were from IPD & 8 were from OPD.71 patients were male & 29 were female. Age ranged from 36-76 years.(mean age 50 yrs) Out of 100 samples ,4 were culture negative. Reasons for this – 1) Patients already on antibiotics & responding to treatment.2) Anaerobes may be present. Various studies of diabetic foot ulcer (DFU) also reported culture negative reports .Sivaraman Umadevi [9] reported 3/105 patients (2.9%) culture negative. Sayed Alavi et al,[10] reported 6/32 (18.75%) culture negative samples.

Total 108 organisms were isolated amongst which 45 were GPC (41.66%) & GNB (58.3%).Various bacteriological studies in DFU reported as follows-Ravishekhar Gadepalli et al[11],2006,AIIMS,New Delhi reported GNB 84.7% &GPC 33.3%. Sayed Alavi et

al[10],2007,Razi Hospital Iran reprted GNB 54.8% & GPC 42.9%Samir Paul et al[12] BIRDEM,Dhaka.(2009)isolated GNB 92.8% & GPC 33.3%.

Out of 63 GNB isolates 21 were confirmed by DDST for ESBL production (33.3%).In present study ,ESBL production was highest in *Citrobacter koseri* (75%), *C.freundii* (57.14%),*E.coli* (50%),*P.mirabilis* (20%),*P.vulgaris* (33.3%),*K.pneumoniae* (12.5%),*Ps.aeruginosa* (8.33%).Prevalence of ESBL producers in various studies in DFU is as follows-Ravishekhar Gadepalli et al[11],2006,AIIMS,New Delhi reported prevalence of ESBL production 44.7% ,(*Proteus spp*.65.3%,*E.coli* 54.5%).Ami Varaiya et al[13],2008,Raheja Hospital ,Mumbai found ESBL production in *E.coli* 48.48%,*K.pneumoniae* 23.13%. Prevalence of ESBL production was reported as 23.13%.Deep et al[14],Govt .Medical College, Amrutsar 2007 reported ESBL production in DFU as 53.25%- *E.coli* 58.6%,*Kpneumoniae* 65.71%,*K.oxytoca* 38.8%,*C.freundii* 85.7%,*C.koseri* 25%,*P.mirabilis* 42.8%,*P.vulgaris* 25%.

Present study shows ESBL producers 33.3%.Prolonged hospital stay, repeated admissions & immunocompromised status in diabetic patients are important factors leading to colonization of ESBL producing organisms.MIC values for Ceftazidime in ESBL positive isolates increased.(range 16µg/ml to 256µg/ml) Deep et al[14] reported MIC of ESBL producing *E.coli* isolates 32-256 µg/ml. Silpi Basak et al,[15] reported MIC values of ESBL producers-*E.coli* 4µg/ml *K.pneumoniae* 256 µg/ml.

13 isolates out of 63 showed AmpC Disk Test positive ,so confirmed as AmpC β-lactamase producers.(20.63%) AmpC production was highest in *K.oxytoca* (50%) while *Ps .aeruginosa* did not show any AmpC production .AmpC β-lactamase production in *E.coli* 20%,*K .pneumoniae* 25%,*C.freundii* 28.57%,*P.vulgaris* 33.3%,*P.mirabilis* 20%. Parul Sinha et al 2008, [7] reported AmpC production in *E.coli* isolates 24%.Singhal et al [16]reported 8% AmpC production, organisms were *E.coli* 6.97%,*Klebsiella spp*.6.18%. Amongst 13 isolates positive for AmpC production, 10 isolates show indentation in AmpC disk test denoting strong AmpC β-lactamase production ,while 3 isolates show flattening indicating weak AmpC production. There are less no. of studies about AmpC production in organisms isolated from diabetic foot ulcer. In this study, One isolate of *P.vulgaris* & One isolate of *C.koseri* was positive for both ESBL & AmpC β-lactamase production (3.17%).Similar results were reported by Singhal et al,[16] about co-existence of both ESBL & AmpC in2 isolates (2.5%).Parul Sinha et al [7]reported 8% isolates positive for both ESBL & AmpC. This co-existence could be because of plasmid mediated AmpC β-lactamase enzymes have been shown to disseminate among *Enterobacteriaceae* along with ESBL sometimes.

ESBL producing organisms were resistant to 3GC.They were sensitive to Ceftazidime-clavulanic acid,Piperacillin-tazobactam.75% isolates showed sensitivity to Amikacin & 100% sensitivity to Imipenem. AmpC producing organisms were resistant to 3GC as well as to Ceftazidime-clavulanic acid. They also show resistance to Cefoxitin. All AmpC producing organisms were 100% sensitive to Imipenem.

CONCLUSSION

Type II diabetic patients often have chronic non-healing foot ulcers due to several underlying factors such as neuropathy, high planter pressure & peripheral arterial disease. Such chronic long standing ulcers are more prone for infection. Prevalence of ESBL producers in gram negative bacilli in type II diabetic foot infections was 33.3% & AmpC β-lactamase production was 20.63%.Multidrug resistance mediated through ESBL & AmpC β-lactamase producing gram

negative bacilli was found in the present study .For ESBL producers Combination of 3rd GC antibiotic with β-lactamase inhibitors like clavulanic acid, tazobactam ,sulbactam can be used. All these multidrug resistant strains were sensitive to Imipenem. .Infection with these multidrug resistant strains increased duration of hospitalization & medical, surgical care cost of the patient. So reporting of ESBL & AmpC β-lactamase producing strains will enable the clinician to select the proper antibiotic at the earliest .There is a need for continuous surveillance of resistant bacteria to provide basis for empirical therapy & to reduce the risk of complications.

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REFERENCES

1. David L. Paterson and Robert A.Bonomo. Extended spectrum β-lactamases:A Clinical Update Clinical Microbiology Reviews. 2005;18(4): 657-86
2. Karen Bush,George A.Jacoby. Updated Functional classification of β-Lactamases..Antimicrob.Agents Chemother 2010;54(3): 969-76
3. George A. Jacoby. 'AmpC β-lactamases' Clinical Microbiology Reviews 2009;22(1): 161-82
4. Singh N.,Armstrong DG,Lipsky B.A. Preventing foot ulcers in patients with diabetes JAMA2005;293(2):217-28
5. Clinical Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: Eighteenth informational suppl:M-100-S-18,28(1) Clinical Laboratory Standards Institute 2008, Wayne P.A.
6. R. S. Miles, SGB Amyes. Laboratory control of Antimicrobial Therapy. Mackie & McCartney Practical Medical Microbiology.J G Colle,A.G.Fraser,B.P.Marmion,A.Simmons.14th ed. Churchill Livingstone 2007 p:151-178
7. Parul Sinha ,Rajni Sharma, Suman Rishi, Raman Sharma, Smita Sood, Deepali Pathak. Prevalence of extended spectrum beta lactamase and Amp C beta lactamase producers among *Escherichia coli* isolates in a tertiary care hospital in Jaipur Indian J. of Pathology & Microbiology 2008; 51(3) ;367-69
8. Dr.B.N.Harish,Mrs.Parveen ,Dr.Ira Praharaj,Dr.Vithiya,Mrs.S.Kavitha. Detection of Extended spectrum beta lactamase producing bacteria (ESβLs) and detection of carbapenemases and AmpC beta lactamases. VIII National Workshop on simple diagnostic methods in Clinical Microbiology JIPMER Pondicherry. 2009 .Standard operating procedure manual: 81-88.
9. Sivaraman Umadevi, Shailesh kumar,Noyal Mariya Joseph, Joshy M.Easow,G.Kandhakumari,Sreenivasan Srirangraj et al. Microbiological study of diabetic foot infections Indian J. of Medical Specialities 2011;2(1):12-17
10. Seyed Mohammad Alavi, Azar D. Khosravi ,Abdulah Sarami, Ahmad Dashtebozorg,Effat,Abasi Montazeri. Bacteriological study of Diabetic Foot Ulcer.Pak. J. Med. Sci. 2007 ;23(1) : 681-84
11. Ravishekhar Gadepalli,Dhavan B.,Sreenivas V,Arti Kapil ,Ammini AC,Choudhary R .A clinico-microbiological study of diabetic foot ulcers in an Indian Tertiary Care Hospital. Diabetic Care, 2006;29 (8) :1727-32
12. Samir Paul, Lovely Barai,Ashraf Jahan,J.Ashraful Ibrahim.A bacteriological study of diabetic foot infection in an urban Tertiary Care Hospital of Dhaka city .Ibrahim Med.Coll.J.2009;3(2):50-54

13. Ami Y. Varaiya, Jyotsana D. Dogra,Manasi H.Kullarni,Pallavi Bhalekar. Extended spectrum β-lactamse producing *Escherichia coli* and *Klebsiella pneumoniae* in diabetic foot infections. Indian J. of Pathology. And Microbiology 2008;(51)(3):368-72

14. Deep, Usha Arora.Extended spectrum beta lactamase – mediated resistance among isolates of family *Enterobacteriaceae*. Indian J. Pathol Microbiol 2007;50(2):458-61

15. Silpi Basak, Sanjay KR,Mallick ,Seema Bose. Extended spectrum β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* isolates in a rural hospital. J. Indian Med. Association.2009; 107(12): 855-58

16. S.Singal ,T.Mathur ,S.Khans, Upadhyay D.J.,S.Chugh,Gaind R .Evaluation of methods for AmpC beta-lactamase in Gram Negative clinical isolates from Tertiary Care Hospitals-Indian J.Med.Microbiology 2005;23(2):120-24.

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