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Incidence of Beta Lactamases in Gram Negative Bacilli in Diabetic foot Infection and the Impact on the Selection of Antimicrobial Therapy

C.S. Vinodkumar[1], H. Srinivasa[2], K.G. Basavarajappa[3] and Nitin Bandekar[4]

[1]Department of Microbiology, S. S. Institute of Medical Sciences and Research Centre, Davangere - 577 005, India.
[2]Department of Microbiology, St. Johns Medical College, Bangalore, India.
[3]Department of Microbiology, S. S. Institute of Medical Sciences and Research Centre, Davangere -57 7005, India
[4]Department of Surgery, S. S. Institute of Medical Sciences and Research Centre, Davangere - 577 005, India.

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Diabetes mellitus is a chronic disorder and affects large segment of population and is a major public health problem. The infection leads to the early development of complication even after a trivial trauma, the disease progresses and becomes refractory to antibacterial therapy. Early diagnosis of microbial infections and screening for mechanism of drug resistance is aimed to institute the appropriate antibacterial therapy and to avoid further complications. Beta lactamases are enzymes responsible for the resistance to beta lactam antibiotics. This study is aimed at the detection of various types of beta lactamases present among the gram negative bacilli isolated from diabetic foot infection. Adult diabetic patients admitted for lower extremity infections from July 2008 to Jan 2010 to the medical wards and intensive care unit of medical teaching hospitals were included in the study. 179 gram negative bacilli were isolated and screened for the presence of extended spectrum beta lactamase, AmpC lactamase, Metallo beta lactamase and confirmed by the respective confirmatory tests. 54.7% produced extended spectrum beta lactamases, 10.1% AmpC beta lactamase and 71.7% strains produced metallo beta lactamases. β-lactamase producers are emerging threat and cause of concern for the clinicians, as it results in the resistance to penicillin, cephalosporins and limits therapeutic options. Screening techniques should be performed routinely to detect these β-lactamase producers so that suitable antimicrobial therapy can be instituted

Keywords: β-lactamases, Diabetic foot infection.

In the past 65 years, antibiotics have been critical in the fight against infectious disease caused by the bacteria and other microbes. Antimicrobial chemotherapy has been a leading cause for the dramatic rise of average life expectancy in the Twentieth Century. However, disease-causing microbes that have become resistant to antibiotic drug therapy are an increasing public health problem. Diabetic wound infections have become hard to treat with antibiotics[1,2,3]. One part of the problem is that bacteria and other microbes that cause infections are remarkably resilient and have developed several ways to resist antibiotics and other antimicrobial drugs. Another part of the problem is due to increasing use, and misuse, of existing antibiotics

* To whom all correspondence should be addressed.
Mob.: +91-9964402525
E-mail: vinodmicro@yahoo.com

in human and veterinary medicine and in agriculture[3]. Nowadays, majority of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. Some organisms are resistant to all approved antibiotics and can only be treated with experimental and potentially toxic drugs[4]. Unless antibiotic resistance problems are detected as they emerge, and actions are taken immediately to contain them, society could be faced with previously treatable diseases that have become again untreatable, as in the days before antibiotics were developed.

Knowledge of resistance pattern of bacterial strains in a geographical area will help to guide the appropriate and judicious antibiotic use and to formulate effective infection control measures. Extended Spectrum β-Lactamases (ESBL), AmpC β-lactamase and Metallo β-lactamase producing organisms pose a major problem for clinical therapeutics[5,6]. The number of reports about incidence of ESBL producing strains among clinical isolates has been steadily increasing over the past few years resulting in limitation of therapeutic options. Initially restricted to hospital-acquired infections, now they have also been isolated from infections in community. Major outbreaks involving β-lactamase positive strains have been reported from all over the world, thus making them emerging pathogens. The routine susceptibility tests done by clinical laboratories fail to detect β-lactamases positive strains and can erroneously detect isolates sometimes to be sensitive to any of the broad-spectrum cephalosporin like cefotaxime, ceftazidime, ceftriaxone and for imipenem or meropenem[7-9].

It is necessary to know the prevalence of β-lactamase positive strains in a hospital so as to formulate a policy of empirical therapy in high risk units where infections due to resistant organisms are much higher. Equally, important is the information on an isolate from a patient to avoid misuse of extended spectrum cephalosporins, which still remain an important component of antimicrobial therapy in high risk wards[10,11]. There is not enough information from the Indian subcontinent regarding the prevalence of β-lactamases mediated resistance among gram negative bacteria in diabetic foot infection. The aim of the present study is to find the prevalence of β-lactamases mediated resistance among gram negative bacteria in diabetic foot infections.

MATERIAL AND METHODS

A prospective study was carried out on 300 diabetic patients with foot ulcer during the period of July 2008 to June 2010 at S.S. Institute of Medical College and Research Centre, Davangere and Chigateri Government General Hospital, Davangere. 179 gram negative bacterial were isolated and identified by standard laboratory techniques[12]. Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates with commercially available discs (Hi-Media, Mumbai) by the Kirby-Bauer disc diffusion method[13]. The results were recorded and interpreted as per CLSI recommendations[14].

Tests for ESBL production

Double disk approximation test for screening

The test organisms were applied on to a Mueller Hinton agar plate by adjusting turbidity to McFarland no 0.5 tube. Antibiotic discs of Amoxicillin / Clavulanic acid (20/10 µg) and cefotaxime (30 µg) were placed at a distance of 15 mm apart and incubated. Organisms that showed a clear extension of cefotaxime inhibition zone towards the disc containing Clavulanate were considered as ESBL producer[15.] The organisms which were screened and found positive for ESBL production were subjected to confirmatory test.

NCCLS phenotypic confirmatory test

Ceftazidime (30 µg) and ceftazidime plus Clavulanic acid (30/10 µg) were placed on Mueller Hinton agar and incubated. Organism was considered as ESBL producer if there was a e" 5mm increase in diameter of Ceftazidime plus Clavulanic disc and that of ceftazidime disc alone[16,17]

Amp C Disk Test

A lawn culture of *E.coli* ATCC 25922 was prepared on MHA plate. Sterile disks (6mm) was moistened with sterile saline (20 µl) and inoculated with several colonies of test organisms. The inoculated disk was then placed 5mm beside a cefoxitin disc. Plates were incubated overnight at 35°C. A positive test was appeared as a flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disc[18-20]. A negative test had an undisturbed zone.

Metallo β-lactamase (MBL) production

Gram negative organisms that showed resistance to Imipenem were selected for MBL production.

Imipenem-EDTA combined disc test

This test was performed according to Yong *et al*. test organisms were inoculated onto Mueller Hinton agar plates as per the CLSI recommendations. Two 10µg imipenem disks were placed on the plate and 10 µl of sterile 0.5 M EDTA solution was added to one of the imipenem disk. The inhibition zones of the imipenem and imipenem plus EDTA disks were compared after inoculation[21]. If the increase in inhibition zone with the imipenem plus EDTA disc was > 7mm than the imipenem disc alone, it was considered as MBL positive.

RESULTS

Percentage of resistance exhibited by 179 gram negative bacilli isolated from diabetic foot infection to various antimicrobial agents is shown in table-1. All the strains were resistant to more than 2 or more drugs hence all the bacteria were designated as multidrug resistance gram negative bacilli (MDRGNB)

All 179 gram negative bacilli were screened for ESβL production, Amp C β lactamase production. 54.7 % of gram negative bacilli were ESβL producers. *Klebsiella pneumonia* (20.7%) was the predominant ESβL producer followed by *Pseudomonas aeruginosa* (17.3%), *Proteus mirabilis* (8.4%), *Acinetobater baumanni* (3.9%),

Table 1. Antibiotic Resistance pattern of Gram negative bacterial isolates from infected foot ulcers in diabetic patients

Antibiotics	*K. pneumoniae* No.(%)	*P.aeruginosa* No.(%)	*P.mirabilis* No.(%)	*P. vulgaris* No.(%)	*A. baumanni* No.(%)	*E .coli* No.(%)	*C. freundii* No.(%)
Ampicillin	43(71.7)	—	19(76.0)	3(100)	08(72.7)	13(100)	11(100)
Amikacin	29(48.3)	25(46.3)	12(48.0)	2(66.7)	04(36.4)	04(30.8)	04(36.4)
Ofloxacin	43(71.7)	33(61.1)	14(56.0)	3(1000	04(36.4)	09(69.2)	03(27.3)
Ciprofloxacin	47(75.0)	35(64.8)	15(60.0)	3(100)	05(45.5)	08(61.5)	04(36.4)
Cephotaxime	47(71.7)	39(72.2)	16(64.0)	2(66.7)	08(72.7)	12(92.3)	07(63.6)
Cefoperazone + Sulbactam	21(35.0)	17(31.5)	10(40.0)	1933.3)	07(63.6)	05(38.5)	04(36.4)
Piperacillin	32(53.3)	35(64.8)	12(48.0)	1(33.3)	08(72.7)	06(46.2)	05(38.5)
Piperacillin + Tazobactam	32(53.3)	09(16.6)	10(40.0)	1(33.3)	08(72.7)	05(38.5)	05(38.5)
Imipenem	16(26.7)	11(20.3)	07(28.0)	1(33.3)	05(45.5)	05(38.5)	02(18.2)

Table 2. Prevalence of ESBL and Amp C producers in Diabetic foot infection

Organisms	No. Tested	ESBL producers		Amp C producers	
		No.	%	No.	%
Klebsiella pneumonia	60	37	20.7	10	5.6
Klebsiella oxytoca	01	-	-	-	
Pseudomonas aeruginosa	54	31	17.3	06	3.4
Proteus mirabilis	28	15	8.4	02	2.1
E .coli	13	06	3.4	-	-
Acinetobacter baumanni	11	07	3.9	-	-
Citrobacter freundii	11	02	1.1	-	-
Morganella morganii	01	-	-	-	-
Total	179	98	54.7	18	10.1

E.coli (3.4%) and *Citrobacter freundii* (1.1%) (Table 2).

10.7% of gram negative bacilli were Amp C producers and Amp C production was seen only in *Klebsiella pneumonia, Pseudomonas aeruginosa and Proteus mirabilis.*

Klebsiella pneumonia (5.6%) was the predominant Amp C producer followed by *Pseudomonas aeruginosa* (3.4%) and *Proteus mirabilis* (1.1%) (Table 2).

MBL producers

Not all gram negative bacteria were tested for MBL production. Only those gram negative bacilli resistant to imipenem (Table 2) were screened for MBL production. 16 out of 60 *Klebsiella pneumoniae* were resistant to imipenem. Similarly 11 out of 54 *Pseudomonas aeruginosa* were resistant. Among 28 *Proteus mirabilis*, 07 were resistant. 05 out of 13 *E.coli* was resistant to imipenem. 05 out of 11 *Acinetobacter baumanni* were resistant and 2 out of 11 *Citrobacter freundii* were resistant to imipenem (Table 3).

Klebsiella pneumonia (26.1%) was the predominant MBL producer followed by *Pseudomonas aeruginosa* (19.6%), *Proteus mirabilis* (13.1%), *Acinetobacter baumanni* (8.7%), *Citrobacter freundii* (2.1%) and *E.coli* (2.1%).

Table 3. Prevalence of Metallo beta lactamase producers in Diabetic foot infection

Organisms	No. of GNB isolated	Imipenem resistant	MBL producers No.	%
Klebsiella pneumonia	60	16	12	26.1
Klebsiella oxytoca	01	-	-	-
Pseudomonas aeruginosa	54	11	09	19.6
Proteus mirabilis	28	07	06	13.1
E .coli	13	05	01	2.1
Acinetobacter baumanni	11	05	04	8.7
Citrobacter freundii	11	02	01	2.1
Morganella morganii	01	-	-	-
Total	179	46	33	71.7

DISCUSSION

The ability to produce β-lactamases enzymes is the major cause of resistance of bacteria to β-lactam antibiotics. Numerous β-lactamases are encoded either by chromosomal genes or transferable genes located on plasmids or transposons[22]. Based on amino acid and nucleotide sequence studies, four distinct classes of β-lactamases have been defined. Class A (Extended spectrum β-lactamases) class B (Metallo β-lactamases), class C (AmpC β-lactamases) and Class D (Cloxacillin hydrolysing β-lactamases)[23,24].

Extended spectrum β-lactamases are plasmid mediated TEM and SHV derived enzymes isolated for first time in Western Europe in mid 1980s[25]. Initially theses enzymes were commonly found in *Klebsiella* species and *E.coli*,[8] but now these enzymes are produced by all the members of Enterobacteriaceae and few other gram negative bacilli[26,27]. These enzymes are capable of hydrolysing broad spectrum cephalosporins and monobactams and inactive against cephamycins and imipenem. In the present study 54.7% of gram negative bacteria were ESBL producers. Few studies in India have reported the prevalence of ESBL in the range of 58% to 68.1%.[9,12, 28,29] Our prevalence rate is lesser than other reports from India and abroad, since the isolates were obtained from infection in diabetic foot infections they might be wide disparity in the prevalence rate of ESBL producing gram-negative bacteria when compared to other reports. *Klebsiella pneumoniae* was the predominant ESBL producer followed by *Pseudomonas aeruginosa* and *Proteus mirabilis.* In addition to the intrinsic resistance to cephalosporins and aztreonam, ESBL producing organism's exhibit co-resistance to many other

classes of antibiotics like quinolones and aminoglycosides resulting in limitation of therapeutic options. In the present study we found such associated resistance with Ciprofloxacin (75.5%). As quinolones are strong selectors of ESBL producers, their use should be restricted as far as possible. Major risk factors for colonization or infection with ESBL producing organisms are long term antibiotic exposure, prolonged hospital stay, high rates of the third generation cephalosporin use and invasive procedures. However in the present study we could not retrieve the previous hospitalization and treatment to reinforce the above arguments. Treatment of ESBL producing strains of Enterobacteriaceae has emerged as a major challenge in hospitalized as well as community patients. There are many factors which determine the choice of antibiotics and the management of diabetic foot infections. Although β-lactamases inhibitors have significant activity against ESBL *in-vitro*, their clinical effectiveness against serious infections due to ESBL producing organisms is controversial. ESBL producing strains might show a false sensitive zone of inhibition in the Kirby Bauer's disc diffusion method. The antibiotics for the treatment include carbapenems, aminoglycoside and β-lactamases inhibitor combinations.

AmpC β-lactamases are clinically important cephalosporinases encoded on chromosomes of many of the Enterobacteriaceae and a few other organisms[20], where they mediate resistance to cephalothin cefazolin, cefoxitin, most of the penicillins and β-lactamase inhibitor[18,19]. In many bacteria Amp C enzymes are inducible and can be expressed at high levels by mutation[19,21]. Over expression confers resistance to broad spectrum cephalosporins. In the present study 10.1% were AmpC producers and *Klebsiella pneumonia* was the predominant Amp C producer followed by *Pseudomonas aeruginosa* and *Proteus mirabilis.* There are no reports to compare the incidence of Amp C mediated resistance among diabetic foot infection in India and aboard.

Metallo β-lactamase (MBL) is a group of carbapenem hydrolysing β-lactamase[30]. They have been reported from many countries, as well as from different parts of Indian subcontinent, particularly in multidrug resistance pathogens like *Pseudomonas aeruginosa* and *Acinetobacter* species. The MBLs are inhibited in-vitro by CuCl3, FeCl3, EDTA and thiol compounds like 2 mercaptopropionic acid, sodium mercaptoacetoic acid and 2 mercaptoethanal, but not by β-lactamase inhibitors like Clavulanic acid, sulbactum or tazobactam[6]. Detection of MBL production in MDR organisms from diabetic foot infection has tremendous therapeutic consequences, as the treatment option for such isolates are aztreonam or potentially toxic polymyxin B and colistin. In the present study not all gram negative bacteria were tested for MBL production. Only those gram negative bacilli resistant to imipenem were screened for MBL production and 71.7% of them were metallo β-lactamase producers. *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus mirabilis* were the predominant MBL producers.

We could not elicit the reason for multidrug resistance in our isolates because we could not extract previous hospitalization details for the same wound in our study subjects. This information could have helped in explaining the reasons for the high prevalence of MDROs in our patients. But one of the reason could be the bacteria isolated may be nosocomial colonizers

In conclusion, the present study highlights the high prevalence of β-lactamases among the multi drug resistant gram negative isolates in diabetic foot infections. It also reflects grim future of the treatment options available for these notorious pathogens. The high incidence of β-lactamases production due to multiple mechanisms in diabetic foot infection is alarming and urgent action needs to be taken from both the therapeutic and infection control perspective. Clinical microbiology laboratories should perform the screening techniques to detect these β-lactamases routinely so that the suitable antimicrobial therapy can be instituted and the dissemination of these isolates may be prevented by employing appropriate control measures.

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