Int J Pharm Bio Sci 2014 July ; 5 (3) : (B) 965 - 973

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

DETECTION OF ESBL AND MBL AMONG GRAM NEGATIVE ENTEROBACTERIACEAE ISOLATES FROM DIABETIC FOOT ULCER IN CHENNAI

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ABSTRACT

Diabetic foot infection (DFI) is poly-microbial in nature, pathogenic microorganism such as *Pseudomonas spp*., *Enterococcus spp*. and *Proteus spp*. are mainly responsible for continuing and extensive tissue destruction with the poor blood circulation of the foot. The present study was aimed to characterize the ESBL and MBL producing non-fermentative and gram negative bacterial isolates from diabetic foot ulcer (DFU) by phenotypic and genotypic methods. Total of 171 pathogenic microorganisms was isolated from 123 DFU pus sample, 41 isolates were found to be non-fermentative and non enterobacteriaceae isolates among these 33 isolates were *Pseudomonas aeruginosa* and 8 isolates were *Acinetobacter baumannii*. ESBL and MBL positivity were found to be 48% (20/41) and 70.7% (29/41) respectively. ESBL gene amplification was done by universal primers such as blaCTX-M, blaTEM and blaSHV. Genotypic ESBL positivity was found to be 2.4%, 31.7%, and 8%forrespective genes such as blaCTX-M, blaTEM, and blaSHV genes. A detailed knowledge of the susceptibility to antimicrobial agents is necessary to facilitate the development of effective strategies to combat the growing problem of resistance especially the ESBL positive strains. There is high percentage of antibiotic resistance found in our isolates; which warrants the use of antibiotic policy as well as proper surveillance programs.

KEYWORDS: **Diabetic foot ulcer, *E. coli*, *K. pneumonia,* ESBL, and MBL**

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INTRODUCTION

According to WHO, Diabetic Foot ulcer (DFU) is an infection, ulcer or destruction of deep tissue associated with neurological abnormalities and various degrees of peripheral vascular disease of the lower limb, collectively known as the diabetic foot syndrome. Foot ulceration and amputation are among the most costly diabetic complications[11].Diabetic foot infection is poly-microbial in nature many studies have reported on the bacteriology of DFU over 25 years, but the results varied with different places[6,14,13and 19]. Earlier studies have shown that *S. aureus* as the major causative organism among DFU but recent investigations revealed a predominance of gram-negative aerobes [3,9,19 and 22].The organisms such as *Pseudomonas spp*., *Enterococcus spp*. and *Proteus spp*. are mainly responsible for continuing and extensive tissue destruction with the poor blood circulation of the foot[19]. Proper management of infections requires appropriate antibiotic selection based on culture and antimicrobial susceptibility results. However, initial management comprises empirical antimicrobial therapy based on susceptibility data extrapolated from studies performed on general clinical isolates[10]. The determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infection. Testing is required not only for therapy but also to monitor the spread of resistant organisms or resistance genes throughout the hospital and community[26]. With the recent reports on increasing incidence of ESBLs and even MBLs production in *P. aeruginosa* worldwide[2,5 and18] especially plasmid mediated ESBL has limited the available antibiotics due to the coexistence of resistance gene[8]. This poses a significant threat to public health in antibiotic regimen[20,24]. Moreover, infections caused by ESBL producing *P. aeruginosa* are associated with increased morbidity, mortality, and health care-associated costs [5].With this background, the present study was aimed to characterize the ESBL and MBL in non enterobacteriaceae isolates from DFU by PCR from the diabetic city of India, “Chennai”.

MATERIALS AND METHODS

The clinical data and characterization of bacterial isolates were published earlier. A total of 171 bacterial isolates were collected from 123 wound swabs collected using sterile cotton swab, by taking fresh pus from diabetic foot ulcers of both in-patients and outpatients attending diabetic foot clinic at KMCH, Chennai. Identification of isolates were done based on the colony morphology, Gram staining, Motility, Catalase test, Oxidase test, Coagulase test, Standard biochemical tests and Oxidation – Fermentation test and other tests. Out of 171 DFU isolates 66(38.59%) were gram-positive and 97 (56.7%) were grams negative bacteria[19]. Out of 41/97 gram negative bacterial isolates were non fermentative and non enterobacteriaceae isolates.

Antibiotic susceptibility test

All the isolates were tested for antibiotic susceptibility by two methods i.e., disc diffusion method and minimum inhibitory concentration (MIC) determination of the micro broth dilution method. All the isolates tested for antibiotic resistant pattern various routinely used drugs. Inoculum of each DFU isolates were prepared from colonies grown on nutrient agar, which had been incubated overnight (18-20 hours) at 37°C in the incubator. Colonies suspended in Mueller Hinton broth (MHB) and adjusted to a turbidity of a 0.5 McFarland standard (1x 10^8 CFU/ml). Antibiotic disc and powder were purchased from Hi Media Ltd., Mumbai, India. Disc diffusion method was followed based on CLSI standards. Micro broth dilution method was made in 96 well microtitre plates. Each well of microtitre plate contained 100µl of respective antibiotic solution followed by bacterial suspension (5µl; 1x10^7 CFU/ml). The quality control strains, *P. aeruginosa* ATCC 27853 used in each run of daily testing. Each batch included a growth control well (no antimicrobial agent) and a negative control well (un-inoculated). After inoculation, each tray covered with a lid to prevent evaporation during incubation. The

micro dilution trays incubated at 37°C for 16-20 hours in ambient incubator prior to reading. MIC/MBC was determined based on the growth observed in the lowest dilution of the well after streaking on MHA plate.

Phenotypic ESBL detection:

ESBL detection by combined disc method

The antimicrobial disks and combination disks (cephotaxime 30 µg, ceftazidime 30 µg, Cefepime 30 µg, cephotaxime 30 µg /clavulanate 10 µg, ceftazidime 30 µg /clavulanate 10 µg and Cefepime30 µg /clavulanate 10 µg) the disc were placed on each plate, the plates were incubated at 37°C and were examined after 16-18 hours of incubation. After incubation, each plate examined, the diameters of inhibition zone were measured. An organism was interpreted as the ESBL producer if there was an increase of ≥5 mm in inhibition zone of the combination disk when compared to the corresponding cephalosporin disk, and then they were considered as ESBL positive[4].

MBL Detection by combined disk method

MBL detection by combined disk (Imipenem10mcg, and Imipenem 10mcg/0.1 M anhydrous EDTA 10µL/disk) placed on lawn cultured MHA plate. Imipenem procured from BD disk, Germany. An increase in zone diameter of 4mm around the IPM-EDTA disk compared to that of the IPM disk alone considered as positive for MBL production[8].

Molecular detection of ESBL genes

β-lactamase genes (*bla*TEM, *bla*SHV, and *bla*CTX-M) were detected by PCR using reverse and forward primer pairs with boiled suspension of bacterial cells as DNA template. All the PCR mix was made in sterile 0.2ml PCR tubes and the following thermal protocol (Table1) has been created in the gene Amp-9700 PCR instrument (ABI-USA), to detect the amplified ESBL genes showed in Table-4. Amplified product were detected by electrophoresis by 2% agarose gel with ethidium bromide (50µg/ml) was made in 0.5X TAE buffer. 10µL of the PCR product mixed with 2µL of gel loading dye and PCR products has been resolved at 100V for 20mins. 5µL of 100bp DNA ladder (Gibcobrl-USA) was used to detect the size of the fragment in the gel. The gel documented in the Bio-rad Gel documentation system; the image saved and represented in the results section(Lim *et al.,*2009).

Table 1

The PCR mixture made to a total volume of 10µL with the following components

Components	concentration	Volume (µL)	Final concentration	Product purchased
PCR Buffer with $MgCl_2$	10X	1	1X	NewEngland Biolab
dNTP	2.5mM	1	250µM	Takara Japan
Fwd Primer	2µM	0.4	80nM	SigmaOligos Bangalore
Rev Primer	2µM	0.4	80nM	SigmaOligos Bangalore
Taq DNA Polymerase	5U/µL	0.05	0.25U/10µL Rtx	NewEnglandBiolab
#Bacterial culture as Template	-	#2µL	-	-
Sterile Distilled water	-	5.15	-	-

***The above components have been made as the master mix for the PCR of multiple samples.**

 # The 2µL template was added separately in each PCR tubes and the other components have been made as the master mix and

8 µL of the mix added to each template to carry out the multiple reactions.

Table2
Primer sequences and PCR conditions

Gene/Primer	Oligonucleotide sequence	Thermal condition	Size of product
CTX-MU1 CTX-MU2	ATGTGCAGYACCAGTAARGT TGGGTRAARTARGTSACCAGA	1 cycle of 7min at 94°C; 35 cycles of 50 sec at 94°C, 40 sec at 50°C, 1min at 72°C; 1 cycle of 5min at 72°C	593
TEM-F TEM-R	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	1 cycle of 5 min at 96°C; 35 cycles of 1min at 96°C, 1min at 58°C, 1min at 72°C; 1 cycle of 10 min at 72°C	867*
SHV-F SHV-R	GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	1 cycle of 5min at 96°C; 35 cycles of 1min at 96°C, 1min at 60°C, 1min at 72°C; 1 cycle of 10 min at 72°C	867*

"*"- both TEM and SHV are same number of bas pair.

RESULTS

Total of 41 non fermentative and non-enterobacteriaceae members were found in 123 diabetic foot ulcer pus specimen such as *P. aeruginosa* (33) and *A. baumannii* (8). Following antibiotic resistant pattern were observed by disc diffusion,for cephotaxime 39.02% (16/41), ceftazidime 68.29% (28/41) and cefepime 46.34% (19/41). Whereas other beta-lactams like aztreonam, ticarcillin and piperacillin/tazobactam showed range of 80-95% if resistance and imipinem showed 36% of resistance. Based on the disc diffusion test three cephalosporin (i.e., cephotaxime, ceftazidime and imipenem) group of antibiotic were chosen for micro-broth dilution method.*P. aeruginosa* showed 54.54% (18/33) of resistance and 24.24% (8/33) of intermediate resistance for cephotaxime, 63.63% (21/33) and 6.60% (2/33) were resistant and intermediate resistant for ceftazidime respectively. In case of imipenem, 13/33 isolates tested were resistant, another 7/33 isolates were intermediately resistant. *A. baumanii* we have observed resistance and intermediate resistance to all three drugs for *A. baumannii* tested by micro broth dilution method. We compared resistant pattern of non-enterobacteriaceae gram-negative isolates, ceftazidime showed higher percentage of resistance in disc diffusion 68.29% (24/41) than the MIC method 58.53% (24/41). Cephotaxime MIC studies showed 22/41, 11/41 resistant and intermediate resistant respectively; they compared with disc diffusion. Which, showed the minimum number of resistant and intermediate resistant isolates 39.02 %(16/41) and 46.34% (19/41) (Table 3). All the 41 non-fermentative gram-negative isolates were subjected to ESBL screening by single disc synergy test (Table-4). The antibiotic combination of cephotaxime + clavulanate, ceftazidime + clavulanate and cefepime + clavulanate showed ESBL positivity of 20/41, 15/41 and 1/41 respectively. The result of MBL detection by phenotypic were method shown in Table-4, we have observed MBL positivity in both resistant and sensitive isolates of non-fermentative gram-negative bacteria29/41 were MBL positive and 12/41 found to be negative MBL production by phenotypic method. Among 41non-enterobacteriaceae isolates only one was CTX-M positive.31.7 % (13/41) of non-enterobacteriaceae isolates were found to be positive for the TEM gene. 4/41 non-enterobacteriaceae was positive for SHV gene (Amplicon size: 867; Photo-2). 2/41 non enterobacteriaceae isolates yielded more than one ESBL gene positivity. When compared phenotypic and genotypic ESBL positivity. 48% phenotypic cephotaxime+CLA positive isolate (20/41) showed 31.7% (13/41) of blaTEM positive 8% of SHV producer. Same way ceftazidime+CLA and cefepime+CLA compared with blaCTX-M, blaTEM and blaSHV showed varied results (table-4).

Table 3
Results of Disc Diffusion and MIC for Non-Enterobacteriaceae*(n=41)

Disc diffusion		Minimum Inhibitory concentration											
		Cephotaxime			Total	Ceftazidime			Total	Imipenem			Total
		R*	I#	S$		R*	I#	S$		R*	I#	S$	
Cephotaxime	R*	12	2	2	16								
	I#	8	8	3	19								
	S$	2	1	3	6								
Ceftazidime	R*					19	4	5	28				
	I#					2	1	1	4				
	S$					3	0	6	9				
Imipenem	R*									9	3	3	15
	I#									2	4	0	6
	S$									5	1	14	22
Total		**22**	**11**	**8**	**41**	**24**	**5**	**12**	**41**	**16**	**8**	**17**	**41**

R*-Resistance, I#-Intermediate, S$-Sensitive and Non-Enterobacteriaceae*- includes*P. aeruginosa* and *A. baumanii*

Table 4
Results of phenotypic and genotypic ESBL for Non-Enterobacteriaceae*

Phenotypic ESBL and MBL detection		Genotypic ESBL gene detection								
		CTX-M		Total	TEM		Total	SHV		Total
		P	N		P	N		P	N	
Cephotaxime+CLA	P	1	19	**20**	13	7	**20**	4	16	**20**
	N	0	21	**21**	0	21	**21**	0	21	**21**
Total		**1**	**40**	**41**	**13**	**28**	**41**	**4**	**37**	**41**
Ceftazidime+CLA	P	1	14	**15**	13	2	**15**	4	11	**15**
	N	0	26	**26**	0	26	**26**	0	26	**26**
Total		**1**	**40**	**41**	**13**	**28**	**41**	**4**	**37**	**41**
Cefepime+CLA	P	1	0	**1**	1	0	**1**	1	0	**1**
	N	0	40	**40**	12	28	**40**	3	37	**40**
Total		**1**	**40**	**41**	**13**	**28**	**41**	**4**	**37**	**41**
Imipenem+EDTA	P	1	28	**29**	13	16	**29**	4	25	**29**
	N	0	12	**12**	0	12	**12**	0	12	**12**
Total		**1**	**40**	**41**	**13**	**28**	**41**	**4**	**37**	**41**

R*-Resistance, I#-Intermediate, S$-Sensitive and Non-Enterobacteriaceae*- includes P. aeruginosa and A. baumanii

DISCUSSION

Diabetes mellitus recognized to be common in Indians of the Asian subcontinent. Currently, 50.8 million Indians have diabetes. The projections indicate that India will have the largest number of diabetic patients by the year 2030A.D. [11] Approximately 15–20% of persons with diabetes will develop a DFU in their lifetime[9,11] In our study, 123 wound swabs were collected from diabetic foot ulcers of both in-patients and outpatients attending diabetic foot clinic in a tertiary care hospital, Chennai between 2007 September to 2008 October. In the present study, 171 organisms were isolated from 123 patients and an average of 1.33 organisms per case found. This is slightly higher than the findings by Vishwanathan *et al.,* 2002 in their study culture yielded an average of 1.21 per case. Poly-microbial

nature of diabetic foot infection was observed in our study as well as other studies from different part of the world[9,11,13and 21]. However Dhanasekaran *et al.,* 2003, documented 84% of diabetic foot ulcers are mono-microbial condition. Among 171 isolates obtained, 66-gram positive isolates, 97-gram negative isolates, and remaining were *candida albicans*[8]. Studies from Western countries show that Gram-positive aerobes are the predominant organisms isolated from DFI[6,3,14,and10]. Studies by Viswanathan *et al.,*[21] from South India, reported 35% gram-positive pathogens isolated and 65% gram-negative ones, these finding emphasizes the high prevalence of gram-negative pathogens in Southern India. Three large diabetes research centers (India, Germany, and Tanzania) have obtained very similar results. Wheat *et al.,*[24] and Shankar *et al.,*[17] also reported Gram-negative aerobes to be the most frequently isolated pathogens (51.4%), followed by Gram-positive aerobes (33.3%) and anaerobes. In contrast, two recent Indian studies have shown a preponderance of Gram-negative aerobes. Gadepalli *et al.,*[9]in their study on 80 ulcer specimens, recovered 183 isolates, of which 28.7% were Gram-negative and only 13.8% gram-positive also anaerobes and fungal isolates. Three MIC and disc diffusion methods were compared for cephotaxime, ceftazidime, and imipenem for members of non-Enterobacteriaceae. Ceftazidime showed higher percentage of resistance in disc diffusion 68.29% (24/41) than MIC 58.53% (24/41). Cephotaxime MIC showed 22/41, 11/41 resistant and intermediate resistant respectively; when compared with disc diffusion it showed minimum no. of resistant and intermediate resistant isolates.

The present study found ESBL positivity in 36.58% of non enterobacteriaceae isolates for ceftazidime-ceftazidime+clavulanic acid. Cephotaxime-cephotaxime + clavulanic acid found to be 2.43%.Which differs from Jiang *et al.,*[25] they showed 20/34 isolates and 1/34 isolates for ceftazidime+clavulanic acid and cephotaxime+clavulanic acid respectively. Other ESBL type namely MBL under functional class B or D carbapenemase genes, leads to increased carbapenem resistance in this group[2, 15, 18]. Along with one or more ESBL, that can confer ESBL to cephalosporin. Rapid acquisition of carbapenemase genes from *P. aeruginosa* and other non-fermenters may not be easily accomplished due to low rates of transfer, maintenance, and expression of these genes in the Enterobacteriaceae[5].MBL-producing gram-negative organisms have now been reported in many geographic regions[8]. Their ability to rapidly disseminate within an institution and lead to poor outcomes when infection ensues is a concern, and therefore, early laboratory detection is of great clinical importance. Unfortunately, the phenotypic appearance of MBL-carrying organisms varies depending on the bacterial host, with increasing reports of carbapenem-susceptible isolates, primarily *Enterobacteriaceae* such as *Klebsiella* spp. and *E. coli* possessing MBL genes[5,2, and15]. Importantly, the combined disc method is highly sensitive at detecting carbapenem-susceptible MBL-producing isolates, a concerning phenotype that is being described with increased frequency, Such organisms often carry hidden MBL genes, whereby the microbiologist and the clinician remain unaware of their presence within an institution. Such a scenario creates the potential for untoward clinical and infection control consequences and is by no means unique to MBLs. In our study, 48/97 gram negative bacteria were found to be sensitive to Imipenem by MIC method. Out of 48 Imipenem sensitive isolates 21 were MBL producing, among these 70.73% (29/41) was non-enterobacteriaceae. The blaCTX-M is among the most prevalent and widely disseminated genes in the clinical bacterial population in India as shown by 8 and 5. We have analyzed the ESBL genes by PCR using universal primers and we found blaCTX-M positivity in 73.21 % (41/56) of Enterobacteriaceae and 2.4% of non-enterobacteriaceae as the most prevalent ESBL gene followed by 25% (14/56) Enterobacteriaceae and 31.7 % (13/41) isolates of non-enterobacteriaceae for blaTEM. Least common type is SHV, which showed 7/56, and 1/41 isolates of enterobacteriaceae and non-

enterobacteriaceae respectively positive for the same.

Infections with MDR organism several antibiotics, and resistant can be treated with extended spectrum antibiotics for longer durations. As a result, duration of hospital stay can be longer and their treatment can be more costly. Furthermore, mortality from infections with MDR is twice as high as mortality from infections with microorganisms sensitive to antibiotics[2].There is high degree of antibiotic resistance found in our isolates, which could be due to the treatment with broad spectrum antibiotics might lead to selective survival advantage of pathogen. The antimicrobial resistance pattern was similar to the recent studies done in India and outside[22and 16]. Gram-negative bacteria that are regarded as normal flora of the skin may cause severe tissue damage in diabetics and should never be disregarded as insignificant in diabetic foot ulcers. The erosion of effective antimicrobials[27] continues as we witness the increased frequency of resistance to all drugs—in particular, the fluoroquinolones and carbapenems, which are often the drugs of last choice. With the relative absence of new antimicrobials coming to market and with new threats arising from the gram-negative bacteria, however, the number of drug options leaves us perilously close to none or only a single effective agent for some life-threatening infections. Hundreds of Beta-lactam-degrading enzymes are rapidly undermining the mainstay penicillin's and late-generation cephalosporin agents. The increase in metallo-beta-lactamases, which are active against carbapenems and most other Beta-lactams, is an alarming new development[5,2 and15].

CONCLUSION

The accumulation of antibiotic resistance in bacteria is beyond any doubt, a practical demonstration of the Darwinian rule of the survival of the fittest; thus, this resistance poses the serious problem of treatment failure. Extensive knowledge about antibiotics susceptibility is vital. The information enables decipher the potential strategies to combat resistance menace. ESBL positive strains are major hurdle for finding a break through drug resistance. There is high percentage of antibiotic resistance found in our isolates; which warrants the use of antibiotic policy as well as proper surveillance programs.

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