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DETECTION OF DRUG RESISTANCE IN ENTEROBACTERIACEAE ISOLATES FROM DIABETIC FOOT ULCER

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ABSTRACT

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 lower limb, collectively known as the diabetic foot syndrome. Foot ulceration and amputation are economically most expensive diabetic complications. Diabetic foot infection is poly-microbial in nature, many studies have reported on the bacteriology of DFU over 30 years, but the results varied with different places. The present study was aimed to characterize the enterobacteriaceae isolates from diabetic foot ulcer. 123 DFU pus samples were collected from tertiary care hospital Chennai. 56 isolates were found to be enterobacteriaceae isolates among these 97 gram negative isolates. Enterobacteriaceae showed wide range of resistance to many of the drugs tested. imipenem, cephotaxime and cefepime showed 25 % (14/56), 60.07% (34/56) and 55.36% (31/56) of resistance respectively. ESBL positivity showed 76.78% (43/56). Whereas aztreonam, and trimethoprim showed higher percentage of resistance 85.71%, and 75% respectively. A detailed knowledge of the susceptibility to antimicrobial agents is necessary to facilitate the development of effective strategies to treat diabetic foot infections, especially the ESBL infections.

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

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INTRODUCTION

Diabetes is fast gaining the status of a potential epidemic in India with more than 62 million diabetic individuals currently diagnosed with the disease[21]. According to Wild et al. the prevalence of diabetes is predicted to double globally from 171 million in 2000 to 366 million in 2030 with a maximum increase in India. It is predicted that by 2030 diabetes mellitus may afflict up to 79.4 million individuals in India. Around 15 -30% of diabetic people are affected due to diabetic foot ulcer in their life time[28]. Diabetic Foot ulcer (DFU) is an infection, ulcer or destruction of deep tissue associated with neurological abnormalities[4] and various degrees of peripheral vascular disease of lower limb, collectively known as the diabetic foot syndrome. Foot ulceration and amputation are among the most costly diabetic complications[7]. Diabetic foot infection is poly-microbial in nature, many studies have reported on the bacteriology of DFU over 30 years, but the results varied with different places. Recent studies from India predominance of gram negative bacteria are increased recently[17, 25, 9, 3]. The organisms such as *Pseudomonas spp.*, *Klebsiella spp.* and *Proteus spp.* are mainly responsible for continuing and extensive tissue destruction with the poor blood circulation of the foot[14, 12, 2]. 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 [6, 26]. 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[17]. Many genera of gram-negative bacteria possess a naturally occurring, chromosomally mediated β-lactamase. One of the most clinically important groups of beta lactamases is the extended-spectrum Beta lactamases (ESBL) [5, 19]. According to the Infectious Diseases Society of America, *E. coli* and *K. pneumoniae* expressing ESBLs are on the hit list of six microbial pathogens of concern, the choice of inappropriate antibiotic therapy for patients harboring an ESBL producing isolate correlated with increased mortality in patients[8, 22, 11]. With this background, the present study was aimed to characterize the ESBL and MBL in enterobacteriaceae isolates from DFU.

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 a diabetic foot clinic at KMCH, Chennai. Identification of DFU bacterial isolates were done based on standard procedures. Out of 171 DFU isolates 66(38.59%) were gram-positive and 97 (56.7%) were grams negative bacteria. Out of 41/97 gram negative bacterial isolates were non fermentative and non enterobacteriaceae isolates.

Antibiotic susceptibility test

Enterobacteriaceae isolates were tested for antibiotic susceptibility by disc diffusion method and micro broth dilution method. 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 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[1].

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[15].

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[23].

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 were made in sterile 0.2ml PCR tubes and the following thermal protocol [Table-1] 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[15].

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	New England 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	NewEngland Biolab
#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.

Table 2
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 AND DISCUSSION

Total of 56 enterobacteriaceae members was ere isolated from 123 diabetic foot ulcer pus samples such as *K. pneumonia*e (7), *K. oxytoca* (4), *E. coli* (11), *C. freundii* (2), *C. koseri* (1), *E. aerogenes* (2), *P. vulgaris* (21), and *P. mirabilis* (8). They showed a wide range of resistance to many of the drugs tested. Imipenem, cephotaxime and cefepime showed 25 % (14/56), 60.07% (34/56) and 55.36 % (31/56) of resistance respectively, whereas aztreonam and trimethoprim showed higher percentage of resistance 85.71%, and 75% respectively. Wheat et al., 1986[27]and Shankar et al., 2005[18] 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., 2006[10] 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. Studies from Malaysia have also reported a predominance of gram-negative bacteria (52%) in patients with DFI, the most common pathogens isolated being Proteus spp., *K. pneumoniae, E. coli* and *E. cloacae*[16]. The reasons for the differences observed in the prevalence of Gram-negative bacilli in DFI between diabetic patients from eastern and western countries remain largely unknown. However, environmental factors such as sanitary habits, e.g. use of water for peri-anal wash (ablution) after defecation leading to contamination of hands with fecal flora, are proposed to be responsible for increased Gram-negative infections in the developing world [29]. 56 enterobacteriaceae isolates were tested for MIC by micro broth dilution method, a total of 21.42% (12/56) and 46.42% (26/56) were intermediate resistant and resistant to cephotaxime. For ceftazidime 21.42% (12/56) and 55.35% (31/56) were intermediate resistant and resistant, respectively. Imipenem showed

23.21% (13/56) and 21.42% (12/56) of intermediate resistant and resistance, respectively. We compared resistant pattern of enterobacteriaceae gram-negative isolates, depicts the observations of comparison of the results of disc diffusion and MIC for cephotaxime, ceftazidime, Imipenem for members of enterobacteriaceae. In case of cephotaxime, higher percentage of resistance was observed in disc diffusion 60.71% (34/56) than the MIC 46.42% (26/56). 31/56 and 12/56 enterobacteriaceae isolates were resistant and intermediate resistant to ceftazidime MIC. It showed varied results with disc diffusion (Table 3).Few, author reports have 50% sensitivity pattern to Imipenem[20], however, our study showed 25 % (14/56) of enterobacteriaceae resistant to imipenem. All the 56 enterobacteriaceae 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 44/56, 43/56 and 23/56 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 gram-negative bacteria. 41/56 enterobacteriaceae isolates showed MBL positivity and the rest 15/56 found to be negative for MBL. Among 56 enterobacteriaceae isolates 73.21 % (41/56) were ctx-M positive (Amplicon size is 593bp) and likewise when the same set of isolates were subjected to TEM (14/56) and SHV (7/56) (Amplicon size: 867; Fig. 1). In our study, the prevalence of multi resistant bacterial strains also portends the possibility of longer periods of hospitalization for patients as healing may be compromised when bacteria are highly resistant to antimicrobials. The prevalence of ESBL producing gram-negative isolates was in incremental with the reports of Hartemann et al., 2008 (Hartemann-Heurtier & Senneville, 2008). There is a high degree of antibiotic resistance found in our isolates, which could be due to the treatment with broad spectrum antibiotics might lead to a selective survival advantage of pathogen. The antimicrobial resistance pattern was similar to the studies done in India and outside[16,24]. 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.

Table 3
Results of Disc Diffusion and MIC for Enterobacteriaceae* (n=56)

Disc diffusion		Minimum Inhibitory concentration											
		Cephotaxime			Total	Ceftazidime			Total	Imipenem			Total
		R*	I#	S$		R*	I#	S$		R*	I#	S$	
Cephotaxime	R*	26	7	1	34								
	I#	0	5	4	9								
	S$	0	0	13	13								
Ceftazidime	R*					31	5	0	36				
	I#					0	7	4	11				
	S$					0	0	9	9				
Imipenem	R*									3	5	6	14
	I#									5	2	8	15
	S$									4	6	17	27
Total		26	12	18	56	31	12	13	56	12	13	31	56

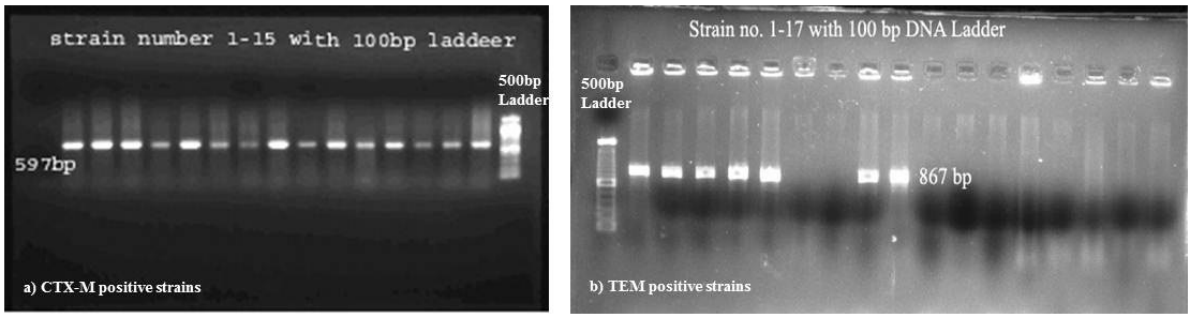
R-Resistance, I#-Intermediate, S$-Sensitive and Enterobacteriaceae*- includes E. coli, K. pneumoniae, P. mirabilis, P. vulgaris, K. oxytosa*

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	31	13	44	11	33	44	6	38	44
	N	0	12	12	3	9	12	2	10	12
Total		31	25	56			56			56
Ceftazidime+CLA	P	28	15	43	10	33	43	6	37	43
	N	3	10	13	4	9	13	2	11	13
Total				56			56			56
Cefepime+CLA	P	16	7	23	8	15	23	1	22	23
	N	15	18	33	6	27	33	7	26	33
Total				56			56			56
Imipenem+EDTA	P	21	20	41	10	31	41	7	34	41
	N	10	5	15	4	11	15	1	14	15
Total				56			56			56

R*-Resistance, *I*#-Intermediate, *S*$-Sensitive and Enterobacteriaceae*- includes E. coli, K. pneumoniae, P. mirabilis, P. vulgaris, K. oxytosa

Figure 1
Agarose gel showing amplicons of CTX-M(a) and TEM (b) DFU bacterial isolates



CONCLUSION

According to the Darwin's rule from On the Origin of Species in 1859, the concept of evolution through natural selection and consequently the ability to adapt bacteria slowly started to accept antibiotics. 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. The prevalence of MDR organisms is alarmingly high in the diabetic foot patients in India because of indiscriminate use of antibiotics. There is a 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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