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Research Article

ISOLATION AND DRUG RESISTANCE OF AEROBIC BACTERIAL ISOLATES IN DIABETIC FOOT ULCERS WITH CARBAPENEMS IN A TERTIARY CARE CENTRE IN SOUTH INDIA

Vandana Vijayeta Kiro[1] and Selvi[2]

[1]Department of Laboratory Medicine, AIIMS, New Delhi - 110029
[2]Department of Microbiology, Director- Institute of Microbiology, MMC, Chennai. 600003

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A B S T R A C T

Background and Objective: As prevalence of diabetes mellitus is increasing so are its complications. This research was done in our tertiary care centre to study the aerobic bacterial profile of diabetic foot ulcer and their resistant pattern focusing on Carbapenemase producers. **Material and Methods**: Sample from 50 patients were processed for determining aerobic bacterial profile phenotypically followed by E- Test and Modified Hodge test. PCR was done to find incidence of bla$_{kpc}$ and *bla*$_{NDM}$. **Results**: 45 pathogenic aerobic bacteria were isolated. Gram positives were 10(22.2%) and Gram negative were 35 (77.7%). The Carbapenem resistant organisms confirmed by E-Test - 2(0.04%) were Modified Hodge test positive. PCR was done for these two isolates to detect the genes specified. **Conclusion**: Apart from empirical treatment with broad spectrum antibiotics; culture and sensitivity tests with rationale surgical procedures to prevent further spread of infection and amputations is suggested to be mandatory owing to the increasing antibiotic resistance.

INTRODUCTION

Diabetes mellitus ;a chronic disorder has affected a large segment of population all over the world .The World Health Organization (WHO) declared India to have exceeded other countries in having the maximum number of diabetics which is around 62 million.[1]It has been projected to increase to 80 million by the end of 2030, giving India status of being called the "Diabetic Capital of the World".[1]

The longevity of the diabetic population is increasing which has led to discovery of novel complications related to this chronic ailment.[2, 3]Among the complications of Diabetes, foot problems are the most common cause of non -traumatic limb amputation.[2] There is a 10-fold greater risk of hospitalization for soft tissue and bone infections in diabetics than non-diabetics.

Several studies have been reported on bacteriology of Diabetic Foot Ulcers(DFU s) over the past 30 years but results have shown variations and are often contradictory.[4] Studies which were done in 1970s demonstrated Gram positive cocci with Staphlylococcus aureus as the main pathogen and aerobic Gram negative bacilli in chronic and previously treated wounds. In recent years ESBLs and MRSA are causing substantial problem.[5]

Corresponding author:* **Vandana Vijayeta Kiro
Department of Laboratory Medicine, AIIMS, New Delhi - 110029

Antibiotic resistance in diabetic foots has become a major concern as there are very few or no alternatives left for the treatment. There is a dearth of data on MDROs and especially evolving carbapenem resistance; if any from this part of the world. This study is mainly focussed on resistance in carbapenems in pathogens associated with Diabetic foot ulcers in our tertiary care centre.

Resistance in carbapenems is mainly mediated by Metallo-β-lactamases. Around ten years back the genes encoding MBL were mainly present in the non-fermenting Gram negative bacterias like Pseudomonas aeruginosa and Acinetobacter species.

Now several studies done suggests that MBLs have disseminated at an alarming rates to members of family Enterobacteriaceae which has been seen in epidemics of *bla*$_{KPC}$ clones in USA, Europe and the worldwide epidemic with *bla*$_{NDM}$ producing Gram negative bacterias. Though initial antibiotic therapy for most patients must be selected empirically, it should be largely based on the assessment of severity and knowledge of the local microbial epidemiology.

MATERIAL AND METHODS

A cross-sectional study was done from January 2016 to August 2016 in our tertiary care centre. Ethical clearance was obtained by the Institutional Ethical Committee (IEC) at our college. Cultures of infected diabetic foot ulcer from 50 patients visiting our hospital were processed.

The infected area of the foot was cleansed thoroughly with normal saline while debridement and sample was collected thereafter by two swabs in a sterile test tube or pus in sterile syringe. The samples were subjected for direct gram staining, AFB staining and culture. AFB staining was done by Kinyouns method.

Turbidity if observed in thioglycollate broth with tissue samples were processed. Primary culture plates used were Nutrient agar, Blood agar and MacConkeyagar. Growths were then further processed for biochemical reactions and antibiotic susceptibility testing was done according to CLSI guidelines (M100S, 26th edition).[6] The pathogens were identified and accordingly antibiotics were tested and reported. Resistant pathogens were identified by the phenotypic confirmation methods mentioned in CLSI (M100S, 26th edition). Pathogens which were resistant to carbapenems were further subjected to genotypic study for ruling out any incidence of bla_{KPC} and bla_{NDM}.

Antibiotic susceptibility testing-Antibiotics susceptibility testing was done by Kirby- Bauer's Disc Diffusion method using Meuller -Hinton Agar medium. Staphylococcus aureus ATCC 25923 ,Escherichia coli ATCC25922 and Pseudomonas aeruginosa ATCC 27853 were used as control strains. The drugs discs were purchased from HiMedia, Mumbai.

E -Test: 0.5 McFarland standard suspension of the isolate was swabbed onto Mueller Hinton Agar plate. E-strip of Imipenem was aseptically placed at the middle of the plate. Incubation was done for 18 to 24 hours at 37˚C and next day the MIC was noted directly from the scale on the top of the strip at the point where the ellipse intersected the scale. MIC determined with this technique generally agree well with MICs generated by standard broth or agar dilution methods.[7,8] Two pathogens which were found to be resistant to Imipenem or Meropenem in the disk diffusion test were subjected to the E-Test.

Modified Hodge test - 0.5McFarl and standard suspension of E.coli ATCC 25922 in broth was diluted to 1:10 and then inoculated onto MHA plate and left to dry for 3-10 minutes. Then meropenem disc was placed on the centre of plate . Using a 10µl loop, 3-5 colonies of test strain and QC organisms grown overnight on a nutrient agar plate were inoculated in a straight line out from the edge of the drug disc. The plate was then incubated at 35˚C for 16-20 hours. Following incubation we examined the plate for enhanced growth around the test or QC organism streak at the intersection of the streak and the zone of inhibition.

Detection of carbapenemase encoding genes: $bla_{KPC}$and bla_{NDM} by PCR. Polymerase chain reaction was performed for identification of bla_{KPC} and bla_{NDM}using PureFast® Bacterial DNA minispin purification kit purchased from Helini Biomolecules, Chennai, India.

RESULTS

From January 2016 to August 2016, 50 patients suffering from diabetic foot ulcers were included in the study. The age group of patients involved in the study were from 40-80 years. The mean age of patients included in this study was 57.06 years with (SD 10.08). Males 32(64%) were affected more than females 18 32%). The number of patients seen in IP were 31 (62%) and OP 19 (38%). 39 patients (78%) did not know about their family history while 9 (18%) patients had family history and 2 (4%) were sure of having no family history.

The duration of diabetes as observed was from 1 year to 25 years with mean of 6.94 years. No mortality was noted.

Patients who were on oral hypoglycemic drugs were more 32 (64%) that on insulin18(32%). Irregularity in treatment was found in 44(88%) of the patients.

Wagners Grading: Maximum patient affected were in Grade II- 24 (48%) followed by Grade III -21 (42%) and Grade IV - 5 (10%). No cases were seen in Grade 1 and Grade 5.

Aerobic bacterial Identification and their antibiotic resistant pattern - In this study done in 50 patients; 45 pathogenic aerobic bacteria were isolated. In 10 (20%) patients there was no aerobic bacterial growth. Details of the isolated organisms and their resistance pattern is shown in Table 1 (Gram positive) ,Table 2 (a) & Table 2(b) (Gram negatives) . Two isolates Proteus mirabilis and Klebsiella pneumoniae were carbapenem resistant and also MBL producers shown in Fig. 1 . PCR done for both resistant isolate for gene detection - bla_{KPC} and bla_{NDM} showed negative results which is depicted in Fig. 2.

Clinical intervention: Adequate draining and thorough debridement with suggested antibiotics was followed for all patients. Patients found with carbapenem resistant underwent foot amputation (carbapenem resistant Klebsiella pneumoniae) and below knee amputation (carbapenem resistant Proteus mirabilis)

Table 1 Gram positives antibiotic resistant pattern

Antibiotics	Enterococcus faecalis (10)	
	Sensitive %	Resistant %
Penicillin	30	70
Ampicillin	50	50
Ciprofloxacin	60	40
High Level Gentamicin	100	0
Vancomycin	100	0

Table 2 (a) Gram negatives antibiotic resistant pattern (S- Sensitive, R- Resistant)

Antibiotics	Klebsiella (14)				Proteus (13)			
	Klebsiella pneumoniae(10)		Klebsiella oxytoca(4)		Proteus vulgaris(11)		Proteus mirabilis(2)	
	S%	R%	S%	R%	S%	R%	S%	R%
Gentamicin	70	30	25	75	9	91	0	100
Amikacin	80	20	25	75	45.4	54.5	0	100
Ciprofloxacin	10	90	0	100	9	91	0	100
TMP-SX	10	90	0	100	27.2	72.8	0	100
Cefotaxime	20	80	25	75				
Ceftazidime					18.1	81.9	0	100
Imipenem	100	25	75	0	100	0	50	50
Meropenem	100	25	75	0	100	0	50	50
Doxycycline	40	60	50	50				
Pip/Tz	90	10	50	50	72.7	27.3	50	50
Tigecycline	100	0	100	0	_	_	_	_
Polymixin B	100	0	100	0	_	_	_	_
Colistin	100	0	100	0	_	_	_	_

Table 2 (b) Gram negativeantibiotic resistant pattern (S - Sensitive, R - Resistant)

Antibiotics	Pseudomonas aeruginosa(5)		Escherichia coli (2)		Citrobacterfreundii (1)	
	S%	R%	S%	R%	S%	R%
Gentamicin	60	40	50	50	0	100
Amikacin	60	40	50	50	0	100
Ciprofloxacin	40	60	0	100	0	100
Trimethoprim-Sulfamethoxazole	–	–	0	100	0	100
Cefotaxime	–	–	0	100	–	–
Ceftazidime	20	80	–	–	0	100
Imipenem	100	0	100	0	100	0
Meropenem	100	0	100	0	100	0
Doxycycline	–	–	50	50	100	0
Piperacillin/ Tazobactam	40	60	100	0	100	0
Tigecycline	–	–	100	0	100	0
Polymixin B	100	0	100	0	100	0
Colistin	100	0	100	0	100	0

Table 3 Carbapenem resistant isolates

Pathogens(35/45)	Carbapenem resistant isolates	
	No.	%
Proteus vulgaris(11)	0	–
Klebsiella Pneumoniae(10)	1	2.8
Pseudomonas aeruginosa(5)	0	–
Klebsiellaoxytoca (4)	0	-
Proteus mirabilis(2)	1	2.8
Escherichia coli(2)	0	–
Citrobacterfreundii (1)	0	–
Total	2	4.44

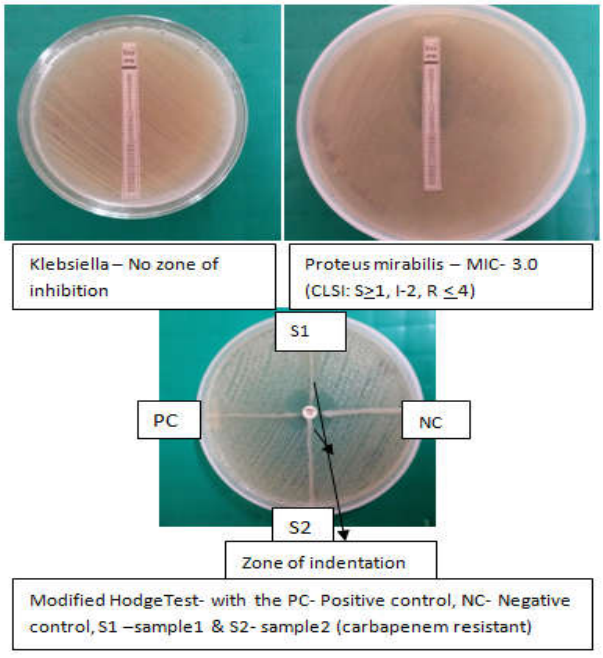

Fig 1 E-Test & Modified Hodge Test

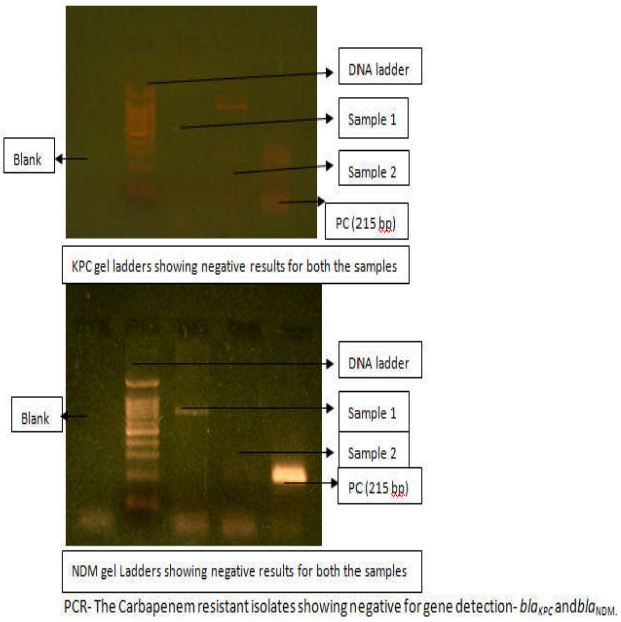

Fig 2 PCR done to detect the bla_{KPC}&bla_{NDM} genes

DISCUSSION

Many studies are there on epidemiology of bacterial foot ulcers but few on evolving carbepenem resistance.

Studies done on aerobic bacterial isolates done by SanjeethSasheedharan *et al* [9]at a parallel time in another part of this city states Gram negative to be predominant (58.5%) which does correlate with our study; Gram negatives being 77.7% . The most frequently isolated bacteria in their study were Staphylococcus aureus (26.9%), followed by Pseudomonas aeruginosa (20.9%). Of the 58.5% Gram negative pathogens, 16.5% were Enterobacteriaceae resistant to carbapenems. Among these isolates, 4 (25%) were positive for bla_{NDM}gene. Among the rest, 18.6% were carbapenem-resistant Pseudomonas, among which 4 (36.3%) were bla_{NDM} positive. Among the Staphylococci, 23.7% were methicillin-resistant Staphylococcus aureus. In contrast to this study our study showed Klebsiella species as most frequent (31.1%) followed by Proteus species(28.8%) Enterococcus (22%), Pseudomonas aeruginosa (11.1%), Escherichia coli (0.04%) and Citrobacter freundii (0.02%). Only two isolates (4.44%) were resistant to carbapenem and of these none were positive for $bla_{NDM}$and bla_{KPC}. Studies done by Gadepelli *et al*.,[10]2006; Zubair *et al*.,[11] 2011 had in their findings also more of Gram negatives than Gram positives.

According to study done by Sudhir K. Jain and Rashmisnata Barman in a tertiary care centre in North-East India,[12] atotal of 185 bacterial isolates were obtained from 150 persons with diabetic foot ulcers. Among the isolates, Gram-negative bacilli were isolated in 112/185 (61%) and Gram-positive cocci in 73/185 (39%) cases. The most common isolate was Staphylococcus spp. 46 (25%), followed by Escherichia coli (20%) and Enterococcus spp. (15%). The antibiotic sensitivity profile of the bacteria was also studied. Among the isolates, 59/112 (53%) of the Gram-negative bacilli were extended spectrum beta-lactamase producers, 19/46 (41%) were methicillin-resistant Staphylococcus aureus, and 5/27 (19%)

were vancomycin-resistant Enterococcus. Carbapenem resistance was11% and 28% among Enterobacteriaceae and Pseudomonas respectively. Further evaluation on carbapenem was not done.

According to a study done in South West China [13]from 2014 -2017, the dominance of Gram negatives (51%) was seen rather than the Gram positives(36.9%). This finding does match with our study and is also contrary to study done in their Southern part of China from 2009 - 2014 where Gram positives were 54%. Incidence of carbapenem resistance in Enterobacteriaceae was 0.4% and in Pseudomonas - 6%. MRSA was20% and VRE - 3%. No further study was done on carbapenem resistant isolates.

In a study done by SM Sekhar, N Vyas, and C Mukhopadhyay[14] from June 2011 to December 2011 in Manipal (India); out of the 108 specimens of the diabetic foot lesions isolated prevalence of Gram-negative organisms (56%, 84/150) was found to be more than Gram-positive organisms (44%, 66/150). However, Staphylococcus aureus was the most frequent pathogen (28%, 42/150). Acinetobacter was completely resistant to all the common antibiotics tested. Carbapenem resistance was 4% among Gram-negatives that too only seen in Acinetobacter. MRSA was4%.No data on VRE.

In other studies among Enterobacteriaceae members, Citrobacter and Proteus were found to be dominant genera, followed by Klebsiella. We found Gram-negative bacteria to be more prevalent than Gram-positive bacteria, as has been observed in other earlier studies from India (Gadepalli *et al*., 2006; Zubair *et al*., 2011) [10,11]

In our study ;Conventional Polymerase chain reaction was done to find incidence of KPC gene(rare gene in India) and NDM gene (not so rare gene in India) in the two carbapenem resistant and MHT positive isolates was negative for both. Genetic study on multi drug resistant isolates have been done in diabetic foot ulcers in North India, but not for the above mentioned two genes.[15]A study done by Rachanasolanki *et al* [16] in India has revealed presence of KPC (15) and NDM(49) in growths from various clinical samples. In a study done in southern India; NDM was detected in 4 patients from a tertiary care centre.[17] In a study done by Anjana Shenoy K., Jyothi E.K. & R. Ravikumarin south India[18], 34 isolates out of 74 MDR GNB were positive for blaNDM-1.

In a study done by Mohan *et al*[19]; presence of NDM-1 gene but absence of KPC gene was noted from the clinical isolates. In their study, out of 166 isolates which were phenotypically carbapenemase producers ;66 isolates were negative e for all the four genes (bla VIM, bla IMP, bla NDM and bla KPC) tested by PCR.

CONCLUSION

Uncontrolled Diabetes with progressing age along with reduced care in foot favours impending infections leading to high cost of hospitalization and further negligence results in disarticulation of toes and amputations of foot and legs thus increasing morbidity. As in India knowledge about importance of regularity in treatment of Diabetes is poor, progression to complications is inevitable and patient often tend to land up in severe infected foot ulcers; harbouring resistant pathogens while hopping from one hospital to another. This study done for bacterial isolation and their antibiotic profile unearths Gram negatives to be most important etiological agents at present in ourcentre. Apart from other higher antibiotics; Piperacilin /Tazobactam showed least resistance favouring its use for high grade diabetic ulcers as it covers aerobic, anaerobic and is a good β-lactamase inhibitor. However; it does not rule out the necessity of culture and sensitivity tests with rationale surgical procedures to prevent further spread of infection. The genotypic test done on those two isolates for bla_{KPC} and bla_{NDM} by conventional PCR proves absence of these genes in the those two resistant isolates. Resistant strain genotypic detection and further sequencing was limited to these two genes because of financial constrain. Furthermore other molecular studies need to be done to detect the resistance mechanisms. This evidence -based study will definitely lead to a well guided approach to the antibiotic management of foot ulcers in diabetics in our centre. Using evolving technologies in detection of resistance will also help in infection control in health care.

Competing Interests: None

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