Original Article

Detection of ESBL and plasmid-mediated AmpC beta lactamases among the Gram-negative bacterial isolates in diabetic foot ulcer infections

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ABSTRACT

Background and Aim: Foot ulcers are a significant complication of diabetes mellitus and are usually poly-microbial. Aerobic Gram-negative bacilli are isolated in higher frequency with increasing grade of ulcers, and development of drug resistance is a cause of concern. The aim of our study is to identify the extended spectrum β-lactamases (ESBL) and AmpC β-lactamases among the Gram-negative aerobic bacterial isolates in the different grades of diabetic foot ulcers. **Materials and Methods:** Pus samples from 104 male and female diabetic patients presenting with grade I to grade V foot ulcers were cultured according to the standard microbiological procedures and antimicrobial sensitivity performed by the Kirby-Bauer disc diffusion method as per CLSI guidelines. MIC_{50} of all isolates for Ceftazidime was detected by the agar dilution method. The Gram-negative bacterial isolates were further tested for ESBL and AmpC β-lactamases by the modified double disc synergy test and combined disc method. AmpC disc test was used for the confirmation of AmpC production. **Results:** Forty-seven isolates exhibited resistance to Ceftazidime phenotypically and 54 isolates had MIC greater than 2 μg/mL. ESBL production was observed in 55.55% of the isolates. AmpC production was seen in 19 isolates (35.18%). No mechanism of cephalosporin resistance was explainable for 5 isolates. **Conclusion:** The combined disc method was more sensitive in the detection of ESBLs, while the AmpC disc test was an easy way to detect AmpC beta lactamases and can be used for routine screening.

Key words: AmpC, diabetic foot, ESBL, MIC

INTRODUCTION

Foot ulceration in diabetics occurs as a result of trauma in the presence of neuropathy and/or peripheral vascular disease with infection occurring as a secondary phenomenon. Factors like high plantar pressures, impaired wound healing due to tissue hypoxia, hyperglycemia and impaired perfusion lead to foot ulceration, gangrene and finally, amputation if appropriate intervention is not applied.

Its well documented that diabetic foot ulcers are poly-microbial involving aerobic and anaerobic organisms.[1,2] While many Western studies[3,4] have reported Gram-positive cocci as the predominant isolate, Indian data has shown aerobic Gram-negative bacilli being isolated in more numbers

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Access this article online	
Quick Response Code:	Website: www.caijournal.com
	DOI: 10.4103/2225-6482.159229

especially in complicated foot infections.[5,6] Development of multidrug resistance and resistance mechanisms like extended spectrum β-lactamases (ESBLs) and AmpC beta lactamases have become prevalent in this group of organisms. The ESBL genes generally result from point mutations in the genes of broad-spectrum β-lactamase Ambler class A enzymes, such as TEM-1, TEM-2 or SHV-1. They are usually located in conjugative mega plasmids, which often carry genes responsible for resistance to other antibacterial drugs, making it extremely difficult to treat infections caused by bacteria that produce these enzymes. Along with ESBLs, plasmid-mediated Ambler class C cephalosporinases (or Bush group 1 cephalosporinases) have been found in clinical isolates of the Enterobacteriaceae. These enzymes can produce resistance to cephamycins, extended spectrum cephalosporins and aztreonam, and unlike class A ESBLs, β-lactamase inhibitors do not inhibit these bacteria. Knowledge of the antibiotic resistance pattern is hence crucial for the proper choice of antimicrobials in the treatment of a limb-threatening diabetic foot ulcer infection.

Many Indian studies have not been done on the ESBL and AmpC enzymes in bacterial isolates of diabetic foot ulcers. This study was hence undertaken to identify the prevalence of ESBL and AmpC β-lactamases producing Gram-negative aerobic bacterial isolates in different grades of diabetic foot ulcers.

MATERIALS AND METHODS

Pus samples from 104 male and female diabetic patients attending the Medicine Department, in the age range of 20 to 90, having type 1 or type 2 diabetes and presenting with grade I to grade V foot ulcers were collected and processed. Thirty outpatients and 74 in-patients were included in the study.

Ulcer grading was done using the Meggitt-Wagner Classification of diabetic foot ulcers. After local debridement of devitalized tissue, sample collection was then done by scrapings of the ulcer base and by using sterile cotton swabs. Two swabs were collected, one for Gram stain and the other for aerobic culture. Pus aspirates were done when there was presence of any deep abscess. Samples were taken immediately to the laboratory and processed according to the standard microbiological procedures and antimicrobial sensitivity performed as per CLSI guidelines.[7] Antibiotic sensitivity was performed on Mueller-Hinton agar plates by the Kirby-Bauer disc diffusion method using antibiotic discs obtained from HiMedia Laboratories Pvt. Ltd, Mumbai. MIC_{50} of all isolates for Ceftazidime was put up according to the agar dilution method [Figure 1]. The Gram-negative bacterial isolates were further tested for ESBL and AmpC production.

Detection of ESBL and AmpC

Two methods were employed to detect ESBL production. As per the CLSI guidelines, any isolate showing a zone of inhibition <22 mm or an MIC ≥2 μg/mL for Ceftazidime should be tested for ESBL. Thus fifty-four isolates that met these criteria were tested for ESBL.

Screening for ESBL and ampC β-lactamases

We simultaneously tested for ESBL and AmpC β-lactamases by a modified double disc synergy test (DDST) [Figure 2].

Modified DDST

A lawn culture of test strain on Mueller-Hinton agar was exposed to a disc of Ceftazidime (30 μg) and a disc of amoxiclav (augmentin) (20 μg amoxicillin/10 μg clavulanic acid) arranged in pairs. The discs were arranged so that the distance between them was approximately twice the radius of the inhibition zone produced by Ceftazidime tested on its own. A cefoxitin (30 μg) disc was also placed at a distance of 20 mm from the Ceftazidime disc. The test isolate was considered to produce ESBL, if the zone size around the antibiotic disc increased toward the Augmentin disc. Isolates showing reduced susceptibility to Ceftazidime and cefoxitin or blunting of the Ceftazidime adjacent to the cefoxitin disc were considered as screen positive for AmpC [Figure 3].

Combined disc method

In the combined disc method (CDM), [Figure 4] we used a modified procedure of CLSI procedure, in that a Cefaperazone disc (75 μg) and a Cefaperazone-Sulbactam disc (75-30 μg) were used. An increase of >5 mm zone diameter for the Cefaperazone-Sulbactam disc compared to the Cefaperazone disc was interpreted as production of ESBL.

Confirmatory method for AMPC beta lactamases

AmpC DISC TEST: All the fifty-four isolates were checked for AmpC production by the AmpC disc test [Figure 5]. A lawn culture of ATCC *Escherichia coli* 25922 was prepared on MHA plate. Sterile discs (6 mm) were moistened with sterile saline (20 μl) and inoculated with several colonies of the test organism. The inoculated disc was then placed beside a cefoxitin disc (30 μg) almost touching it. The plates were incubated at 35°C overnight.

Interpretation

A flattening or indentation of the cefoxitin inhibition zone for ATCC *E. coli* in the vicinity of the test disc was interpreted as a positive test. An undistorted zone was taken as negative test.

Statistical analysis

Percentage and proportion method was used for the analysis of MIC for Ceftazidime, ESBL and AmpC producers and their antibiotic sensitivity pattern. Chi-square test was used to compare the DDST with CDM.

RESULTS

Forty-seven isolates exhibited resistance to Ceftazidime phenotypically (zone size <18 mm) and 54 isolates had MIC

greater than 2 μg/mL. All the members of Enterobacteriaceae had an MIC_{50} of 128 μg/mL except for *Proteus vulgaris* (64 μg/mL). Morganella spp. and the non-fermenter GNBs, Pseudomonas spp. and Acinetobacter spp. had higher MIC_{50} of 256 μg/mL.

ESBL production was observed in 28.84% of the study subjects and 27.52% of the total isolates. The CDM detected 66.66% ESBLs among the Ceftazidime-resistant strains compared to 55.55% by the DDST. ESBLs were more prevalent in *Proteus mirabilis* (38.88%), Klebsiella spp. (30.76%) and *E. coli* (24%) while no ESBL production was seen in Citrobacter spp.

Screening for AmpC was positive in 53.70% of Ceftazidime-resistant isolates, (29/54) but confirmed AmpC production was seen only in 19 isolates (35.18%). Only one isolate each of Acinetobacter spp., Citrobacter spp. and *Proteus penneri* were obtained and all were AmpC producers. *E. coli* showed significant (40%) AmpC production. Both ESBL and AmpC were found in two isolates of *E. coli* and *P. mirabilis* while one isolate each of *Klebsiella pneumoniae*, Acinetobacter spp. and *P. penneri*. No mechanism of cephalosporin resistance was explainable for 5 isolates.

DISCUSSION

Diabetic foot infection may begin superficially in an ulcer or crack in the skin, but may spread to involve the deep tissues including tendons and bones. The potential for a wound to become infected is determined by two main factors: The microbial contamination of the wound and the person's resistance to that contamination. Generally, diabetic foot tends to have a polymicrobial infection involving aerobic, anaerobic and fungal agents. Gram-negative bacilli were the predominant isolates in our study (78.98%). The resistance patterns now prevalent among Gram-negative organisms include resistance to extended-spectrum cephalosporins and penicillin due to production of ESBLs and AmpC β-lactamases. Though initially reported in *Escherichia coli* and *K. pneumoniae*,[8] these enzymes have been detected in other bacterial species like *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia marcescens*, *Citrobacter diversus*, *Providencia stuartii*,

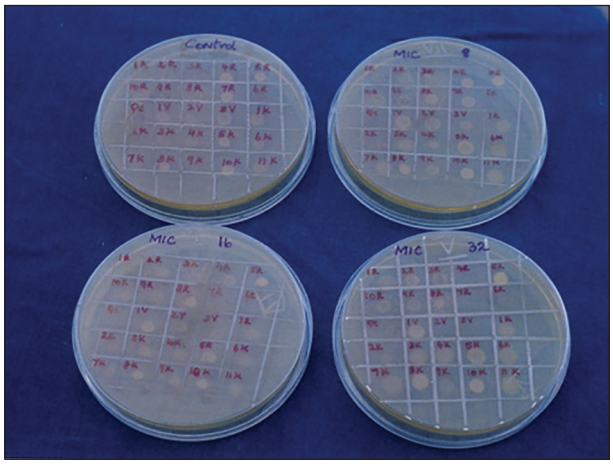

Figure 1: Minimum inhibitory concentration of ceftazidime for the gram negative isolates by agar dilution method

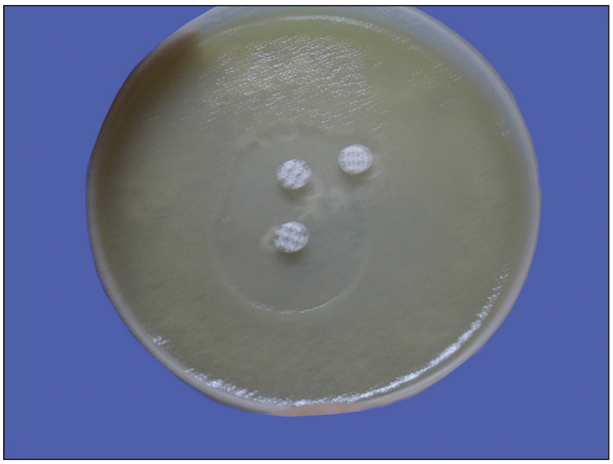

Figure 2: Double disc synergy test

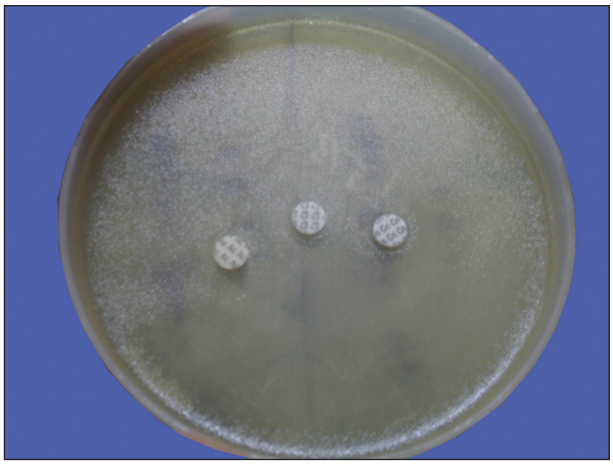

Figure 3: AmpC screening test

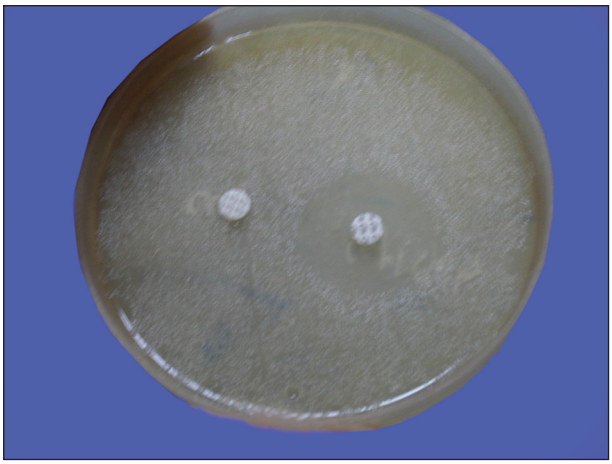

Figure 4: Combined disc method for ESBL detection

P. mirabilis, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Burkholderia cepacia and Acinetobacter spp.* Presence of ESBL-producing organisms has been reported to significantly affect the course and outcome of an infection.

There are various methods of ESBL detection, ranging from phenotypic tests like DDST, phenotypic confirmatory disc diffusion test (PCDDT), automated systems like Vitek 2 and molecular methods for detection of the resistance genes. Though the automated and molecular methods give an accurate identification, they are expensive and technically demanding. Hence many small laboratories especially in developing countries will have to rely on the phenotypic methods for detection of these resistance mechanisms.

Ceftazidime MIC

Ceftazidime is considered the best substrate for detection of TEM and SHV ESBLs and therefore we performed the minimum inhibitory concentration for Ceftazidime by agar dilution method for screening for ESBL detection. We found that most of the Gram-negative isolates had an alarmingly high MIC_{50} of > 128 μg/mL. [Table 1] In the case of *Morganella morganii*, *P. aeruginosa* and Acinetobacter spp., the MIC_{50} was even higher at 256 μg/mL. In a similar study by Jog *et al.*,[9] the MIC of ceftazidime ranged from 16 μg/mL to 256 μg/mL in ESBL-positive strains. This increase in MIC could probably be due to the "inoculum effect" among ESBL-producing strains where MICs of broad-spectrum cephalosporins increase if the inoculum increases.[10]

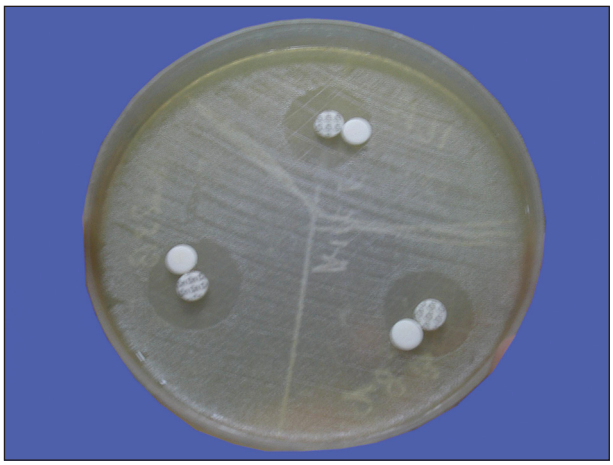

Figure 5: AmpC disc test for confirmation of ampC

Table 1: Minimum inhibitory concentration of ceftazidime for the GNB isolates

Organisms	64 μg/mL (*N*)	128 μg/mL (*N*)	256 μg/mL (*N*)	Total (*N*)	MIC_{50} μg/mL	MIC_{90} μg/mL
E. coli	2	7	1	10	128	128
K. pneumoniae	1	4	1	6	128	128
K. oxytoca	0	1	0	1	128	128
Citrobacter koseri	0	1	0	1	128	128
P. mirabilis	5	15	4	24	128	256
P. vulgaris	1	1	0	2	64	128
P. penneri	0	1	0	1	128	128
M. morganii	0	1	2	3	256	256
P. Aeruginosa	0	1	4	5	256	256
Acinetobacter baumanii	0	0	1	1	256	256
Total	9	32	13	54		

Comparison of DDST with CDM for ESBL detection

In our study we employed two methods, the modified double disc approximation method using Ceftazidime and amoxiclav and CDM using Cefaperazone alone and Cefaperazone-Sulbactam disc. We used Cefaperazone/sulbactam (1:1) as it is a unique combination of third-generation cephalosporin that is more stable to β-lactamases than penicillin with inhibitor at the highest available ratio. Among all β-lactam inhibitor combinations tested, Cefaperazone-sulbactam has revealed the highest activity against ESBL-producing organisms. Its superior activity is probably attributed to the improved stability of Cefaperazone and to the high concentration of the inhibitor component-Sulbactam.[11] Though we found that CDM (66.66%) was more effective in the detection of ESBL producers compared to DDST (55.55%), it was not statistically significant (p value >0.05). Similar results were obtained with other studies done by Dhara *et al.*,[12] and Goyal *et al.*[13] Dhara *et al.*,[12] had also found the phenotypic confirmatory disc diffusion test to be more sensitive than DDST in the detection of ESBLs. Also, they found that modified DDST using piperacillin-tazobactam identified ESBLs, which were not detected when amoxyclav was used. This difference could probably be due to the fact that clavulanate, which is used in DDST, may act as an inducer of AmpC production. High-level expression of AmpC, especially in species or strains that produce a chromosomally encoded inducible AmpC β-lactamase like *Enterobacter spp.*, *Serratia spp.*, *Providencia spp.*, *Aeromonas* spp., *M. morganii*, *C. freundii*, *Hafnia alvei*, and *P. aeruginosa* have been found to mask the detection of ESBLs. Tazobactam and Sulbactam are much less likely to induce AmpC β-lactamases and are therefore preferable inhibitors for ESBL detection tests with these organisms.[11]

The prevalence of ESBLs in diabetic foot ulcers have been varying. In our study, ESBL production was highest among *P. mirabilis* (38.88%) followed by Klebsiella spp. (30.76%) and *E. coli* (24%) while Varaiya *et al.*,[14] had reported 48.38% of *E. coli* and 51.61% of *K. pneumoniae* as ESBL producers. Kapil *et al.*,[6] have also reported 54.5% of *E. coli* isolates as ESBLs.

AmpC detection

The other resistance mechanism that we simultaneously screened for while detecting ESBL production was the presence of AmpC β-lactamases [Table 2]. Like detection of ESBLs, various methods like EDTA disc test[15], modified three-dimensional test[16] and boronic acid test[17] have been employed for the detection of AmpC β-lactamases. We used the AmpC disc test to confirm the presumptive AmpC producers based on a study by Singhal *et al.*,[18] which showed concordant

results between the AmpC disc test and the modified three-dimensional test. The AmpC disc test was able to identify 19/29 cefoxitin-resistant isolates. Though many studies have been done, not many have been able to clearly define the phenotypic tests to differentiate between chromosomal mediated and plasmid-mediated AmpC enzymes. *K. pneumoniae*, *K. oxytoca and P. mirabilis* do not have chromosomal-mediated AmpC enzymes. Hence, it can be assumed that the AmpC mechanism of resistance detected in 9/16 (56.25%) isolates of *Klebsiella spp. and P. mirabilis* by the disc test are plasmid-mediated. Other β-lactamases and impermeability of the cell wall porin channels should be considered for the cefoxitin resistance in the remaining isolates (10/29).[19] The sensitivity of detection of AmpC can be increased by the addition of EDTA to the discs as in the EDTA disc test.

Regarding the sensitivity pattern of the ESBL and AmpC producing strains [Table 3], all the isolates were sensitive to Imipenem (100%). Piperacillin and amikacin was found to be effective in >90% of *E. coli* isolates. Klebsiella spp. and *P. mirabilis* were better sensitive to Cefoperazone−sulbactam (83.33%) compared to 70% in *E. coli* and 66.66% in *M. morganii*. Only one isolate of Citrobacter spp. was isolated, and it was sensitive to amikacin, ofloxacin and gentamicin. Proteus species exhibited moderate to poor sensitivity to all the drugs. Acinetobacter spp. was resistant to all drugs except Imipenem and Cefaperazone−sulbactam. Pseudomonas spp. also showed 80% sensitivity to piperacillin but was poorly sensitive to all the other antimicrobials. All the isolates were poorly sensitive to gentamicin and co-trimoxazole.

Production of ESBL and AmpC-Clinical outcome in diabetic foot

In many hospitals, β-lactam/β-lactamase inhibitor compounds, such as piperacillin-tazobactam or ampicillin-sulbactam, are considered first line therapy for complicated diabetic foot infections. ESBL production limits the use of third-generation cephalosporins, all β-lactams and aztreonam. With AmpC, even cephamycins and β-lactamase inhibitors are not useful. Also, plasmid-mediated transfer of AmpC means faster dissemination and higher prevalence of multiple drug resistance. Treatment has to entirely rely on carbapenems, excessive use of which leads to higher prevalence of carbapenemases. Second, the poly-microbial nature of diabetic foot infection leads to easier microbial communication and faster spread of resistance mechanisms. Rand *et al.*, in their comparative study on clinical outcomes of bacteremic patients with and without AmpC production had found that patients with AmpC isolates tended to go for longer hospital stay.[20] This is of significance in diabetic foot isolates because foot ulceration tends to go in for chronicity, and infection with AmpC isolates would imply greater morbidity.

Limitations of our study

Though we had used two methods for detection of ESBL, usage of other antibiotics like cefotaxime, ceftriaxone, aztreonam or cefpodoxime in addition to Ceftazidime would have significantly improved the sensitivity of ESBL detection. Also, further confirmation of the AmpC positive isolates by molecular methods like AmpC multiplex PCR

Table 2: Percentage of ESBL and AmpC producers by different methods

Organism	Number of isolates (*N*)	Isolates with ESBL Phenotype		Isolates with AmpC Phenotype	
		DDST *N* (%)	CDT *N* (%)	Screening test *N* (%)	Confirmatory test *N* (%)
E. coli (25)	10	6 (60)	6 (60)	4 (40)	4 (40)
Klebsiella spp. (14)	7	5 (71.42)	5 (71.42)	2 (28.57)	1 (14.28)
C. koseri (1)	1	0 (0)	0 (0)	1 (100)	1 (100)
P. mirabilis (36)	24	14 (58.33)	17 (70.83)	14 (58.33)	8 (33.33)
P. vulgaris (5)	2	1 (50)	1 (50)	1 (50)	1 (50)
P. penneri (1)	1	0 (0)	1 (100)	1 (100)	1 (100)
M. morganii (9)	3	2 (66.66)	2 (66.66)	1 (33.33)	1 (33.33)
P. aeruginosa (17)	5	2 (40)	3 (60)	2 (40)	1 (33.33)
A. Baumanii (1)	1	0 (0)	1 (100)	1 (100)	1 (100)
Total (109)	54	30 (55.55)	36 (66.66)	29 (53.70)	19 (35.18)

Table 3: Sensitivity pattern of the ceftazidime resistant isolates

Sensitivity pattern of ceftazidime resistant isolates	Ak *N* (%)	G *N* (%)	Of *N* (%)	Cs *N* (%)	Cfs *N* (%)	Pc *N* (%)	Co *N* (%)	I *N* (%)
E. coli (10)	9 (90)	2 (20)	6 (60)	7 (70)	7 (70)	9 (90)	1 (10)	10 (100)
K. pneumoniae (6)	4 (66.66)	2 (33.33)	4 (66.66)	4 (66.66)	5 (83.33)	4 (66.66)	1 (16.66)	6 (100)
K. oxytoca (1)	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	1 (100)
C. koseri (1)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	1 (100)
P. mirabilis (24)	17 (70.83)	7 (29.16)	14 (58.33)	6 (25)	20 (83.33)	15 (62.5)	2s (8.33)	24 (100)
P. vulgaris (2)	1 (50)	0 (0)	1 (50)	2 (100)	2 (100)	1 (50)	0 (0)	2 (100)
P. penneri (1)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)
M. morganii (3)	1 (33.33)	1 (33.33)	2 (66.66)	1 (33.33)	2 (66.66)	2 (66.66)	0 (0)	3 (100)
P. aeruginosa (5)	2 (40)	0 (0)	2 (40)	2 (40)	4 (80)	4 (80)	0 (0)	5 (100)
A. baumanii (1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)
Total (54)	37 (68.51)	13 (24.07)	31 (57.40)	23 (42.59)	44 (81.48)	37 (68.51)	4 (7.4)	54 (100)

would have helped in characterization of the chromosomal AmpCs, especially in *E. coli*.

CONCLUSION

In conclusion, the ESBLs were more prevalent than AmpC β-lactamases and co-production of both the β-lactamases was seen in diabetic foot infections. As most of the strains were sensitive to Imipenem and Cefaperazone-sulbactam, these can be used for treatment of limb-threatening infections in ESBL-producing organisms. In AmpC producers, the treatment options will get narrowed down to only carbapenems. Hence routine screening for ESBLs and AmpC should be done for effective treatment. The CDM using Cefaperazone-sulbactam has been found to be effective in the detection of ESBLs while the AmpC disc test is an easy way of detection of AmpC β-lactamases.

ACKNOWLEDGEMENT

We are greatly indebted to Dr. T.S. Vijayalakshmi, Dr Sheela Doris and Dr. Sujatha for their valuable guidance and support.

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How to cite this article: Ranjini CY, Rangasamy VR. Detection of ESBL and plasmid-mediated AmpC beta lactamases among the Gram-negative bacterial isolates in diabetic foot ulcer infections. Community Acquir Infect 2015;2:57-62.
Source of Support: Nil, **Conflict of Interest:** None declared