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Prevalence of metallo-β-lactamases producers among carbapenem-resistant *Acinetobacter baumannii* strains isolated from diabetic foot ulcers

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The aim of this work was to detect the prevalence of metallo-β-lactamases (MBL) producers among carbapenem-resistant *Acinetobacter baumannii* strains isolated from diabetic foot ulcers. Out of 290 samples of diabetic foot ulcers, 99 strains of *A. baumannii* (33%) were isolated and identified by conventional culture methods. Antibiotic sensitivity pattern of the isolated *A. baumanni* strains was done by disc diffusion method. For *A. baumanni* strains that was resistant to imipenem and meropenem, MBL production was screened by potentiated disc test and confirmed by multiplex polymerase chain reaction for bla_{IMP} and bla_{VIM} genes. Out of 99 strains of *A. baumannii*, 26 (26%) were found resistant to imipenem and/or meropenem. Of these 26 carpabenem resistant *A. baumannii* strains 9 (34.61%) were positive for MBL by potentiated disc test, and 6 strains (23.07%) were positive for bla_{VIM} or bla_{IMP} by multiplex PCR; where bla_{VIM} gene was detected in 4 strains (15.38%) and bla_{IMP} was detected in 2 strains (7.69%). The antimicrobial susceptibility profile for the isolated *A. baumanni* strains showed that the highest sensitivity was to meropenem (74.4%), imepenem (76.55%), amikacin (65%) and the lowest sensitivity was to ceftazidime (11%) and ciprofloxacin (12%). Rapid dissemination of carbapenem-resistant isolates in diabetic foot ulcers is worrisome and calls for judicious use of antibiotics. bla_{VIM} and bla_{IMP} genes have a role in carbapenem-resistant in the community. More studies are needed to differentiate MBL from non-metalloenzymes producers.

Key words: Diabetic foot, *A. baumanni*, metallo-β-lactamases.

INTRODUCTION

Foot ulcers are among the leading causes of morbidity in diabetics and are the most common indication for admission in this population (Azer et al., 1999). Devitalized tissue is the site where the bacteria responsible for the non-healing ulcers inflict damage. Infectious agents are associated with amputation of the

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infected foot if not treated promptly (Logerfo et al., 1984). *Acinetobacter baumannii* has emerged as an important opportunistic Gram-negative bacteria in health care institutions globally, as it resists desiccation, is hard to eradicate and has numerous intrinsic and acquired mechanisms of drug resistance. Production of carbapenamases, which hydrolyse carbapenems, the drugs with high efficacy and broad spectrum of activity against this organism, has been a cause of worry to the clinician and the microbiologist (Peleg et al., 2008).

Carbapenamases are β-lactamases, which include serine-β-lactamases (KPC, OXA, GES, etc.) and metallo-β-lactamases (MBLs). The latter require metal ion zinc for their activity, which is inhibited by metal chelators like EDTA and thiol-based compounds but not by sulbactam, tazobactam and clavulanic acid (Karthika et al., 2009).

MBL production is typically associated with resistance to aminoglycosides and fluoroquinolones, further compromising therapeutic options. Among the seven types of MBL genes described throughout the World, bla_{IMP} and bla_{VIM} are the most common (Karthika et al., 2009).

The genes responsible for MBL production may be chromosomal or plasmid mediated and pose a threat of horizontal transfer. The introduction of carbapenems into clinical practice was of great help in the treatment of serious bacterial infections caused by β-lactam resistant bacteria and is the drug of choice for the infection caused by cephalosporin-resistant Gram negative infections (Varaiya et al., 2008).

The aim of the present study was to detect the prevalence of MBL producers among carpabenem-resistant *A. baumannii* strains isolated from diabetic foot ulcers with antimicrobial susceptibility pattern of *A. baumannii* strains isolated from diabetic foot ulcers.

MATERIALS AND METHODS

Over a one-year period from June 2012 to May 2013, 99 isolates of *A. baumannii* were isolated from patients with diabetic foot ulcers admitted to surgery department in Tanta University Hospital. The specimens were collected, transported and processed in microbiology laboratory without any delay. This work had the approval of the ethical committee in Faculty of Medicine, Tanta University and a written consent from all participants.

Inclusion criteria

Diabetic patients with duration of 10-15 days of diabetic foot infections, with no antibiotic therapy for one week before the study were used.

Exclusion criteria

Patients treated with antibiotics before admission for this attack of infection and chronic diseases other than diabetes.

Microbiological study

Culture specimens were obtained at the time of admission, after the surface of the wound had been washed vigorously with saline, and followed by debridement of superficial exudates. Specimens were obtained by scraping the ulcer base or the deep portion of the wound edge with a sterile curette. The soft tissue specimens were promptly sent to the laboratory for microbiological study (Shanker et al., 2005).

The isolates of *A. baumanni* were identified by conventional culture methods which are confirmed by biochemical reactions. Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates with commercially available discs (Hi-Media, Mumbai) by disc diffusion method. The results were recorded and interpreted as per Clinical and Laboratory Standard Institute (CLSI) recommendations (CLSI, 2012). The routine antibiotic sensitivity tests were put up for amikacin (30 µg), gentamicin (10 µg), amoxycillin (10 ug), tobramycin (10 µg), cefoperazone (75 µg), cefepime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), ceftizoxime (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), meropenem (10 µg), chloramphenicol (30 µg), piperacillin/tazobactam (100/10 µg) and colistin (10 µg). Isolates were considered to be carbapenem resistant when the zone of inhibition around imipenem and meropenem discs was ≤13 mm.

MBL production

Screening for the detection of MBL was done by disc potentiation test with EDTA-impregnated imipenem discs and EDTA-impregnated meropenem discs and confirmed by multiplex PCR (Hemalatha et al., 2005).

Disc potentiation test methods

Test organism was inoculated onto plates of Mueller-Hintonagar plate (opacity adjusted to 0.5 McFarland opacity standards). A 0.5-m EDTA solution was prepared by dissolving 186.1 g of disodium EDTA $2H_2O$ in 1000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving. Two 10-mg imipenem discs and meropenem discs were placed on the plate; 5 ml of EDTA solution was added to one of the disceach. The inhibition zones of the imipenem and imipenem-EDTA discs and meropenem and meropenem-EDTA discs were compared after 16-18 h of incubation at 35 8°C. An increase in the zone size of at least 7 mm around the imipenem-EDTA disc and meropenem-EDTA discs was recorded as an MBL-positive strain (Leek et al., 2003***)***.

PCR

Detection of the *bla*-IMP and *bla*-VIM genes was carried out using primers as described by Mostachio et al. (2009). *bla*-IMP primers: *bla*-IMP-F (5′-GAATAGAATGGTTAACTCTC-3′) *bla*-IMP-R(5′-CCAAACCACTAGGTTATC-3′) and *bla*-VIM primers: *bla*-VIM-F (5′-GTTTGGTCGCATATCGCAAC-3′) *bla*-VIM-R(5′-AATGCGCAGCACCAGGATAG-3′) were used.

A total of 4-5 identical colonies of *A. baumannii* were re-suspended in 500 µl of sterile saline in 1.5 ml Eppendorf tube. This was boiled at 100°C for 10 min, centrifuged at 8000 rpm for 5 min and the supernatant containing DNA was used for further processing. The PCR mixture used was as follows: 1 µl DNA template in a 49 µl mixture containing 10 mMTris/HCl (pH 8.8), 50 mMKCl, 4 mM $MgCl_2$, 200 µM each dNTP (FermentasGenetix Biotech Pvt. Ltd., New Delhi), 1 µl of each of the forward and

Table 1. Demographic and clinical characteristics of patients with diabetic foot infections.

Patients characteristic	No. (%)
Sex: Male	51 (51.51%)
Sex: female	48 (48.48%)
Age (years) Mean ± SD	49 ± 16.8
Clinical characteristics	
Duration of DM (years) Mean ± SD	12 ± 10.2
DM foot duration (days) Mean ± SD	12 ± 10.2
Duration of hospital stay	22 ± 5.58
Medications:	
OHA	60
Insulin	33
Wagner's classification:	
W1	34 (34.3%)
W2	21 (21.4%)
W3	15 (15.6%)
W4	19 (19.04%)
W5	Nil
Diabetic compliations	
Peripheal neuropathy	49 (49.35%)
Nephropathy	18 (18.44%)
Retinopathy	15 (15.22%)
Coronary artery disease	17 (17.32%)
Treatment	
Antibiotic alone	75
Surgical intervention	24

reverse primers (Bangalore genei) and 1 unit Taq DNA polymerase (FermentasGenetix Biotech Pvt.Ltd.,New Delhi). The PCR conditions included: initial denaturation at 94°C for 5 min followed by 33 cycles each of 94°C for 25 s, 53°C for 40 s and 72°C for 50 s, followed by a single final elongation step at 72°C for 6 min. The PCR product of 188 bp for *bla*-IMP and 382 bp for *bla*-VIM was visualized by 1.5% agarose gel electrophoresis containing ethidium bromide, 0.5 µg/ml (Bangalore Genei).

RESULTS

Out of 290 cases of diabetic foot ulcers attending surgery department, Tanta university hospital; 99 *A. baumannii* strains were isolated and were eligible for the study including 51 (51.51%) male patients and 48 (48.48%) with mean age±SD (49 ± 16.8 years). The mean duration of diabetes in the patients of the study was 12 ± 10.2 years and the mean duration of diabetic foot ulcers was 12 ± 10.2 days. Table 1 shows demographic and clinical characteristics of patients of the study.

Table 2. Antibiotic sensitivity pattern of *A. baumanni* strains isolated from diabetic foot ulcers.

Antibiotics	Sensitive no. (%)
Amikacin	65 (65.6)
Gentamicin	13 (13.13)
Tobramycin	14(14.14)
Cefoperazone	9(9.09)
Cefepime	16 (16.16)
Ceftazidime	11 (11.1)
Ceftriaxone	13 (13.3)
Ciprofloxacin	12 (12.2)
Piperacilline/tazobactam	23 (23.33)
Imepenem	76 (76.5)
Meropenem	74 (74.4)
Amoxycilline	25 (25.4)
Colistin	43 (43.4)
Chloramphenicol	24 (24.4)

The result of the study shows that 26 (26.26%) *A. baumanii* strains out of 99 strains were resistant to at least one or both carbapenem tested. Out of these, 22 (84.61%) were resistant to both imipenem and meropenem, 3 (11.53%) to meropenem and one strain (3.84%) to imipenem alone. Among 99 *A. baumanni* strains isolated from diabetic foot ulcers, Amikacin showed the highest level of sensitivity (65.6%), followed by colistin (43.3%). 9.09% only were sensitive to cefoperazone, 23.33% were sensitive to piperacillin/tazobactam. Table 2 shows antibiotic sensitivity pattern of *A. baumanni* strains isolated from diabetic foot ulcers.

The results of the study show that out of 26 carpabenem resistant *A. baumannii* strains 9 strains (34.61%) were positive for MBL by potentiated disc test, and 6 strains (23.07%) were positive for bla_{VIM} or bla_{IMP} by multiplex PCR; where bla_{VIM} gene was detected in 4 strains (15.38%) and bla_{IMP} was detected in 2 strains (7.69%). Figure 1 shows Agarose gel electrophoresis showing positive amplification of 382 and 188 base fragments specific for bla_{VIM} and bla_{IMP} respectively.

DISCUSSION

Diabetic foot ulcer is the most common complication requiring hospitalization among diabetic patients (Logerfo et al., 1984 and Bridges et al., 1994). It is also the most common cause of non-traumatic lower extremity amputations (El-Tahawy et al., 2000). Physicians have an

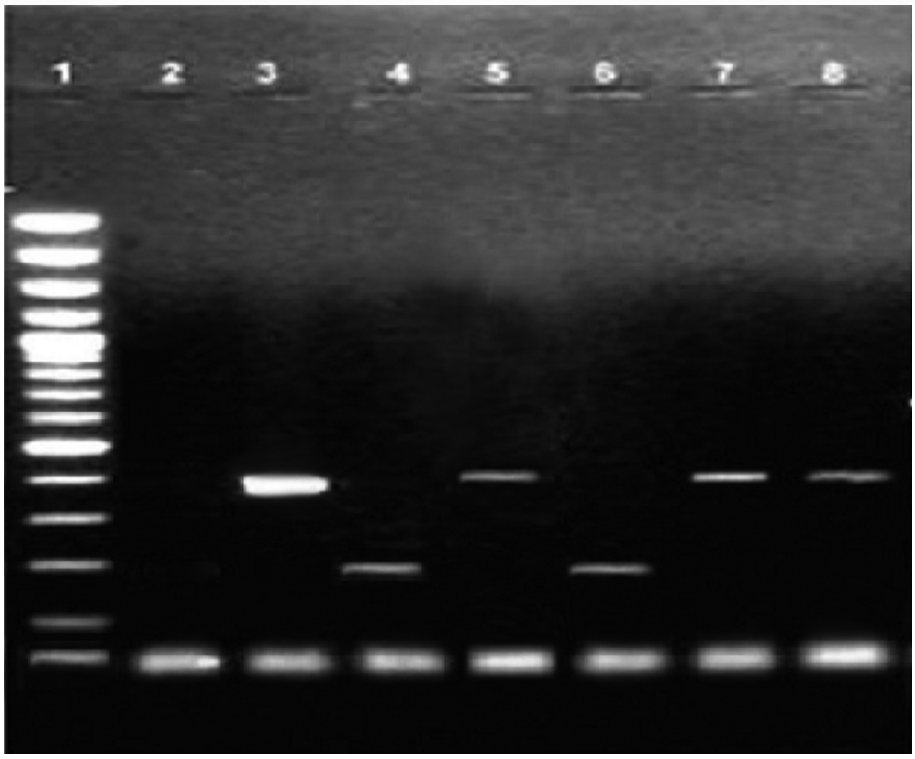

Figure 1. Agarose gel electrophoresis showing positive amplification of 382 and 188 base fragments specific for *bla*VIM and *bla*IMP, respectively. Lane 1, 100-1500 bp ladder; Lane 2, negative control; Lane 3, control *bla*VIM (382 bp); Lane 4, control *bla*IMP (188 bp). Lane 6, test strain (188 bp); Lanes 5, 7 and 8, test strain (382 bp).

important role in prevention, early diagnosis, and management of diabetic foot complications. Management, however, entails an extensive knowledge of the major risk factors for amputation and preventive maintenance with special reference to drug resistance in bacteria (Logerfo et al., 1984; El-Tahawy et al., 2000).

MBLs have been identified from clinical isolates in members of *Enterobacteriaceae, Pseudomonas aeruginosa* and *Acinetobacter* spp. over the past few years (Walsh et al., 2005; Kohlenberg et al., 2009). Strains producing these enzymes have been responsible for nosocomial outbreaks that have been accompanied by serious and prolonged infections. MBLs are powerful carbapenemases and can hydrolyze a wide variety of β-lactams, including penicillins, cephalosporins and carbapenems. Since the initial isolation of carbapenem resistant *A. baumannii* producing *bla*-IMP-1 and *bla*-VIM-1 type MBL in Japan and Italy, respectively (Lee et al., 2003). Clinical isolates of these strains have been identified worldwide. CLSI document (2009) has no guidelines for detecting MBLs, however, it has recommended modified Hodge test for detection of carbapenamases but in members of *Enterobacteriaceae* only.

The present work was carried out over a period of one year that extended from June 2012 to May 2013 on 290 patients with diabetic foot ulcers attending department of surgery in Tanta University Hospital. Out of 290 clinical samples, *A. baumanni* strains could be isolated from 99 (34.13%) cases. This percentage was in agreement with Gadepalli et al. (2006) who could isolate *A. baumanni* from 40% of cases of diabetic foot ulcers.

The results of antimicrobial susceptibility profile for the 99 *A. baumanni* strains isolated in this study showed that the highest sensitivity was to Meropenem (74.4%), Imepenem (76.55%), Amikacin (65%) and the lowest sensitivity was to Cefoperazone (9.09%), Ceftazidime (11%) and Ciprofloxacin (12%). The results showed that 9 strains (9.09%) were only sensitive to cefoperazone, and 23 strains (23.33%) were sensitive to piperacillin/-tazobactam. The study of Umadevi et al. (2011) showed that the majority of *Acinetobacter* spp. were susceptible to piperacillin-tazobactam (83%), imipenem (67%) and trimethoprim-sulfamethoxazole (67%), while being less susceptible to gentamicin (17%), amikacin (50%), ciprofloxacin (67%), tetracycline (50%), ceftriaxone (33%) and ceftazidime (33%). In accordance with the results of this study, the result of Shanker et al. (2005) showed that

76.4% of the isolated *A. baumanni* strains in their study were sensitive to Amikacin, but in reverse to the results of this study they found that 100% of the isolated *A. baumanni* strains were sensitive to Imepenem and ciprofloxacin and 76.5% of the strains were sensitive to piperacillin/tazobactam

Carbapenems are used for treating serious infections caused by multidrug-resistant Gram-negative bacilli. Resistance to carbapenems is due to decreased outer membrane permeability, increased efflux systems, alteration of penicillin-binding proteins, and the production of carbapenem hydrolyzing enzymes, that is, carbapenemases. The resistance may also be due to the production of metallo-β-lactamases (MBL); such resistance can be chromosomally encoded or plasmid mediated (Gladstone et al., 2005)

The results of the present study showed that 26 (26.26%) *A. baumanii* strains out of 99 strains were resistant to at least one or both carbapenem tested. Out of these 26 carpabenem resistant strains, 22 (84.61%) were resistant to both imipenem and meropenem, 3 (11.53%) to meropenem only and one strain (3.84%) to imipenem alone. In various studies across the world, varying rates of resistance (4-60%) have been reported for imipenem and meropenem. Among the Indian workers, Gladstone et al. (2005) reported 14.2%, whereas Taneja et al. (2003) reported 36.4%.

As regard the EDTA-imipenem-microbiological assay, which differentiates metalloenzymes from non-metalloenzymes, in *A. baumanii* strains, by this assay, we could confirm 9 isolates (9.3%) as MBL producers and 15 (32.56%) as non-metalloenzyme producers among the 26 screen test positives. However, of the 9 MBL positives only 4 (15.38%) showed presence of *bla*-VIM and 2 showed presence of *bla*-IMP (7.69%) and none of the 15 non-metalloenzyme producers showed presence of either *bla*-IMP or *bla*-VIM. Karthika et al. (2009) who found *bla*-VIM MBL gene only in 7 (16.28%) of the 43 screen test positive isolates reported *bla*-IMP-1 in 42% of *A. baumannii.* while *Amudhan* et al. (2001) reported *bla*-VIM in 46.55% with and both *bla*-IMP and *bla*-VIM in only one isolate of *A. baumannii*. The non-demonstration of *IMP* and VIM genes in 20 of our 26 screen test positive isolates could be either due to presence of unidentified MBL gene, limitation of the primer set used either with regards to picking up the variant IMP/VIM gene or because of presence of MBL genes other than IMP/VIM, presence of other enzymes [OXA like (Ambler class D) carbapenamases AmpC β-lactamases] or other mechanism of carbapenem resistance, namely; loss of porins, increase in efflux pump activity alteration in penicillin binding proteins (PBPs), Singh et al. (2009). "False" positivity of disc potentiation test method (three isolates) against the PCR, could be due to presence of either other uncommon MBL encoding gene like SIM, SPM, GIM, AIM or variants of IMP and VIM (Singh et al., 2009). Very high MBL positivity of 95.2 and 88% in *P. aeruginosa* using disc potentiation test method has been reported by Singh et al. (2009) and Jain et al. (2011), respectively. However, Quinones-Falconi et al. (2009) using PCR as a gold standard reported only 3.5% MBL positivity in *P. aeruginosa* using disc potentiation test method. Galicia et al. (2009) have reported 3.4% MBL positivity in *P. aeruginosa* using disc potentiation test method with excellent specificity but poor sensitivity of the test. We did not come across any study on EIM in *A. baumannii*

Conclusion

Rapid dissemination of carbapenem-resistant isolates in diabetic foot ulcers is worrisome and calls for judicious use of antibiotics .The. bla_{VIM} and bla_{IMP} genes have a role in carbapenem resistance in the community. More studies are needed to differentiate MBL from non-metalloenzymes producers.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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