Phenotypic and genotypic assays for detecting the prevalence of metallo-β-lactamases in clinical isolates of *Acinetobacter baumannii* from a South Indian tertiary care hospital

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Nosocomial infections caused by *Acinetobacter baumannii* often prove difficult to treat owing to their multiple drug resistance. Carbapenems play a pivotal role in the management of severe *Acinetobacter* infections. However, reports of carbapenem resistance have been increasing alarmingly due to production of a variety of carbapenemases including metallo-β-lactamases (MBLs). This study investigated by both phenotypic and genotypic assays the prevalence of MBLs in a total of 55 *A. baumannii* strains isolated from a South Indian tertiary care hospital. Random amplified polymorphic DNA (RAPD) genotyping and antimicrobial susceptibility testing for nine clinically relevant antibiotics was done for characterization of isolates. Phenotypic expression of MBLs was examined by a simple double disc synergy (DDS) test, and the presence of the most frequent MBL coding genes, bla_{IMP1} and bla_{VIM2}, was checked by PCR. RAPD analysis generated six clusters of isolates and there was very little correlation between RAPD clusters and resistant profiles. Most of the isolates showed complete or high resistance to imipenem (100 %), meropenem (89 %), amikacin (80 %), cefotaxime (89 %) and ciprofloxacin (72 %). In addition, 44 % of isolates showed a high MIC level ($\geqslant$16 μg ml^{-1}) for meropenem. Thirty-nine isolates (70.9 %) were positive for MBL production by the DDS test while bla_{IMP1} gene amplification was seen only in 23 isolates (42 %). Interestingly, none of the isolates showed amplification of bla_{VIM2}. Further investigations on DDS-positive/PCR-negative isolates by spectrophotometric assay showed MBL activity in most of the isolates, suggesting involvement of other genes. The high incidence of isolates possessing MBL activity in the present study represents an emerging threat of complete resistance to carbapenems among *Acinetobacter* spp. in India.

INTRODUCTION

Acinetobacter baumannii, one of the most important nosocomial pathogens with multiple drug resistance (MDR), is of great concern because of its intrinsic and acquired resistance mechanisms, limiting the treatment options (Navon-Venezia *et al.*, 2005). Carbapenems are the drugs of choice for *A. baumannii* infections and are often used as a last resort (Yano *et al.*, 2001). However, carbapenem-resistant *A. baumannii* producing carbapenemases has been isolated increasingly in recent times (Jones *et al.*, 2005) and in particular a variety of IMP and VIM type metallo-β-lactamases (MBLs) have been detected and grouped under the class B carbapenemases (Jeong *et al.*, 2006; Yum *et al.*, 2002; Da Silva *et al.*, 2002). Since the first descriptions of MBLs in *Acinetobacter* spp., IMP and VIM types have been found to have a wide geographical occurrence (Walsh *et al.*, 2005; Jones *et al.*, 2005). Recently, a new type of MBL, SIM-1, has also been reported (Lee *et al.*, 2005). MBL genes also have the propensity to disseminate quickly to other species of Gram-negative bacilli (Navon-Venezia *et al.*, 2005; Peleg *et al.*, 2005). Therefore, it is essential to rapidly screen and detect MBLs in *Acinetobacter*, which could help in modifying therapy and initiate effective infection control to prevent further dissemination (Hirakata *et al.*, 1998).

Numerous Indian studies have documented the presence of MBLs in *Pseudomonas aeruginosa* (Jesudason *et al.*, 2005;

Abbreviations: DDS, double disc synergy; MBL, metallo-β-lactamase; MDR, multiple drug resistance; RAPD, random amplified polymorphic DNA.

Mendiratta *et al.*, 2005; Sarkar *et al.*, 2006); however, to our knowledge only one study for phenotypic detection of MBLs in *A. baumannii* has been done in India (Gupta *et al.*, 2006). The aim of this study was to determine both phenotypically and genotypically the prevalence of MBL-producing strains among MDR *A. baumannii* isolated from clinical specimens in this geographical region.

METHODS

Bacterial isolates. All the *Acinetobacter* isolates were obtained from a South Indian tertiary care hospital (Pondicherry Institute of Medical Sciences, Puducherry, India) from various clinical specimens, such as endotracheal aspirates, cerebrospinal fluid, wound swabs, urine and blood culture specimens, from patients admitted to the intensive care and acute medical care units from January to April 2007. All the replicates were excluded from the study. A total of 55 *Acinetobacter* isolates were identified and grouped into the *Acinetobacter calcoaceticus–baumannii* complex (*Acb* complex) using phenotypic tests as described elsewhere (Gerner-Smidt *et al.*, 1991; Prashanth & Badrinath, 2000; Kenchappa & Sreenivasmurthy, 2003). A simple molecular method, namely amplified rDNA restriction analysis (ARDRA), was used to further identify genomic groups within the *Acb* complex as described elsewhere (Chandra *et al.*, 2002). ARDRA identified all the isolates from our collection as *A. baumannii*.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed for nine different therapeutically relevant antibiotics by the Kirby–Bauer disc diffusion method according to Clinical Laboratory Standards Institute guidelines (CLSI, 2006). Antibiotics tested included amikacin (10 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), cefepime (30 μg), imipenem (10 μg), meropenem (10 μg) and netilmicin (30 μg). *A. baumannii* ATCC 19606^T was used as control. Isolates showing MDR were further tested for MBL production. MDR was defined as resistance to two or more drugs or drug classes of therapeutic relevance (Prashanth & Badrinath, 2004; Navon-Venezia *et al.*, 2005).

RAPD typing. Genotyping of *A. baumannii* isolates was performed using random amplified polymorphic DNA (RAPD) analysis for 48 isolates using arbitrary hexamers (H series) obtained from Operon Technologies. The PCR mixtures (10 μl) contained 1 μl PCR buffer with 1.5 mM $MgCl_2$, 50 μM each dNTP, 50 pmol primer, 1 U *Taq* polymerase (Bangalore Genei) and 50 ng DNA template. PCR amplifications were performed using a thermal cycler (Eppendorf) as follows: (i) initial denaturation step of 5 min at 94 °C; (ii) 35 cycles of PCR, with each cycle consisting of 30 s at 94 °C, 45 s at 45 °C and 2 min at 72 °C; and (iii) a final extension step of 5 min at 72 °C. Amplified products from the isolates were analysed by electrophoresis on 1.2 % (w/v) agarose gels, stained with ethidium bromide and the banding patterns were analysed using the Quantity One Gel Doc 2000 (Bio-Rad) computer software system.

MIC determination and MBL detection. The MIC for meropenem was determined by the agar dilution method as recommended by the CLSI (2006). The organisms were considered susceptible to meropenem if the MIC was $\leqslant 4$ μg ml^{-1} and resistant if the MIC was $\geqslant 16$ μg ml^{-1} (CLSI, 2006). For phenotypic detection of MBLs among the *A. baumannii* strains, we used the imipenem EDTA-double disc synergy (DDS) test developed by Yong *et al.* (2002). All the MBL-positive isolates were repeatedly checked for reproducibility. All the isolates were checked for MBL genotypically by PCR for the presence of the predominant genes bla_{IMP1} and bla_{VIM2}. PCR conditions and bla_{IMP1} and bla_{VIM2} primers used for PCR amplification were according to previously described studies (Poirel *et al.*, 2000; Yum *et al.*, 2002). Isolates which were positive for MBL by the DDS test but PCR-negative were further tested for enzyme activity by spectrophotometry. The spectrophotometric assay for assessing MBL activity was performed as described previously (Edwards *et al.*, 1998; Lauretti *et al.*, 1999).

RESULTS AND DISCUSSION

In recent years, there have been numerous reports on MDR *A. baumannii* from hospital settings in India (Prashanth & Badrinath, 2005, 2006; Sinha *et al.*, 2007; Joshi *et al.*, 2003b). In our earlier studies, we characterized isolates obtained mainly from intensive care units (Prashanth & Badrinath, 2000, 2005), while this study attempted to determine the resistance among isolates obtained from various other medical wards also. In particular, 36 strains were isolated from wards outside the intensive care unit. Antibiotic susceptibility testing showed that the majority of the isolates were resistant to three or more antibiotics (Table 1). While all the isolates were resistant to imipenem, 89 %, 80 %, 89 % and 72 % of isolates were resistant to meropenem, amikacin, cefotaxime and ciprofloxacin, respectively. *A. baumannii* isolates also showed moderate resistance to ceftriaxone (42 %) and ceftazidime (36 %). Unfortunately, these are the antibiotics that are currently being prescribed in the hospital. However, a few antimicrobials, those that were not used frequently in the hospital, such as cefepime and netilmicin, continued to show low resistance, i.e. 30 % and 27 %, respectively. Such observations have also been witnessed by other investigators wherein susceptibility is attributed to decreased usage of the antimicrobial (Manikal *et al.*, 2000).

RAPD analysis of the *A. baumannii* isolates examined showed fragments ranging from 500 to 1500 bp (Fig. 1). An UPGMA dendrogram (data not shown) constructed using binary data generated six clusters from the isolates (designated A–F), revealing the genetic relatedness among the isolates. Two major clusters depicted in the dendrogram, namely D and F, comprised 17 and 13 isolates, respectively. Cluster E had a total of nine isolates. The A, B and C clusters constituted only a few isolates (one to five isolates). RAPD results did not correlate with those of the antibiotypes since our isolates showed highly divergent resistance profiles and only a few correlations could be made in this regard. A few isolates belonging to certain clusters (A: P31, P49, P51; C: P39, P56; E: P46, P47; F: P15, P16) had identical resistograms (Table 1). Interestingly, cluster D had seven isolates showing two kinds of identical resistograms specific for the site of isolation [D1 (P5, P53, P55 all from endotracheal aspirates) and D2 (P32, P33, P36, P38 all from wounds)]. Such associations were also found in clusters C (wound) and E (urine), suggesting that distinct clones may be responsible for specific disease or infection caused by this organism. Although the discriminatory power of PFGE genotyping was found to be higher than that of PCR-based techniques, the present study

Table 1. Origin, resistogram, MBLs and RAPD cluster of all the isolates of *A. baumannii*

Strain ID	Site of isolation*	Resistogram†	Metallo-β-lactamase			Imipenem hydrolysis – spectrophotometric assay‡	RAPD cluster
			DDS test	(PCR) bla_{IMP1}	(PCR) bla_{VIM2}		
P1	Urine	Ca, Ce, Ci, Cf, Cpm, I, M	+	+	−	−	D
P2	Wound	Ak, Ca, Ce, Cf, I, M	−	−	−	−	C
P3	Wound	Ak, Ce, Cf, I, M	+	+	−	−	D
P4	Wound	Cf, Ci, I, M, Nt	+	+	−	−	D
P5	ETA	Ak, Ce, Cf, I, M	−	+	−	−	D
P6	ETA	Ak, Ce, Cf, I, M	−	+	−	−	D
P9	Wound	Ce, Cf, I, Nt, M	−	+	−	13.2 nmol§	D
P10	Wound	Ak, Ca, Ce, Cf, I, M, Nt	−	+	−	−	C
P11	Wound	Ak, Ce, Cf, Ci, I, M	−	+	−	−	D
P12	ETA	Ak, Ce, Cf, I, Nt, M	−	+	−	−	D
P13	Wound	Ak, Ce, Cf, Ci, Cpm, M, I	−	−	−	−	F
P14	Wound	Ak, Ce, Cf, Ci, Cpm, I, M, Nt	+	−	−	48.0 nmol	F
P15	Wound	Ak, Ca, Ce, Ci, Cpm, M, I	+	−	−	51.0 nmol	F
P16	ETA	Ak, Ca, Ce, Ci, Cpm, I	+	−	−	53.6 nmol	F
P17	ETA	Ca, Ce, Cf, Ci, Cpm, I, M	−	+	−	−	F
P18	Wound	Ak, Ce, Cf, Ci, I, M	+	+	−	−	F
P19	CSF	Ak, Ce, Cf, I, M, Nt	+	−	−	52.8 nmol	F
P20	Wound	Ak, Ca, Cf, I, M, Nt	ND	−	−	−	F
P21	Wound	Ak, Cf, Ce, I, M, Nt	ND	−	−	−	F
P22	Wound	Ak, Ca, Ce, Cf, Ci, Cpm, I, M	+	+	−	−	E
P23	Wound	Ca, Ce, Cf, Ci, Cpm, I, M	+	+	−	−	F
P24	ETA	Ak, Ca, Ce, Cf, I, M	+	+	−	−	E
P25	Blood	Ca, Ce, Ci, Cpm, I, M	+	+	−	−	E
P26	Blood	Ak, Ca, Ce, Ci, Cpm, I, M	+	+	−	−	E
P27	ETA	Ak, Ce, Cf, I, M	−	−	−	1.9 nmol§	E
P28	Urine	Ak, Ca, Ce, Cf, I, M	+	−	−	58.6 nmol	F
P29	Urine	Ak, Ca, Ce, Cf, Ci, I, M, Nt	+	−	−	60.0 nmol	B
P30	Wound	Ak, Ce, Ci, Cpm, I, M	+	−	−	57.2 nmol	C
P31	ETA	Ak, Ce, Cf, I, M	+	−	−	60.1 nmol	A
P32	Wound	Ak, Ce, Cf, I, M, Nt	+	−	−	53.9 nmol	D
P33	Wound	Ak, Ce, Cf, I, M, Nt	+	−	−	No activity	ND
P34	CSF	Ak, Ce, Cf, Cpm, I, M, Nt	+	−	−	46.3 nmol	D
P35	BT	Ak, Ce, Cf, Cpm, I, M, Nt	−	−	−	−	D
P36	Wound	Ak, Ce, Cf, I, M, Nt	+	−	−	56.0 nmol	D
P37	CSF	Ak, Ca, Ce, Ci, I, M, Nt	+	−	−	56.4 nmol	D
P38	Wound	Ak, Ce, Cf, I, M, Nt	+	−	−	42.7 nmol	D
P39	Wound	Ak, Ce, Cf, Ci, I, M	+	−	−	41.6 nmol	C
P40	Blood	Ak, Ca, Ci, Cpm, I, M	+	−	−	41.8 nmol	D
P41	Sputum	Ak, Ca, Ce, Ci, Cpm, I, M	+	−	−	No activity	E
P42	Urine	Ak, Ce, Ci, Cpm, I	+	−	−	43.1 nmol	F
P43	Wound	Ce, Cf, Ci, I, M	+	−	−	49.2 nmol	F
P44	ETA	Ce, Cf, Ci, Cpm, I	+	−	−	49.2 nmol	ND
P45	Wound	Ce, Ci, I	+	−	−	42.5 nmol	E
P46	Urine	Ca, I, M	+	−	−	43.1 nmol	E
P47	Urine	Ca, I, M	+	−	−	54.8 nmol	E
P48	CSF	Ak, Ce, Cf, I, M	+	−	−	57.0 nmol	ND
P49	ETA	Ak, Ce, Cf, Ci, I, M	+	−	−	41.6 nmol	A
P50	ETA	Ak, Ce, I, M	+	−	−	54.8 nmol	ND
P51	ETA	Ak, Ce, Cf, Ci, I, M	+	+	−	−	A
P52	ETA	Ak, Ce, Cf, I	+	+	−	−	ND
P53	ETA	Ak, Ce, Cf, I	+	+	−	−	D
P54	Wound	Ak, Ce, I, M	−	+	−	−	ND
P55	ETA	Ak, Ce, Cf, I, M	+	+	−	−	D
P56	Wound	Ak, Ce, Cf, Ci, I, M	−	+	−	−	C
P57	ETA	Ak, I, M	−	+	−	−	ND

ND, Not determined.

*BT, Bone tissue; CSF, cerebrospinal fluid; ETA, endotracheal aspirate.

†Ak, Amikacin; Ca, ceftazidime; Ce, cefotaxime; Ci, ceftriaxone; Cf, ciprofloxacin; Cpm, cefepime; I, imipenem; M, meropenem; Nt, netilmicin.

‡Imipenem hydrolysed min^{-1} $(mg\ protein)^{-1}$.

§Negative control.

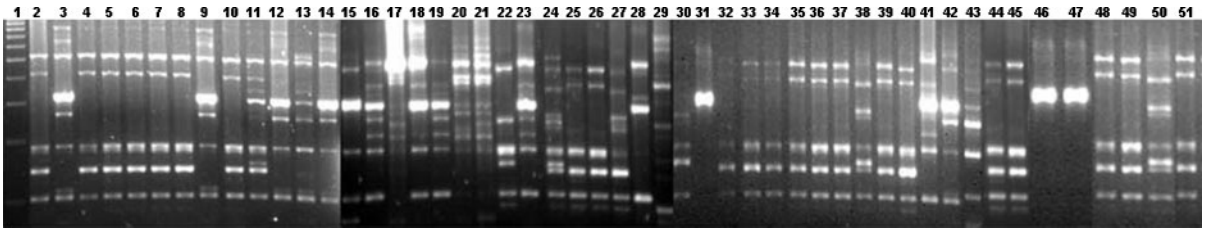

Fig. 1. RAPD fingerprinting of representative clinical isolates of *A. baumannii* including the reference strain *A. baumannii* ATCC 19606^T. Lanes: 1, 500 bp molecular mass marker (Bangalore Genei); 2–14 and 16–51, clinical isolates of *A. baumannii* (1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 45, 46, 47, 49, 51, 53, 55, 56 and 57). Lane 15, *A. baumannii* ATCC 19606^T.

employed RAPD because of its simplicity and feasibility (Liu & Wu, 1997). PFGE, though more efficient in analysing *Acinetobacter* epidemiology in complex endemic settings (Vila *et al.*, 1996; Liu & Wu, 1997; D'Agata *et al.*, 2001), is cumbersome as well as expensive and many less well-equipped laboratories may not be able to use this technique routinely.

Carbapenems are the drugs of choice for nosocomial *Acinetobacter* infections in India. However, in recent years, it has been reported that there is reduced susceptibility to imipenem (Taneja *et al.*, 2003; Joshi *et al.*, 2003a; Sinha *et al.*, 2007; Sinha & Srinivasa, 2007). Interestingly, many of these reports have documented only moderate resistance to imipenem (Taneja *et al.*, 2003; Sinha & Srinivasa, 2007). The present study showed high levels of imipenem and meropenem resistance among *A. baumannii* isolates. Forty-four per cent of isolates were resistant to meropenem with MIC levels ranging from 16 μg ml^{-1} to 128 μg ml^{-1}. However, there was low agreement among the disc diffusion and MIC results for meropenem. Meropenem MIC results showed 60 % of isolates having moderate to complete resistance, while the disc diffusion test showed 89 % resistance. In the present study, 29 % of isolates detected as resistant by disc diffusion were found to have their MICs in the sensitive range. A similar observation was witnessed in a recent study from India (Sinha & Srinivasa, 2007).

Detection of resistance in pathogens by molecular testing is gaining momentum in India. Many Indian studies have checked for carbapenem resistance in bacteria other than *A. baumannii* by looking for the presence of extended-spectrum β-lactamase and MBL genes by PCR (Mendiratta *et al.*, 2005; Sarkar *et al.*, 2006). Amid various MBL-encoding genes thus far discovered, bla_{IMP} and bla_{VIM} appear to be the most clinically important due to their ability to spread among other major pathogens (Da Silva *et al.*, 2002). In India, recently a high percentage of *P. aeruginosa* strains producing MBL has been reported (Jesudason *et al.*, 2005; Mendiratta *et al.*, 2005; Sarkar *et al.*, 2006). A similarly significant increase in the percentage of *A. baumannii* isolates (70.9 %) positive for MBL production by the EDTA-DDS test was observed in the present study, which is in contrast to the 7.5 % reported in the only other Indian study on MBL production in *A. baumannii* (Gupta *et al.*, 2006), reflecting the evolving scenario in India.

PCR revealed amplification of an 862 bp fragment corresponding to the bla_{IMP1} gene in 42 % of isolates and no isolates showing the presence of bla_{VIM2} (510 bp) when tested for MBL genes. Thirteen isolates were positive for MBLs by both the DDS assay and bla_{IMP1} amplification. There were 10 isolates that were PCR-positive, but negative in the DDS test. Since bla_{IMP} gene cassettes have been associated with integrons, it is possible that bla_{IMP} alleles could be carried by *Acinetobacter* strains but do not phenotypically express the IMP β-lactamases (Da Silva *et al.*, 2002). Besides, the DDS test showed 26 isolates positive for MBL which did not show amplification for bla_{IMP1}. These isolates were further tested for MBL activity by spectrophotometry, which was positive for 24 isolates wherein cell extracts caused breakdown of imipenem. Specific activity ranged from 41.4 to 60 nmol imipenem hydrolysed min^{-1} (mg protein)$^{-1}$, compared to 1.9–13.2 nmol imipenem hydrolysed min^{-1} (mg protein)$^{-1}$ by the two MBL-negative isolates used as controls (Table 1). The remaining two DDS-positive isolates displayed no enzyme activity. In the presence of EDTA, hydrolysis measured by spectrophotometry was greatly reduced. These results suggest that other genes might be responsible for encoding MBLs. The DDS test thus proved to be quite reliable and easy to perform.

The carbapenem-resistant strains with no MBL detectable by the DDS test in this study may possess other enzymes mediating carbapenem resistance, such as OXA-type lactamases (class D) or AmpC β-lactamases, and/or other mechanisms such as outer-membrane permeability and efflux mechanisms that were not checked. The isolates that were MBL-positive by the DDS test but negative for either bla_{IMP1} or bla_{VIM2} amplification may have variant bla_{IMP} or bla_{SIM} genes. MBL genes are mostly detected in class 1 integron structures and these integrons are detected in a high percentage of *Acinetobacter* isolates (Seward, 1999; Gallego & Towner, 2000). The bla_{VIM-2} gene cassette has been identified throughout the world as part of class 1

integrons and is noted to be the most prevalent carbapenemase gene (Poirel *et al.*, 2000; Yum *et al.*, 2002; Walsh *et al.*, 2005). Surprisingly, bla_{VIM-2} was not detected in our *A. baumannii* isolates. Interestingly, one of the latest studies showed bla_{VIM-2} only in imipenem-resistant *Acinetobacter* genomic species 13TU isolates, and not in imipenem-resistant *A. baumannii* isolates, which produced the OXA type of β-lactamase (Lee *et al.*, 2007). Possibly there may be specific genotypic traits against antimicrobial agents in our *A. baumannii* isolates too that could lack bla_{VIM-2}. By acquiring various kinds of resistance mechanisms, *A. baumannii* has developed into one of the most difficult hospital pathogens to control and treat. The alarming increase in the frequency of MBLs presents an emerging threat of complete resistance to the useful drugs against *Acinetobacter* spp. in India. Hence restrained and careful use of antibiotics, as well as strict hygiene practices, are critical for preventing the emergence of complete resistance and spread of this pathogen in this country.

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