

Multiple drug resistant carbapenemases producing *Acinetobacter baumannii* isolates harbours multiple R-plasmids

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Received October 4, 2012

Background & objectives: The nosocomial human pathogen *Acinetobacter baumannii* has high propensity to develop resistance to antimicrobials and to become multidrug resistant (MDR), consequently complicating the treatment. This study was carried out to investigate the presence of resistant plasmids (R-plasmids) among the clinical isolates of *A. baumannii*. In addition, the study was performed to check the presence of common β -lactamases encoding genes on these plasmids.

Methods: A total of 55 clinical isolates of *A. baumannii* were included in the study and all were subjected to plasmid DNA isolation, followed by PCR to check the presence of resistance gene determinants such as *bla*_{OXA-23}, *bla*_{OXA-51}, *bla*_{OXA-58} and *bla*_{IMP-1} on these plasmids that encode for oxacillinase (OXA) and metallo- β -lactamase (MBL) type of carbapenemases. Plasmid curing experiments were carried out on selected isolates using ethidium bromide and acridine orange as curing agents and the antibiotic resistance profiles were evaluated before and after curing.

Results: All the isolates were identified as *A. baumannii* by 16SrDNA amplification and sequencing. Plasmid DNA isolated from these isolates showed the occurrence of multiple plasmids with size ranging from 500bp to ≥ 25 kb. The percentage of *bla*_{OXA-51} and *bla*_{OXA-23} on plasmids were found to be 78 and 42 per cent, respectively and 20 isolates (36%) carried *bla*_{IMP-1} gene on plasmids. Significant difference was observed in the antibiograms of plasmid cured isolates when compared to their parental ones. The clinical isolates became susceptible to more than two antibiotic classes after curing of plasmids indicating plasmid borne resistance.

Interpretation & conclusions: Our study determined the plasmid mediated resistance mechanisms and occurrence of different resistance genes on various plasmids isolated from MDR *A. baumannii*. The present findings showed the evidence for antibiotic resistance mediated through multiple plasmids in *A. baumannii* clinical isolates. This indicates towards a need for preventive measures to avert the dissemination of plasmid resistance determinants in clinical environments.

Key words *Acinetobacter baumannii* - carbapenem resistance - R-plasmids

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Acinetobacter baumannii is responsible for various nosocomial infections that mainly affect critically ill patients in intensive care units (ICUs) and its most striking manifestation is endemic and epidemic occurrence as multiple drug resistant (MDR) strains in the hospitals across the globe^{1,2}. Generally, carbapenems are the most active agents against *A. baumannii*, but carbapenem-resistant *A. baumannii* (CRAB) isolates are becoming increasingly common where the predominant resistance mechanism involves the expression of β -lactamases¹ (either metalloenzymes or class D enzymes). Regardless of recent progress in knowledge of antibiotic resistance machinery in *A. baumannii*, very little is known about the genetic factors driving the isolates towards multiple-drug resistance. Though many genetic means of resistance to multiple classes of antibiotics are known to exist in this organism, the major ones appear to be intrinsic or acquired via plasmids, integrons, and transposons¹. However, the mechanisms of the dissemination of mobile elements bearing β -lactamase genes between *Acinetobacter* spp. are not fully understood. Many earlier investigations have given special emphasis for plasmid-mediated transferable antibiotic resistance³. Transfer of R-plasmids from *A. baumannii* to other nosocomial pathogens can create complications during the treatment. Moreover, recognition of plasmid transfer is crucial for control of outbreaks caused by MDR nosocomial pathogens. Lack of sufficient reports from India on the molecular basis of resistance in *A. baumannii* necessitated this study. We examined the presence of R-plasmids in the clinical isolates of *A. baumannii* and checked for the existence of different resistance genes on these plasmids.

Material & Methods

This study was done at Department of Biotechnology, School of Life Sciences, Pondicherry University, Puducherry.

Bacterial isolates: Overall, 55 consecutive *A. baumannii* isolates were obtained from Pondicherry Institute of Medical Sciences hospital, Puducherry, India, from various clinical specimens like endotracheal aspirates, cerebrospinal fluid, wound swabs, urine and blood culture specimens from patients mostly admitted in ICUs and acute medical care units during 2008-2009. The isolates were characterized to the species level using standard microbiological tests and simplified phenotypic tests as described elsewhere⁴ and their identification was reconfirmed using 16s rDNA amplification and sequencing⁵.

Plasmid DNA isolation: The plasmids from *A. baumannii* were isolated by following the standard procedure with slight modifications³. Briefly, a single colony of the bacteria was inoculated into 10 ml of Luria Bertani (LB) broth and incubated overnight at 37°C. The cells were harvested by centrifuging at 13600 g for 5 min and the pellet was resuspended in 700 μ l of solution I [50 mM glucose, 25 mM Tris Cl (pH 8.0), 10 mM EDTA; autoclaved and stored at 4°C] and mixed thoroughly by vortexing. Subsequently, 1 ml of solution II (0.2N NaOH, 1% SDS; freshly prepared from the stocks of 1N NaOH and 10% SDS) was added and the preparations were stored in ice for 5 min, followed by addition of 500 μ l of ice-cold solution III [5M potassium acetate (60 ml), 11.5 ml glacial acetic acid (3M K⁺; 5M CH₃COO⁻) in 28.5 ml distilled water; stored at 4°C]. This preparation was mixed and stored in ice for 3 to 5 min and then centrifuged at 12000 g for 5 min and the supernatant was transferred to a fresh tube. Subsequently, 6 μ l of RNase (Sigma, USA) (10 mg/ml) was added and the solution was incubated at 37°C for 30 min. Equal volumes of saturated phenol:chloroform (1:1 v/v) solution was added to this preparation, mixed well and centrifuged at 12000 g for 10 min. The resulting supernatant was transferred carefully into a fresh tube. Subsequently, twice the volume of ice-cold isopropanol was added to these tubes and stored at 4°C for overnight. The contents were centrifuged at 12000 g for 5 min, the supernatant was discarded and the pellet was rinsed well with 70 per cent ethanol, air-dried and resuspended in 100 μ l of TE buffer (10 mM Tris HCl (pH 8.0), 1 mM EDTA, (pH 8.0)).

Transformation experiments: Conjugation experiment was carried out as described elsewhere⁷ using *A. baumannii* isolates as the donor and *Escherichia coli* strain HB101, which was susceptible to most of the antibiotics tested, as the recipient. Plasmid transformation was checked through plasmid isolation followed by electrophoresis. Any trans-conjugants obtained were tested for antibiotic resistance to check for any acquired resistance. *i.e.* looking for resistance in the recipient strain for a particular antibiotic, which has already recorded resistance in the donor strain.

Plasmid-curing experiments: Ten clinical isolates that showed resistance to more than eight antibiotics including carbapenems were selected for further analysis by plasmid curing experiments. Acridine

orange and ethidium bromide (EtBr) (Hi-Media, India) were used as curing agents and their minimal inhibitory concentrations (MICs) were determined for the isolates and the highest / sub-inhibitory concentration that permitted slight growth was used for plasmid curing (Acridine orange – 640 to 2560 µg/ml; EtBr – 320 to 5120 µg/ml). Curing broth was prepared by diluting curing agents to sub-inhibitory concentration using LB broth to a final volume of 10 ml. The test cultures were inoculated into the above curing broth, incubated for 48 h and plated on LB agar. The cured cultures were subjected to antibiotic susceptibility testing by inoculating them into 5 ml Mueller-Hinton (MH)-broth and incubating until the turbidity reaches 0.5 McFarland standard. Subsequently, antimicrobial susceptibility was checked by disc diffusion method as per Clinical Laboratory Standard Institute (CLSI) guidelines⁸ for different antibiotics, which were previously used to test before curing. MIC values of meropenem were also checked for cured isolates. Phenotypic metallo-β-lactamase (MBL) detection was carried out using the imipenem EDTA double disc synergy (DDS) test⁹. Production of oxacillinases was analyzed according to the method described by Zarilli *et al*¹⁰, wherein the assays were performed in Mueller-Hinton agar (MHA) plates with and without 200 mM of NaCl.

PCR screening for carbapenemase genes: All the isolated plasmids were checked for the presence of different genes encoding carbapenemases through PCR. The presence of predominant MBL gene namely *bla*_{IMP-1} and the most common carbapenem-hydrolyzing class D β-lactamases coded by oxacillinase (OXA) type genes such as *bla*_{OXA-23}, *bla*_{OXA-51} and *bla*_{OXA-58} were investigated. Primer sequences used for *bla*_{IMP-1}, *bla*_{OXA-23}, *bla*_{OXA-51} and *bla*_{OXA-58} amplifications are given in Table I. The in-house strains bearing all these four resistant determinants, confirmed by amplification and

DNA sequencing, were used as positive controls for PCR. The PCR composition and reaction conditions employed were according to our earlier investigation¹¹. All the MBL and oxacillinase positive isolates by both the methods were repeatedly checked for reproducibility.

Results

Majority of *A. baumannii* isolates were obtained from the wound specimen (n=24; 44%) whereas 29 per cent of isolates were from endotracheal aspirates (n=16). The remaining isolates were from urine (n=6) of patients with urinary tract infection (UTI), and from CSF (n=5) of patients suffering from secondary meningitis. Many wound infection patients have also shown blood culture positive for *A. baumannii*, when subsequent second sample was analyzed (n=13). However, at first only four isolates were directly from blood culture of patients suffering from blood stream infection (n=4) and were counted separately. All the 55 isolates were identified to be *A. baumannii* based on 16S rDNA sequencing followed by BLAST analysis.

Presence of multiple R-plasmids: Plasmid profiles generated from the *A. baumannii* clinical isolates were analyzed and majority of the isolates had at least one plasmid. Of the 55 isolates tested, 50 (94.5%) contained plasmids. Isolates with identical plasmid profile were pooled in the same plasmid group and the variable number of plasmids (ranging from 1 to 9) with different molecular weights in each isolate enabled the identification of 10 plasmid profiles without the need for endonuclease digestion. The sizes of the plasmids were in the range of 2 to >25 kb (Fig. 1). Genotyping data of all the *A. baumannii* isolates that was previously performed and reported had generated six random amplified polymorphic DNA (RAPD) clusters (designated I-VI) revealing highly divergent distinct

Table I. Details of primers used for the detection of carbapenemase genes in this study and their amplicon sizes

Target gene	Primer	Amplicon size (bp)	Source/Reference
<i>bla</i> _{IMP-1}	Forward Primer - 5' CTACCGCAGCAGAGTCTTTGC 3' Reverse Primer - 5' GAACAACCAGTTTTGCCTTACC 3''	640	Present study
<i>bla</i> _{OXA-23}	Forward Primer - 5' GATGTGTCATAGTATTTCGTCGT 3' Reverse Primer - 5' TCACAACAACCTAAAAGCACTGT 3'	1058	11
<i>bla</i> _{OXA-51}	Forward Primer - 5' CCAAATCACAGCGCTTCAAAA 3' Reverse Primer - 5' CCCATCCAGTTAACCAGCCTAC 3''	625	Present study
<i>bla</i> _{OXA-58}	Forward Primer - 5' CGATCAGAATGTTCAAGCGC 3' Reverse Primer - 5' ACGATTCTCCCCTCTGCGC 3''	529	Present study
Synthesized by authors in their laboratory			

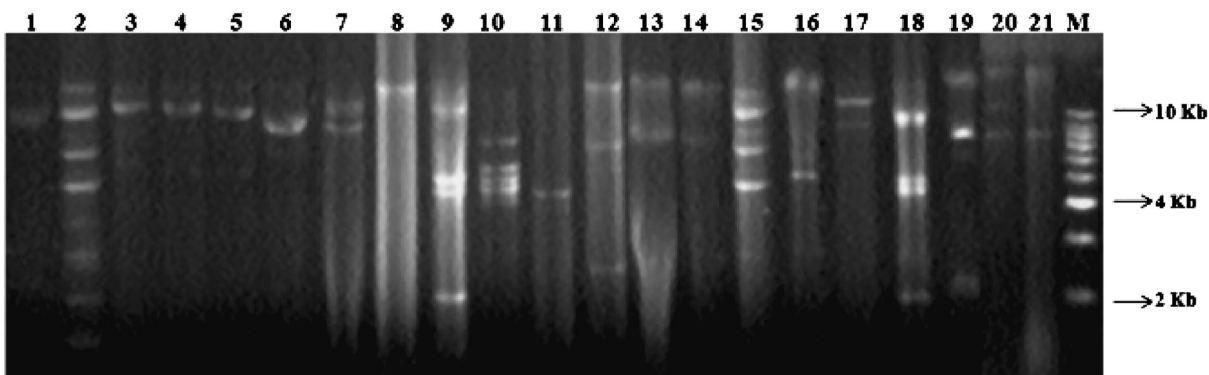


Fig. 1. Plasmid profiles of representative MDR *A. baumannii* isolates bearing single and multiple numbers of plasmids. (a) Lanes 1, 3-7, 17 (strain IDs P1, P4, P5, P6, P9, P11, P35) depict strains containing two plasmids of sizes greater than ≥ 10 Kb and lane 16 containing two plasmids, one is of 5Kb and another of ≥ 10 Kb size; (b) Lanes 13, 14 & 21 depict strains (P20, P3 & P23) containing two plasmids, one ≥ 10 Kb and another of 8Kb size; (c) Lane 2 depicts strain (P2) containing multiple plasmids (n=9); (d) Lane 8 depicts strain (P12) harbouring single plasmid with molecular weight greater than 10Kb; Lane 11 depicts strain (P16) harbouring single plasmid with molecular weight of 5Kb; (e) Lanes 9, 10 and 18 represents strains (P13, P14, P48) harbouring four plasmids of size varying from 2Kb to 10Kb; (f) Lane 12, 19 and 20 (strain IDs P17, P28, P22) contains three plasmids of size varying from 2Kb to >10 Kb; (g) Lane 15 shows strain (P10) containing five plasmids of size varying from 5Kb to >10 Kb.

clones¹². Four main groups of isolates comprised a total of 11 isolates showed the presence of three or more plasmids ranging from 500 bp to ≥ 25 kb with one common high molecular weight plasmid of size ≈ 12 or ≥ 25 kb (Table II). One major cluster of 23 isolates contained plasmids more than 5 kb except one isolate *i.e.* P43 which also had a 2 kb plasmid. The remaining group of 16 isolates contained only one plasmid with sizes ranging from 5 to ≥ 25 kb. Isolates such as P2 and P18 had highest number of plasmids. Multiple plasmids were common in majority of the isolates and only 16 isolates had single plasmid of high molecular-weight (>10 kb). Transformation experiments performed for native plasmid transformation showed that *E. coli* HB101 failed to acquire resistance to any of the antibiotics for which donor *A. baumannii* was resistant.

Resistance profiles after plasmid curing: Ten representative MDR clinical isolates were tested for ‘resistance’ properties by plasmid curing using known sub-inhibitory concentrations of curing agents and showed significant shift from resistance to susceptibility in nine of them when tested by disc-diffusion method. For instance, four isolates (P2, P22, P34 & P41) became susceptible to more than two antibiotics after EtBr curing. Conversely, P37 became resistant to imipenem and ceftriaxone after EtBr treatment, which was earlier susceptible. Most of the isolates became susceptible to more than two antibiotics when treated with acridine orange, and susceptibility to seven antibiotics (Table III). P12 showed four-fold reduction in its MIC for meropenem, P24 had a three-fold decrease, while P2 and P22 showed two-fold reductions in their MIC for meropenem after curing. Only P19 and P37 showed contrasting results wherein both became resistant to carbapenems and ceftriaxone after acridine orange curing.

Table II. Details of multiple R-plasmids harbouring *A. baumannii* isolates

Isolate ID	Total number of plasmids	Size range
P2 and P18	9	500 bp to ≥ 25 kb
P10	5	5 kb to ≥ 10 kb
P13, P14, P48	4	2 to 10 kb
P17, P28, P46, P50, P53	3	2 to ≥ 25 kb
23 isolates	2	5 to ≥ 25 kb
Remaining 16 isolates	1	5 to ≥ 25 kb

Presence of OXA type and MBL genes: The results showed complete absence of *bla*_{OXA-58} both on the chromosome and plasmids. PCR results showed the presence of *bla*_{OXA-51} and *bla*_{OXA-23} on plasmids in around 39 (78%) and 21 (42%) isolates, respectively out of total 55 isolates tested. Twenty isolates (40%) had plasmids bearing *bla*_{IMP-1} gene (Fig. 2). PCR amplification and sequencing of IS element *ISAbal* from representative isolates (n=6) confirmed the presence of *ISAbal*

Table III. Details of origin, resistogram before and curing experiments, MBL & carbapenem-hydrolysing oxacillinases (CHDL) presence on plasmids and random amplified polymorphic DNA (RAPD) cluster of ten representative isolates of *A. baumannii*

Sl. no.	Isolate	Site of isolation	Curing results	Resistogram			Phenotypic tests		PCR screening of plasmids			RAPD cluster
				EtBr	Acridine orange	Double Disk Synergy test for MBL	Phenotypic detection of oxacillinase	<i>bla</i> _{IMPI}	<i>bla</i> _{OXA-51}	<i>bla</i> _{OXA-58}	<i>bla</i> _{OXA-23}	
1	P2	Wound	R to S R to In	NOR IPM, AMK, CIP	CIP IPM, NOR	-	+	-	+	-	+	III
2	P5	ETA	R to S R to In	- -	IPM, AMK, CIP, NOR	-	+	-	+	-	+	IV
3	P12	ETA	R to S R to In	MEM, CAZ CRO, NOR	MEM, CAZ, NOR IPM, CRO, FEP, CHL	-	+	+	+	-	+	IV
4	P19	CSF	R to S R to In	IPM NOR	IPM MEM	+	+	-	+	-	+	VI
5	P22	Wound	R to S R to In	CRO, FEP, AMK, NOR -	- -	+	-	+	+	-	+	V
6	P24	ETA	R to S R to In	- -	IPM, CRO, CAZ, AMK, NOR	+	-	+	+	-	+	V
7	P34	CSF	R to S R to In	IPM, AMK CRO, CAZ	IPM, AMK CRO, CAZ, FEP, CIP	+	+	-	-	-	+	IV
8	P35	BT	R to S R to In	MEM IPM	MEM IPM, CIP	-	+	-	+	-	+	IV
9	P37	CSF	R to S R to In	- -	- -	+	+	-	+	-	+	IV
10	P41	Sputum	R to S R to In	CAZ, AMK -	- FEP, CIP	+	-	-	+	-	+	V

S, sensitive; R, resistant; In, intermediate; BT, bone tissue; CSF, cerebrospinal fluid; ETA, endotracheal aspirate; MEM, meropenem; IPM, imipenem; CRO, ceftriaxone; FEP, cefepime; CAZ, ceftazidime; AMK, amikacin; Ce, cefotaxime; CIP, ciprofloxacin; CHL, chloramphenicol; NOR, norfloxacin; Ebr, ethidium bromide; MBL, metallo- β -lactamase

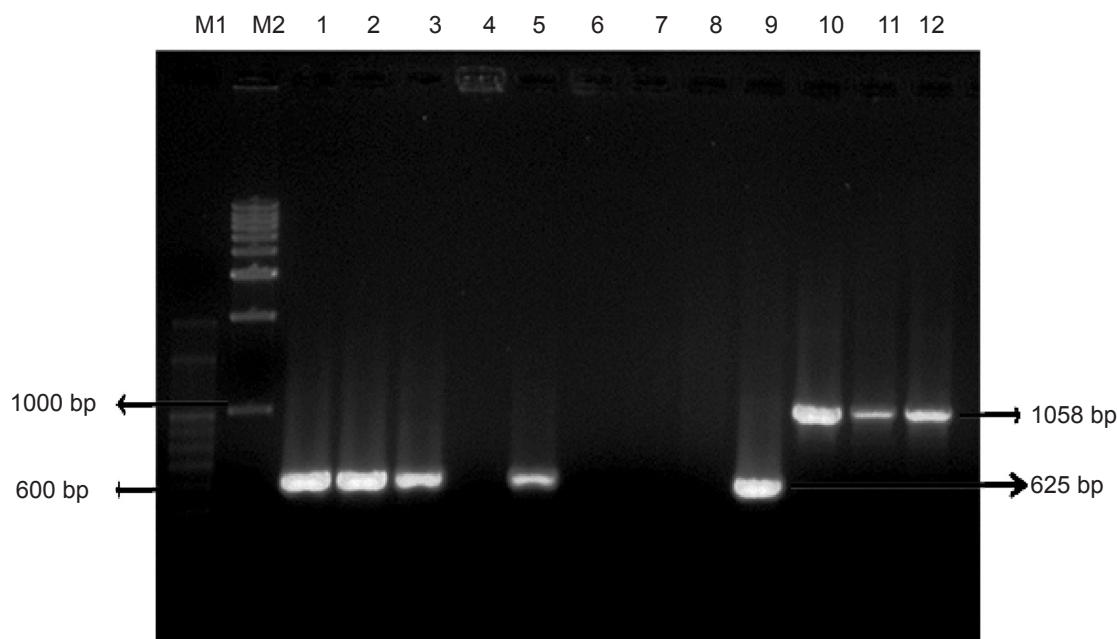


Fig. 2. PCR amplification of *bla*_{OXA-51} and *bla*_{OXA-23}. Lane M1 - 100bp DNA ladder, Lane M2 - 1kb DNA ladder, Lane 1, 2, 3, 5 & 9 Positive amplification of *bla*_{OXA-51} (Isolate ID: P24, P25, P26, P28, P37); Lane 4, 6, 7 & 8 (P27, P29, P33, P36) Isolates showing no amplification of *bla*_{OXA-51}; Lane 10-12 - Positive amplification of *bla*_{OXA-23} (P2, P19, P41).

upstream of OXA-carbapenemase genes, retaliating the association between OXA-genes and *ISAbal*. There was no association between the results of genotypic and phenotypic assays used to detect β -lactamases.

Discussion

Acinetobacter genus appears to have high propensity to develop resistance in response to new antibiotics rapidly, unlike more “traditional” clinical bacteria². The success of *A. baumannii* is attributed to its ability to respond quickly to new antibiotic challenges. Mobile genetic elements like plasmids and transposons play an important role in the biology of most prokaryotic organisms, and *Acinetobacter* species is no exception¹³. Plasmid profiling has been used to study the epidemiology of *Acinetobacter* in the past and >80 per cent of *Acinetobacter* isolates appear to carry multiple indigenous plasmids with variable molecular sizes¹. Study conducted by Poirel *et al*¹⁴ conclusively inferred plasmids/integrans playing an important role in antibiotic resistance, endemic and epidemic behaviour of *A. baumannii*. Almost all our isolates harboured 1 to 2 high molecular weight plasmids (> 10 kb), which is in accordance with earlier observations. For instance, 63 kb plasmid pIP1841 of *A. baumannii* harbouring a gene, which confers resistance to aminoglycosides is

self-transferable to other *A. baumannii*, *A. haemolyticus* and *A. lwoffii* but not to *E.coli*¹⁵.

All the isolates in this study were resistant to three or more antibiotics and plasmid curing experiments attributed resistance properties to one or more plasmids present in them. Two earlier Indian studies have documented *A. baumannii* bearing high molecular weight plasmids ranging from 5 to 66 kb^{7,16}. One of them reported five different types of plasmids with a maximum of three plasmids in *A. baumannii* isolates with molecular size 1.5 to 40 kb and it also revealed that *Acinetobacter* spp. isolated from the tribal skin flora had low number of plasmids as compared to clinical and environmental isolates¹². This study also documented that isolates harbouring low-molecular weight plasmids were susceptible to most of the antibiotics, which is contrary to our observations. The authors speculated that these smaller plasmids might be cryptic in nature or code for some other novel properties other than resistance¹⁶.

It is shown that *Acinetobacter* is capable of harbouring large plasmids in multiple numbers and these may be large due to the presence of many transferable integron cassettes in them, which are evidently responsible for resistance. Our PCR results

showed the presence of *bla*_{IMP-1} gene only in larger plasmids. Earlier it has been revealed that the *bla*_{IMP-1} gene normally resides in a plasmid-borne class-1 integron cassette^{1,14}. Numerous studies have noted integron cassettes on larger plasmids, which is likely to be true in our isolates also. The presence of a common large size plasmid (≈25-40kb) among all our isolates possibly indicates the presence of transferable integrons cassettes responsible for resistance.

Plasmid-mediated transfer of *bla*_{NDM-1} gene in Gram-negative bacteria was investigated in a study in which five *bla*_{NDM-1} positive plasmids of different incompatibility groups from clinical isolates belonging to *Enterobacteriaceae* were evaluated for conjugation properties and host specificity. Successful conjugative transfers were obtained between members of enterobacterial spp. when these were used as either donor or recipients. However, none of the five plasmids were transferable to *A. baumannii*¹⁷. Likewise, we failed to obtain transconjugants when *E. coli* HB101 was used as a recipient. Though many intrinsic *Acinetobacter* plasmids reported are non-self-transferable, the almost ubiquitous replicase gene appears to be strongly associated with a potential *tra* locus that could serve as a general system for plasmid mobilization. Consequently, horizontal gene transfer (HGT) of resistance genes among *A. baumannii* strains may not be ruled out¹⁸. Therefore, one can presume that successful resistance determinant transfer occurs between two *Acinetobacter* strains and transfer of plasmids directly from a member of enterobacteriaceae to acinetobacter or vice versa is difficult or a rare event.

Earlier it was noted that EtBr curing led to disappearance of resistance to extended spectrum of β-lactams (ESBL) with the concurrent loss of plasmids from *A. baumannii*⁴ suggesting that ESBL determinants were plasmid-borne. Similarly, a significant shift was observed in our study from resistance to susceptibility as well as loss of plasmids after curing indicating that most of the clinical isolates had plasmids bearing multiple resistance determinants on them. Four of the 10 isolates became susceptible to more than two antibiotics after curing and isolates P2, P22 and P41 became susceptible to three antibiotic classes such as carbapenem, aminoglycoside and quinolone and/or cephalosporin.

Presence of *bla*_{OXA-51}, *bla*_{OXA-23} and *bla*_{IMP-1} was predominantly observed on plasmids in our study and

these resistant determinants can be easily transferred to other isolates of *A. baumannii*. Plasmid-mediated HGT of common *bla*_{OXA-24/40}-carrying plasmids (30-kb repA_AB; 10-kb aci2) coding for imipenem resistance was recently demonstrated in *A. baumannii* and here the *bla*_{OXA-24/40} was also identified in the chromosome¹⁹. OXA-40 present on *Pseudomonas aeruginosa* plasmid showed 100 per cent homology to *bla*_{OXA-24/40} carried by *A. baumannii* plasmid. Further, *A. radioresistens*, a commensal isolated from the skin has been identified as the source for this OXA-40²⁰. Epidemiologically unrelated CRAB isolates belonging to European clonal lineage-II, have been shown to carry an identical OXA-24-encoding plasmid pABVA01²¹. Interestingly, pABVA01 is flanked by conserved inverted repeats which are homologous to flanking regions of other *Acinetobacter* plasmids suggesting their mobilization is through a novel site-specific recombination mechanism.

It has been reported that CRAB bearing *bla*_{OXA-24} on plasmid expressing class-D carbapenemase releases it into outer membrane vesicles during *in vitro* growth that serves as a mechanism of HGT imparting the carbapenem resistance²². A plasmid-borne ISAbal-*bla*_{OXA-51}-like gene along with its additional copy on their chromosomes has been identified in carbapenem resistant *Acinetobacter* genomospecies 13TU and *A. baumannii* wherein this plasmid appeared to be acquired via one-ended transposition (*Tn6080*)²³. One study from India has noted very high carbapenem resistance (imipenem MIC -128 μg/ml) among the isolates that bear *bla*_{OXA-51} on their chromosome²⁴. In our study, plasmid-borne *bla*_{OXA-51} bearing isolates had higher MICs to carbapenems than those bearing *bla*_{OXA-51} only on chromosomes. This could be due to increased gene dosage provided by the higher copy number of associated plasmids.

Multicopy *bla*_{OXA-58} gene has been a source of high-level carbapenem resistance and plasmid borne *bla*_{OXA-58} is apparently the only mechanism of resistance which can be transferred by conjugation^{14,22,23}. Earlier, it was shown that an 11 kb *A. baumannii* plasmid (pTVICU53) harbouring 13-open reading frames (ORFs) including *bla*_{OXA-58} with an upstream insertion of ISAb3 and IS1008 provided two independent promoters for the transcription control of OXA-58²⁵. pTVICU53 transformation was shown to increase the carbapenem MICs by 64 to 256-fold and the deletion of these promoters resulted in drastic reduction of carbapenem MIC. Such findings underscore that *A.*

baumannii might develop carbapenem resistance with a single transformation step, taking up a plasmid containing a genetic construct with a potentially high level of transcription of the OXA-genes²⁵.

In summary, multiple drug resistance in *Acinetobacter* species appears to be a combined effect of lateral gene transfer (plasmids and integrons) and clonal dissemination of MDR clones. This is likely scenario in any hospital settings including ours. Studies on identification of resistant plasmids and deducing their likely mode of transfer are important to control and eradicate persistent endemic and epidemic clones of *A. baumannii*. In addition, stringent infection control measures and their implementation are of prime importance that can prevent further propagation of resistance.

Acknowledgment

Authors thank the Indian Council of Medical Research (ICMR), Government of India, New Delhi, for funding the study (No.5/3/3/14/2007-ECD) and University Grants Commission (UGC), Government of India for financial assistance under Special Assistance Programme (SAP) F.3 -21/2009(SAP-II).

References

- Roca I, Espinal P, Vila-Farrés X, Vila J. The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug-resistant menace. *Front Microbiol* 2012; 3 : 148.
- Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin Microbiol Rev* 2008; 21 : 538-82.
- Maltezou HC. Metallo-beta-lactamases in Gram-negative bacteria: introducing the era of pan-resistance? *Int J Antimicrob Agents* 2009; 33 : 405.e1-7.
- Kenchappa P, Sreenivasamurthy B. Simplified panel of assimilation tests for identification of *Acinetobacter* species. *Indian J Pathol Microbiol* 2003; 46 : 700-6.
- Ertürk A, Çiçek AC, Gümüş A, Cüre E, Şen A, Kurt A, *et al.* Molecular characterization and control of *Acinetobacter baumannii* isolates resistant to multi-drugs emerging in inter-intensive care units. *Ann Clin Microbiol Antimicrob* 2014; 13 : 36.
- Sambrook J, Russell DW. Plasmids and their usefulness in molecular cloning. In: Sambrook J, Russell DW, editors. *Molecular cloning: a laboratory manual*, 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2001. p 1.1-1.163.
- Joshi SG, Litake GM, Ghole VS, Niphadkar KB. Plasmid-borne extended-spectrum beta-lactamase in a clinical isolate of *Acinetobacter baumannii*. *J Med Microbiol* 2003; 52 : 1125-7.
- Clinical and Laboratory Standards Institute (CLSI). *Performance standards for antimicrobial susceptibility testing*, 21st informational supplement (M100-S22). Wayne, PA: CLSI; 2012.
- Yong D, Lee K, Yum JH, Shin HB, Rossolini, GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol* 2002; 40 : 3798-801.
- Zarrilli R, Vitale D, Di Popolo A, Bagattini M, Daoud Z, Khan AU, *et al.* A plasmid-borne bla_{OXA-58} gene confers imipenem resistance to *Acinetobacter baumannii* isolates from a Lebanese hospital. *Antimicrob Agents Chemother* 2008; 52 : 4115-20.
- Jeon BC, Jeong SH, Bae IK, Kwon SB, Lee K, Young D, *et al.* Investigation of a nosocomial outbreak of imipenem-resistant *Acinetobacter baumannii* producing the OXA-23 beta-lactamase in Korea. *J Clin Microbiol* 2005; 43 : 2241-5.
- Uma Karthika R, Srinivasa Rao R, Sahoo S, Shashikala P, Kanungo R, Jayachandran S, *et al.* 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. *J Med Microbiol* 2009; 58 : 430-5.
- Bergogne-Bérézin E, Joly-Guillou ML. Antibiotic resistance mechanisms in *Acinetobacter*. In: Towner KJ, Bergogne-Bérézin E, Fewson CA, editors. *The biology of Acinetobacter: taxonomy, clinical importance, molecular biology, physiology, industrial relevance*. New York: Plenum Press; 1991. p. 83-111.
- Poirel L, Nordmann P. Carbapenem resistance in *Acinetobacter baumannii*: Mechanisms and epidemiology. *Clin Microbiol Infect* 2006; 12 : 826-36.
- Lambert T, Gerbaud G, Courvalin P. Transferable amikacin resistance in *Acinetobacter* spp. due to a new type of 3'-aminoglycoside phosphotransferase. *Antimicrob Agents Chemother* 1988; 32 : 15-9.
- Pardesi KR, Yavankar SP, Chopade BA. Plasmid distribution & antimicrobial susceptibility patterns of *Acinetobacter* genospecies from healthy skin of a tribal population in western India. *Indian J Med Res* 2007; 125 : 79-88.
- Potron A, Poirel L, Nordmann P. Plasmid-mediated transfer of the bla(NDM-1) gene in Gram-negative rods. *FEMS Microbiol Lett* 2011; 324 : 111-6.
- Towner KJ, Evans B, Villa L, Levi K, Hamouda A, Amyes SG, *et al.* Distribution of intrinsic plasmid replicase genes and their association with carbapenem-hydrolyzing class D beta-lactamase genes in European clinical isolates of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2011; 55 : 2154-9.
- Grosso F, Quinteira S, Poirel L, Novais A, Peixe L. Role of common blaOXA-24/OXA-40-carrying platforms and plasmids in the spread of OXA-24/OXA-40 among *Acinetobacter* species clinical isolates. *Antimicrob Agents Chemother* 2012; 56 : 3969-72.
- Poirel L, Figueiredo S, Cattoir V, Carattoli A, Nordmann P. *Acinetobacter radioresistens* as a silent source of carbapenem resistance for *Acinetobacter* spp. *Antimicrob Agents Chemother* 2008; 52 : 1252-6.
- D'Andrea MM, Giani T, D'Arezzo S, Capone A, Petrosillo N, Visca P, *et al.* Characterization of pABVA01, a plasmid

- encoding the OXA-24 carbapenemase from Italian isolates of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2009; 53 : 3528-33.
22. Rumbo C, Fernández-Moreira E, Merino M, Poza M, Mendez JA, Soares NC, *et al.* Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2011; 55 : 3084-90.
23. Chen TL, Lee YT, Kuo SC, Hsueh PR, Chang FY, Siu LK, *et al.* Emergence and distribution of plasmids bearing the *bla*OXA-51-like gene with an upstream IS_{Aba1} in carbapenem-resistant *Acinetobacter baumannii* isolates in Taiwan. *Antimicrob Agents Chemother* 2010; 54 : 4575-81.
24. Tiwari V, Kapil A, Moganty RR. Carbapenem-hydrolyzing oxacillinase in high resistant strains of *Acinetobacter baumannii* isolated from India. *Microb Pathog* 2012; 53 : 81-6.
25. Chen TL, Wu RC, Shaio MF, Fung CP, Cho WL. Acquisition of a plasmid-borne *bla*OXA-58 gene with an upstream IS1008 insertion conferring a high level of carbapenem resistance to *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2008; 52 : 2573-80.

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