

Detection of Carbenicillin Hydrolysing (CARB) Type of ESBL Enzyme in *Acinetobacter baumannii* Strains Isolated from Bacterimic and UTI Patients

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Abstract—Aim of this study was to find out the antibiotic resistance pattern of hospital associate *Acinetobacter baumannii* species isolated from bacterimic and urinary tract infected patients and to determine the type of β -lactamase produce by the isolates.

Six strains of *A.baumannii* were isolated from hospital blood and urine cultures. The genuinity of the isolates was determined by chromosomal transformation assay and by biochemical tests. Preliminary antibiotic sensitivity test was carried out by quick disk diffusion break point assay and MIC to different antibiotics was performed by agar dilution methods. β -Lactamase test was carried out by Macro- Iodometric method and observed on 7.5% PAGE. Substrate hydrolysis in presence and absence of inhibitors; Sulbactam, para-chloromercuribenzoate (p-CMB), Calvulanic acid, Cloxacillin and NaCl were carried out by rapid fixed time method.

A.baumannii isolates were resistant to almost all penicillins (MIC \geq 256 μ g/ml). β -lactamase test was positive for blood isolates. The highest rate of substrate hydrolysis was achieved when Carbenicillin was used as substrate (116 μ mol), while lowest rate of hydrolysis was achieved when Cloxacillin was used as substrate (50.2 μ mol) [$p < 0.05$]. The rate of substrate hydrolysis was adversely affected in presence of Sulbactam, Clavulanic acid and NaCl (3.5, 3.0, 3.6 μ mol) respectively. However, the penicillins hydrolysis was not affected by p-CMB or Cloxacillin. MIC in presence of β -lactamase inhibitors Clavulanic acid and Sulbactam decreased to 25 μ g/ml.

Keywords—*A. baumannii*, antibiotic resistance, β -lactamase, penicillins hydrolysis.

I. INTRODUCTION

THE genus *Acinetobacter baumannii* occupied an increasingly important position as opportunistic pathogen in the hospital environment [1]. One of particular attribute of these strains is the resistance to multiple antibiotics including β -lactams [2]. Screening 100 isolates *Acinetobacter* by Guillou *et al.*, [3] revealed that 81% of the strains were produced two types of β -lactamases (TEM and CARB). *A.baumannii* hospital isolates produce mainly cephalosporinase type enzyme and inhibited by 25mM Clavulanic acid but not by 1mM EDTA or 100mM p-CMB. The β -lactamases produced by *A. lwoffii* ULA-501, *A. baumannii* ULA-187, and *A. baumannii* AC-14 strains were

purified and characterized, their kinetic interactions with several β -lactam molecules, including substrates and inhibitors were studied in detail [4]. Three β -lactamases enzymes were identified and appeared to be cephalosporinases type of β -lactamases with different acylation efficiencies (kcat/Km ratio values), and their hydrolytic activities were inhibited by Benzylpenicillin, Piperacillin, and Cefotaxime, which did not behave as substrates for the enzyme. Carbenicillin was a substrate for the β -lactamase from *A. lwoffii* ULA-501, whereas it acted as a transient inactivator of the enzymes produced by the two *A. baumannii* strains. Clavulanic acid was unable to inactivate the three β -lactamases, whereas Sulbactam behaved as an inactivator only at a high concentration (1 mM) which was difficult to achieve during antibiotic therapy [4].

265 isolates were identified as *Acinetobacter* species by Gaur *et al.*, [5]. These isolates were tested for antibiotic resistance by the disc-diffusion method with 14 antimicrobials, including Meropenem and Imipenem. Thereafter, all *Acinetobacter* species were subjected to MIC for Meropenem. More than 80% resistance to second- and third-generation cephalosporins, aminoglycosides, and quinolones was recorded and discussed. All Carbapenem-resistant/intermediate strains were also resistant to other (≥ 10) antibiotics tested by the disc-diffusion break point method.

In Iran few information are available on antibiotic resistance in *A.baumannii*, Kosroshahi and Sharifi [6] isolated 400 samples from ICU patients in 4 university hospitals in Isfahan, Iran, 15 (3.75%) were belong to *A.baumannii*. Antibiotic sensitivity test revealed 4 (26.6%) isolates were resistant to Imipenem and Meropenem. Similarly, Farhani *et al.*, [7] isolated 60 species of *Acinetobacter* from Shahid Beheshti hospital in Kashan, Iran. Among them 48 isolates were *A.baumannii*, 6 were *A.lwoffii* and 6 were other *Acinetobacter* species. They were resistant to Amikacin, Tobramycin, Ampicillin/Sulbactam and Imipenem.

The present investigation deals with antibiotic resistance pattern and identification of CARB type of β -lactamase enzyme in multiple drug resistant strains of *A.baumannii* isolated from bacterimic and urinary tract infected patients.

II. MATERIAL AND METHODS

Bacterial identification

Six strains of *A.baumannii* were isolated from the hospitals. Two *A.baumannii* BL was blood isolates from bacterimic patient, while, three *A. baumannii* APH isolates were from urinary tract infected patients and one was isolated from

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ventilator in ICU. The genuinity of the isolates were studied preliminary by chromosomal transformation assay [8] using auxotrophic strain of *A.calcoaceticus* BD143 trpE27 and species identification was carried out by biochemical and sugar utilization tests as described by Bouvet and Grimont [9]. The isolated *A.baumannii* were identified to species level by performing the other tests including Gram staining, motility, cultural characteristics on nutrient agar and Cysteine Electrolyte Deficient (CLED) agar, Catalase, Oxidase, hemolytic ability on blood agar, glucose oxidation in Hugh and Leifson medium containing 1% glucose and ability to grow at 44°C.

Antibiotic susceptibility tests

Antibiotic sensitivity of the isolated *A. baumannii* strains was carried out by disc diffusion breakpoint assay and MIC was measured by agar dilution method as described previously by Clinical and Laboratory Standard Institute (CLSI) [1- 10]. Following antimicrobial agents were used in this investigation; Carbenicillin, Cloxacillin, Penicillin-G, Tetracycline, Chloramphenicol, Streptomycin, Amoxicillin, Cefazolin, Cefadroxil, Ciprofloxacin, Cefuroxime, Cefotaxime, Amoxicillin/Clavulanic acid, Ceftazidime, Ampicillin/Sulbactam and Sulfamethoxazole/trimethoprim (Oxoid disks purchased from padtanteb, Tehran, Iran). *A.baumannii* isolates were considered susceptible to above antibiotics if the MIC was $\leq 2 \mu\text{g/ml}$ and resistant if the MIC was $\geq 4 \mu\text{g/ml}$. Standard culture of *A.calcoaceticus* BD413 was used as sensitive bacterium.

β -lactamase test

Production of β -lactamase enzyme in the above strains was carried out by macro-Iodometric test [11]. Briefly, fresh one loopful of overnight culture of *A.baumannii* (CFU= 10^8) in nutrient broth was inoculated into CLED agar (Oxoid) containing 100 $\mu\text{g/ml}$ Ampicillin and incubated at 37°C for 24 hours. 200 $\mu\text{g/ml}$ of the culture was suspended in 1ml 0.05M K-phosphate buffer (pH-7.4) tube containing 5mg penicillin-G solution. To this preparation one drop of freshly prepared starch solution (2%) and one drop of iodine reagent (0.001M I₂ and 0.006M KI) were added. Change of color from blue to colorless within the few seconds indicated positive result. *E.coli* K12 J53.2 was used as negative control.

Extraction of β -lactamase

The enzyme was extracted from the organism by both sonication and freeze –thawing. *A.baumannii* was grown in Luria-Bertani (LB) broth containing 100 $\mu\text{g/ml}$ Ampicillin and incubated at 37°C on shaker incubator (100rpm) for 24 hours. The culture was centrifuged at 8.000rpm at 4°C for 15 minutes and the cell pellet was washed with 0.01M sterile phosphate buffer (pH-8.0). The suspension was sonicated with Lab-sonic sonicator (Germany) followed by freeze – thawing. Sonication was done for 15 seconds with 50% on/off pulsed cycle and freeze – thawing at -70°C for 10 minutes. The sonicated cell fractured were centrifuged at 12.000rpm for 10 minutes and simultaneously observed microscopically to see the disintegrated bacterial cells. Supernatant was taken for further study.

Polyacrylamide gel electrophoresis (PAGE)

Tube gel electrophoresis was performed using non-denaturing 7.5% acrylamide and 0.18% bisacrylamide at pH-8.3. 60 μl of supernatant from crude cell lysate was used to load the gel and electrophoresis was conducted at 30mA for 2 hours in Tris-glycine buffer (pH-7.4). 10 μl of 2% starch and 10 μl of iodine preparation was loaded simultaneously with the preparation. The tubes were submerged in 250ml 0.05M penicillin –G solution. Clear zone of discoloration in the tube within the seconds compared with negative control (without penicillin).

Substrate hydrolysis in the presence and absence of inhibitors. The modification of rapid fixed time β -lactamase assay was employed with reduction of iodine by hydrolysis penicillins with UV/Light spectrophotometer at 450nm. The sonicated solution was diluted with 2.5ml 0.01M phosphate buffer (pH-8.0). To this preparation 0.5ml 200 $\mu\text{g/ml}$ substrate (Cloxacillin, Ampicillin, Carbenicillin, and penicillin-G) was added separately and incubated at room temperature for 30 minutes. In case of β -lactamase inhibitors, 0.5ml of 0.5mM para-chloromercuribenzoate [p-CMB] (Fluka grade), NaCl, 100mM, 200 $\mu\text{g/ml}$ Sulbactam / Clavulanic acid and Cloxacillin 200 $\mu\text{g/ml}$ were added to the crude enzyme preparation 15 minutes before addition of the substrates. The reaction was stopped by addition of 5ml iodine reagent containing 0.32N I₂ and 1.2M KI with rapid stirring at room temperature. Absorbance was measured at 540nm and the results were compared with presence of inhibitors. Simultaneously, a blank-A containing 3ml phosphate buffer (pH-7.5) and 5ml of iodine reagent and blank -B containing 5ml of phosphate buffer (pH-7.5) were run along side of the tests as shown in Table 1.

MIC of A.baumannii to Sulbactam and Clavulanic acid

Sensitivity of organism to penicillins in presence and absence of inhibitors (Clavulanic acid and Sulbactam) was determined by both disc diffusion on Muller-Hinton agar (Oxoid) and broth micro-dilution tests. Similarly, MIC test was performed using different concentration of Amoxicillin and Ampicillin (from 0.01 till 256 $\mu\text{g/ml}$) and fixed concentration of Clavulanic acid and Sulbactam (25 $\mu\text{g/ml}$) in 1ml sterile Muller-Hinton broth.

Statistical analysis

All analyses were performed using SPSS, version 15.0 (SPSSInc, Chicago, IL, USA). Categorical variables were compared using Pearson's chi squared or Fisher's exact test as appropriate. Continuous variables were compared using the Wilcoxon rank-sum test. All p values were two-tailed; $p \leq 0.05$ was considered statistically significant.

II. RESULTS

Six strains of *A.baumannii* were isolated from hospital blood and urine samples and identified to the genus *Acinetobacter* by chromosomal transformation assay using auxotrophic strain of *A.calcoaceticus* BD413trpE27 and biochemical tests. The species colonies on CLED agar were circular, smooth, convex, translucent, mucoid, and nonpigmented. They were gram negative coccobacilli, non motile, oxidase negative, encapsulated and no spore forming.

TABLE I
STEPS WERE USED FOR DETERMINATION OF RATE OF PENICILLINS
HYDROLYSIS BY *A.BAUMANNII* STRAINS ISOLATED FROM
HOSPITALS

Tube No.	Enzyme (ml)	Phosphate buffer (ml)	Inhibitor (ml)	Incubation time (ml)	Reagent (ml)
1	0.5	2.5	0.5	15	5
2	0.5	2.5	0.5	15	5
Blank A	-	3	-	-	5
Blank B	-	3	-	-	5

Among the isolates, strains isolated from blood were resistant to at least 9 antibiotics as shown in Figure 1. Blood isolates were highly resistance to different penicillins. The MIC to Ampicillin, Carbenicillin, Amoxicillin and Penicillin-G was 512 μ g/ml. It also exhibited high degree of resistance to Tetracycline, Chloramphenicol and Sulfametaxazole (MIC range 64-126 μ g/ml), moderately resistant to first generation of cephalosporins like Cefazoline (MIC 75 μ g/ml) and Cephadroxil (MIC 50 μ g/ml), while quite sensitive to third generation of cephalosporins like Cefuroxime and Cefotaxime (MIC \leq 4 μ g/ml) (Fig. 1).

A.baumannii BL54 was positive for β -lactamase and rapid discoloration of iodine starch complex was observed within the seconds. The results were confirmed by PAGE where clear zone of discoloration was observed after addition of Penicillin-G by the organism. The tests were compared with negative controls. Substrate and inhibitor profile of β -lactamase produced by organism are shown in Table 2. The highest rate of hydrolysis of penicillins was achieved when Carbenicillin was used as substrate (116 μ mol) [$p \leq 0.05$], while lowest hydrolysis rate was obtained when Cloxacillin was used as substrate (50.2 μ mol). The rate of substrate hydrolysis was considerably reduced in presence of Sulbactam, Clavulanic acid and NaCl [$p \leq 0.05$] (table 2). However, rate of hydrolysis was not affected by p-CMB or Cloxacillin (80.2 \pm 0.02 and 50.2 \pm 0.01 respectively) [$p \leq 0.05$]. This suggests that β -lactamase produced by *A.baumannii* blood isolate was belonging to CARB family of the enzyme. These results were further followed by MIC (sensitivity) of *A.baumannii* BL54 in presence and absence of β -lactamase inhibitors as shown in figure 2. The MIC of Amoxicillin and Ampicillin in presence of Clavulanic acid and Sulbactam decreased to 25 μ g/ml. While, in the absence of inhibitors, MIC to the above antibiotics were increased to 256 μ g/ml as illustrated in figure 2. This suggests that indeed the isolated enzyme was belonging to CARB family of β -lactamases and differ from previously reported PSE -1 (*Pseudomonas* extended spectrum β -lactamase) type as shown in Table -3.

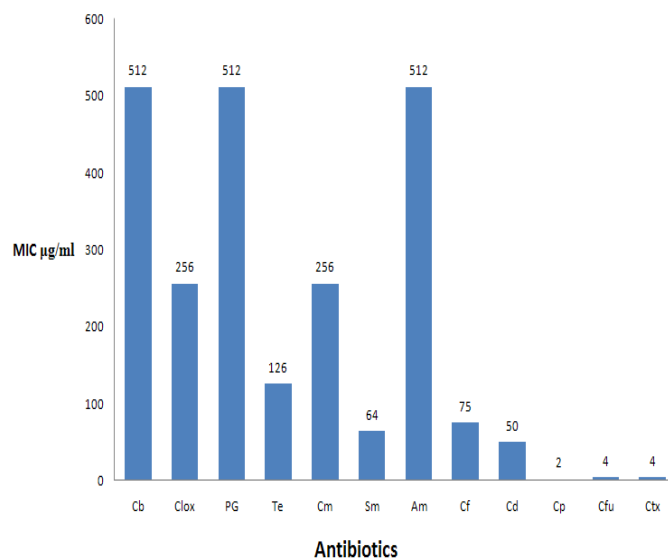


Fig. 1. MIC of different antibiotics (β -lactams) for *A.baumannii* blood isolates.

Abbreviations: Cb= Carbenicillin, Clox = Cloxacillin, PG= Penicillin-G, Te= Tetracycline, Cm=Chloramphenicol, Sm = Streptomycin, Am= Amoxicillin, Cf= Cefazoline, Cd= Cephadroxil, Cp=Ciprofloxacin, Cfu=Cefuroxime, Ctx=Cefotaxime.

TABLE II
THE RATE OF SUBSTRATE (B- LACTAMS) HYDROLYSIS AND
INHIBITOR PROFILE OF *A.BAUMANNII* HOSPITAL ISOLATES

Substrate	MIC (μ g/ml)	β -lactamase inhibitor	Substrate hydrolysis (μ mol)
Ampicillin	512	-	80 \pm 0.04
Ampicillin	512	P-CMB (50mM)	80.2 \pm 0.02
Ampicillin	512	NaCl (100mM)	2.1 \pm 0.01
Ampicillin	512	Sulbactam (200 μ g/ml)	2.5 \pm 0.03
Ampicillin	512	Clavulanic (200 μ g/ml)	2.3 \pm 0.02
Carbenicillin	>512	-	116 \pm 0.02
Carbenicillin	>512	P-CMB (50mM)	116.6 \pm 0.01
Carbenicillin	>512	NaCl (100mM)	3.5 \pm 0.03
Carbenicillin	>512	Sulbactam (200 μ g/ml)	3.0 \pm 0.04
Carbenicillin	>512	Clavulanic (200 μ g/ml)	3.6 \pm 0.01
Penicillin-G	>512	-	83.1 \pm 0.03
Penicillin-G	>512	P-CMB (50mM)	50.6 \pm 0.02
Penicillin-G	>512	NaCl (100mM)	0.002 \pm 0.01
Penicillin-G	>512	Sulbactam (200 μ g/ml)	4.0 \pm 0.04
Penicillin-G	>512	Clavulanic (200 μ g/ml)	4.8 \pm 0.02
Cloxacillin	256	-	50.2 \pm 0.01

Notes: The β -lactamase inhibitors were added 15 min. before addition of the substrate.

$p \leq 0.05$ was considered as significant of association. The above results are mean of two independent experiments

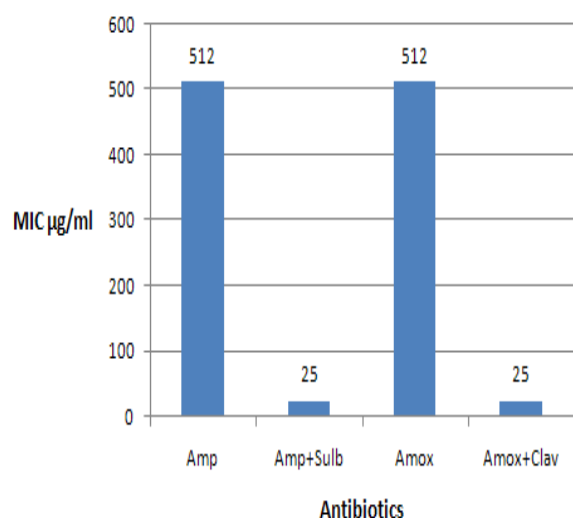


Fig. 2. Comparison between MIC of Ampicillin and Amoxicillin in presence and absence of β -lactamase inhibitors Sulbactam and Clavulanic acid. **Note:** The above results were repeated twice and similar observation was made.

TABLE III
COMPARISON OF β -LACTAMASE PRODUCED BY *A. BAUMANNII* BL54 WITH PSE AND CARB TYPE OF THE ENZYMES BASED ON INHIBITOR PROFILE AND RATE OF HYDROLYSIS OF CLOXACILLIN

β -lactamase type	p-CMB	NaCl	Clavulanic acid	Sulbactam	Cloxacillin hydrolysis
<i>A.baumannii</i> this study	R	S	S	S	50.2
PSE	R	S	R	R	0.0
CARB	R	S	S	S	142

III. DISCUSSION

A. baumannii is intrinsically resistant to most commonly available antibiotics; hence it is able to survive in the hospital environment under hostile conditions and also to colonise in susceptible patients treating with broad spectrum antibiotics. An outbreak of *Acinetobacter* respiratory tract infection resulting from incomplete disinfection of ventilatory equipment was reported by Cefai *et al.*, [12]. Strains that cause infection were liable to be more resistant than colonizing strains. Injudicious use of antibiotics, particularly fluoroquinolones (e.g. Ciprofloxacin) or Carbapenems (e.g. Imipenem) leads to the emergence of more resistant forms of colonising strains. Plasmid mediated β -lactam resistance is very common in *A.baumannii* strains especially those isolated from the hospitals [13]. Various researches conducted for identification of different types of β -lactamase in *Acinetobacter*. Deshpande and Chopade, [14] isolated a plasmid from *Acinetobacter* species which produced cephalosporinase type of β -lactamase and was resistant to Clavulanic acid and Sulbactam. TEM type of β -lactamase which is widely distributed among bacterial populations

reported to be sensitive to both Carbenicillin and Cloxacillin and could not hydrolyse these antibiotics efficiently [15]. In this study a strain of *A.baumannii* isolated from blood sample and was resistant to most penicillin type of antibiotics and produced a CARB type β -lactamase. The enzyme was extracted from the cells and observed on 7.5% polyacrylamide gel electrophoresis. The important characteristic of this enzyme was efficient hydrolysis of Carbenicillin (116 μ mol) and moderate hydrolysis of Cloxacillin (50.2 μ mol). *A.baumannii* isolates showed an impressive inhibitor profile. It was insensitive to hydrolysis of carbenicillin in the presence of p-CMB and Cloxacillin, however, Sulbactam, Clavulanic acid and NaCl substantially inhibited the activity of the enzyme (Clavulanic acid was more effective than the other inhibitors). In previous report in case of *Acinetobacter* [16], it was found that the β -lactamase from *A.calcoaceticus* CCM 5593 hydrolysed cephalosporins better than penicillins. Similarly, Guillou *et al.*, [17] reported the inhibition of β -lactamase activity by Cloxacillin and Sulbactam for various *Acinetobacter* species. Mathew *et al.*, [18] reported that SHV-1 type of β -lactamase enzyme was inhibited by p-CMB when Cephaloridine was used as substrate; however, the enzyme was not inhibited by p-CMB in presence of the Benzylpenicillin. Different studies have already shown that clones of CRAB may spread in a town [19] or even a country [20].

Seven cases of PDR *A. baumannii* from the Thai hospital and 17 cases of MDR and PDR *A. baumannii* [21]. Most (n = 17; 71%) participants had either head trauma or intracranial bleeding and seven (29%) had CNS tumors. Among 23 cases who had neurosurgery, 19 (83%) had craniotomy with external ventricular drain placement; four (17%) had tumor resection; two (9%) had dural grafting, and one (4%) had meningeal prosthesis placement. Risk factors for *A. baumannii* included presence of a CNS IMD (n = 22; 92%), ICU admission (n = 16; 67%), prior antibiotic use (n = 16; 67%), *A. baumannii* colonization (n = 1; 8%) and prolonged hospitalization (mean 22 days; range 4–82 days). The most common antibiotic exposures were cephalosporins (n = 10; 63% of 16 patients), followed by Carbapenems (n = 7; 44%), penicillins (n = 5; 31%), Vancomycin (n = 5; 31%), Fluoroquinolones (n = 5; 31%), Aminoglycosides (n = 4; 25%), and Metronidazole (n = 1; 6%). Analysis of 77 ampicillin-nonsusceptible (resistant plus intermediate categories) strains of *Vibrio cholerae* non-O1, non-O139, isolated from aquatic environment and diarrheal stool [22], showed that all of them produced a β -lactamase an environmental ampicillin-resistant strain from this sample, ME11762, isolated from a waterway in the west region of Argentina, was studied. The nucleotide sequence of the structural gene of the β -lactamase was determined by bidirectional sequencing of a *Sau3AI* fragment belonging to this isolate. The gene encodes a new 288-amino-acid protein, designated CARB-7, that shares 88.5% homology with the CARB-6 enzyme; an overall 83.2% homology with PSE-4, PSE-1 (*Pseudomonas* extended spectrum β -lactamase), CARB-3, and the *Proteus mirabilis* N29 enzymes; and 79% homology with CARB-4 enzyme.

IV. CONCLUSION

From above results it can be concluded that *A. baumannii* hospital isolates were resistant to multiple antibiotics and produced an extended spectrum CARB type β -lactamase. The important characteristic of this enzyme was efficient hydrolysis of Carbenicillin ($\geq 116 \mu\text{mol}$) and moderate hydrolysis of Cloxacillin ($50.2 \mu\text{mol}$). This suggested that the β -lactamase enzyme is penicillinase type. The enzyme exhibited an impressive inhibitor profile. It was insensitive to hydrolysis of carbenicillin in presence of p-CMB and Cloxacillin, however, Sulbactam, Clavulanic acid and NaCl substantially inhibited the activity of the enzyme. Further research is underway in order to determine the mode of expression and regulation of the CARB gene.

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