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Molecular Characterization and Antimicrobial Susceptibility Study of *Acinetobacter baumannii* Clinical Isolates from Middle East, African and Indian Patients

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Abstract

The aim of the present investigation was to characterize the prevalence of extended-spectrum β-lactamases (ESBLs) and metallo β-lactamases (MBLs), and to study the antibiotic susceptibility profile among 250 clinical isolates of *Acinetobacter baumannii*. Phenotypic characterization was carried out by double disc synergy method and the prevalence of ESBLs and MBLs antibiotic resistant determinants were analyzed with Polymerase Chain Reaction (PCR). Susceptibility studies were performed by disc diffusion method according to Clinical and Laboratory Standards Institute guidelines 2009.

Among the two hundred fifty isolates, two hundred nine isolates (83.6%) were positive for ESBLs whereas one hundred sixty seven isolates (79.9%) were positive for both ESBLs and MBLs. Moreover, five isolates (2.3%) which were positive for MBL on disc diffusion test, but negative in PCR showed MBL activity by spectrophotometric assay. Susceptibility study showed that all of the isolates were found to be more susceptible to ceftriaxone plus ethylenediaminetetraacetate plus sulbactam (90-93%), followed by meropenem (50-53%), imipenem (42-45%), cefoperazone plus sulbactam (40-42%), piperacillin plus tazobactam (38-42%) and amoxicillin plus clavulanic acid (28-31%). Among the ESBLs, TEM-types were varied from 82 to 87% followed by SHV-types (67-78%), CTX-M types (60 to 67) and OXA types (51 to 56%) in all of the isolates. Among the MBLs, NDM-1 varied from 40 to 49% followed by IMP-1 (51 to 55%), VIM-1 (55 to 59%) and KPC (47 to 55%) in all of the isolates. Moreover, results of the present study revealed that all of the clinical isolates were susceptible to ceftriaxone plus EDTA plus sulbactam and can be a potent antibacterial agent for the treatment of severe bacterial infections caused by *A. baumannii*.

Keywords: *Acinetobacter baumannii*; Clinical isolates; Susceptibility; Extended-spectrum β-lactamases; Metallo β-lactamases

Introduction

Acinetobacter species are aerobic gram-negative organism being responsible for various types of infections such as pneumonia, urinary tract infection and septicemia [1-4]. The *Acenitobacter* infections have been recognized as an emerging problem and appeared to be associated with high mortality rates throughout the world. Very recently, it has been reported that *Acinetobacter* species accounts for 10% of community-acquired bacteraemia in Kenyan hospital and the prevalence of antibiotic resistance among *Acinetobacter baumannii* isolates in Syria have been increasing in recent years [5,6]. The overall prevalence of nosocomial infections in hospital intensive care units due to *A. baumannii* varies from 2 to 10% [7]. The infections caused by *Acinetobacter* are often treated with cephalosporins including ceftazidime, ceftriaxone, aminoglycosides such as tobramycin and amikacin, carbapenems, and tetracycline. However, to date, most strains of *A. baumannii* have become increasingly resistant to almost all these currently available antibacterial agents [4].

According to a surveillance study conducted at 40 centers in 12 countries revealing a substantial increase in resistance rates in *Acinetobacter* species for meropenem (43.4%) and imipenem (42.5%) [8]. The prevalence of imipenem resistance in *A. baumannii* isolated from a burns unit of United State America was found to be 87% [9]. Similarly, according to a surveillance study conducted in several regions of Greece between 1996 and 2007, *A. baumannii* shows resistant to imipenem up to 85% (ICUs), 60% (medical wards), and 59% (surgical wards) [Greek System for Surveillance of Antimicrobial Resistance (GSSAR): http://www.mednet.gr/whonet/].

In India, it has been demonstrated that approximately 35% *Acinetobacter* species are found to be resistant to carbapenem drugs and the prevalence of carbapenem resistance is increasing greatly in *Acinetobacter* species [3,10]. Among the factors thought to contribute antibiotic resistance development in *A. baumannii,* extended-spectrum β-lactamase (ESBL) production [11], metallo-β-lactamases (MBLs) production [10,11] are predominant. Szabo et al. [11] reported that MBLs cause resistance to all β-lactam antibiotics except monobactam.

In view of the above data, the increasing rate of the antibiotic resistance and its impact on treatment failure compelled us to think a new means by which the increasing mortality rate because of failure of drug therapy can be controlled. We studied the susceptibility of different antibiotics and compared it with a newly launched patent protected drug having a non-antibiotic adjuvant along with β-lactam and β-lactamase inhibitor which altogether termed as ceftriaxone plus ethylenediamine tetraacetic acid disodium (EDTA) plus sulbactam. Therefore, the aim of this study was to characterize the prevalence of ESBLs and MBLs, and to study the antibiotic susceptibility profile among 250 clinical isolates of *A. baumannii* collected from different

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hospitals of India, Kenya, Uganda, and Syria. This work illustrates that ceftriaxone plus EDTA plus sulbactam appear to be most active against both ESBLs and MBLs producing *A. baumannii*.

Materials and Methods

Antimicrobial agents

Ceftriaxone plus EDTA plus sulbactam (Ceftriaxone: Sulbactam :: 2:1 with 10 mM EDTA disodium), amoxicillin plus clavulanic acid (Augmentin, Glaxo Smith Kline, Pharmaceuticals Limited, Mumbai, India), piperacillin plus tazobactam (Zosyn; Wyeth Pharmaceuticals, India, Mumbai), meropenem (Meronem, Astrazeneca Pharma India Limited, Bangalore, India), cefoperazone plus sulbactam (Magnex, Pfizer Limited, Mumbai, India) were used in the study. All the drugs were reconstituted according to the instructions of manufacturer prior to use. Working solutions were prepared using MH broth (Mueller Hinton, Himedia, Mumbai, India) and serial two fold dilutions were made using CAMH (Cation-Adjusted Mueller-Hinton, Himedia, Bombay, India) broth in wells of 96-well plate.

Collection of clinical isolates and their identification

A total of 250 multi-drug resistant clinical isolates of *A. baumannii* collected between January 2010 to April 2012 from different hospitals of India, Kenya, Uganda, and Syria. The isolates were from various clinical specimens such as pus (n=42), blood (n=95), sputum (n=68), urine (n=45). The identity of all strains were reconfirmed by morphologically and conventional biochemical methods [12]. Prior to use, all the samples were inoculated on Mac-Conkey's and blood agar, incubated at 37°C for overnight and colonies were processed. In case of blood sample, blood was incubated at 37°C overnight in brain heart infusion broth. A drop of brain heart infusion broth was inoculated on Mac-Conkey agar and blood agar and incubated at 37°C for overnight. For each isolates, three to five colonies were transferred into 10 ml of cation adjusted Mueller-Hinton broth (CAMHB, Himedia, Mumbai, India) and incubated at 37°C overnight on a rotary shaker at 150 rpm to obtain a planktonic culture in exponential growth phase. This bacterial suspension was used as the inoculum at a concentration of 10^6 colony-forming units (cfu/ml).

Screening of *A. baumannii* clinical isolates for ESBL and MBL

Screening of all clinical isolates was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines [13].

Phenotypic ESBL detection

ESBL production among the clinical isolates was confirmed by phenotypic test [13]. Lawn culture of the organism was made and a disc of 3rd-generation cephalosporins (ceftazidime, cefotaxime and ceftriaxone) and in combination with clavulanic acid disc was placed with 25 mm apart. A ≥ 5 mm increase in a zone diameter for either antimicrobial agents tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL-producing organisms. The clinical isolates which were positive for ESBL by the double disc synergy test but PCR-negative were further tested for ESBL enzyme activity spectrophotometrically as described earlier [14]. *K. pneumoniae* ATCC 700603, (ESBL positive), *Escherichia coli* ATCC 35218 (TEM-1 positive), negative), and *A. baumannii* MTCC 1425 (ESBL negative) strains were used as control throughout the study.

Phenotypic MBL detection

Phenotypic detection of MBLs among the *A. baumannii* clinical isolates was carried out using imipenem (10 µg) and imipenem (10 µg) + EDTA (750 µg) discs as described elsewhere [15]. The test organism was inoculated onto Mueller-Hinton agar (MHA, Himedia, Mumbai, India) and an increase of 7 mm or more in zone diameter in the presence of EDTA compared to imipenem tested alone was considered to be a positive test for the presence of an MBL. The clinical isolates which were positive for MBL by the double disc synergy test but PCR-negative were further tested for MBL enzyme activity spectrophotometrically as described previously [16]. The strains *Klebsiella pneumoniae* BAA-2146, *Klebsiella pneumoniae* BAA 1705 and *Pseudomonas aeruginosa* ATCC 27853 were used as control throughout study.

Preparation of template DNA

5ml of each bacterial culture was centrifuged at 5000 rpm for 5 min at 4°C and resulting pellet was washed once in Tris-EDTA (TE) buffer (Tris-HCl 1.0 M, pH 8.0; EDTA 0.5 M, pH 8.0). After addition of 300 µl of TE buffer, 40 µl of 10% SDS (sodium dodecyl sulfate), 3 µl of 0.5 M EDTA (pH 8.0) incubated for 5 min at 65°C. Following incubation, 750 µl of isopropanol was added and centrifuged at 14000 rpm for 5 min at 15°C. The resulting pellet was resuspended in 500 µl of TE and 2 µl of RNase (10 mg/ml) and incubated at 65°C for 30 min, then added 2 µl of proteinase K (20 mg/ml) and again incubated at 37°C for 15 min. Following incubation, 1 ml of phenol: chloroform (1:1) was added. The upper phase was transferred to another tube and added equal amount of chloroform, shaken well and centrifuged at 14000 rpm for 5 min at 15°C. The supernatant was provided with 40 µl of 5 M Na-Acetate (pH5.2) and 1 ml of ethanol and left at room temperature for 1 h, centrifuged at 7000 rpm for 5 min at 4°C. The DNA pellet was washed with 70% ethanol and suspended in 50 µl of TE buffer. DNA purity and concentration were assayed in a spectrophotometer (260/280).

Detection of ESBLs and MBLs types by PCR

All of the the isolates phenotypically positive for ESBL and MBL were checked for ESBLs and MBLs genotypically by PCR. PCR analysis for β-lactamase and metallo β-lactamase genes was carried out using the previously reported specific oligonucleotide primers shown in the Table 1. All of the primers were procured from Sigma Aldrich Chemicals Private Limited, Banglore, India. For PCR amplifications, about 200 pg of DNA was added to 20 µl mixture containing 0.5 mM of dNTPs, 1.25 µM of each primer and 3.0 U of Taq polymerase (Bangalore Genei) in 1x PCR buffer. Amplification was performed in an Eppendorf thermal cycler (Germany). The amplified products were separated in 1.5% agarose gel containing 4 µl of 10 mg/ml of ethidium bromide. The gel was run at 70 volt for 1 h. The gel images were taken under ultraviolet light using gel documentation system (Bio-Rad, USA). A 100 bp ladder molecular weight marker (Bangalore Genei) was used to measure the molecular weights of amplified products. The images of ethidium bromide stained DNA bands were visualized using a gel documentation system (Bio-Rad, USA).

Antimicrobial susceptibility study

Disc diffusion method: The antimicrobial susceptibility testing of the following drugs were determined by the disc diffusion method according to the Clinical Laboratory Standards Institute guidelines [13]. Antibiotic tested included ceftriaxone plus EDTA plus sulbactam (30:10:15 µg), piperacillin plus tazobactam (100:10 µg), amoxicillin plus clavulanic acid (20:10 µg), cefoperazone plus sulbactam (75:30 µg), imipenem (10 µg) and meropenem (10 µg). The quality control strains were the same which mentioned above. The results were interpreted using the CLSI, 2009 guidelines [13].

Table 1: Oligonucleotides used in the study for each tested genes.

Primer	Primer sequences (5'-3')	Amplicon (base pair)	References
TEM-1	F-TCGGGGAAATGTGCG R-TGCTTAATCAGTGAGGCACC	966	30
TEM-2	F-ATCAGCAATAAACCAGC R-CCCCGAAGAACGTTTTC	516	31
TEM- 50	F-GAAGACGAAAGGGCCTCGTG R-GGTCTGACAGTTACCAATGC	264	32
SHV -1	F- TAAGCGAAAGCCAGCTGTCG R- TTTCGCTCCAGCTGTTCGTC	178	33
SHV-10	F-CCGATAAGACCGGAGTTCGC R-AGTCATATCGCCCGGCAC	248	34
CTXM-9	F-TACCGCAGATAATACGCAGGTG R-CAGCGTAGGTTCAGTGCGATCC	355	35
CTXM- 10	F- CGTGCTTTGTAAAAGTAGCAG R- CATGATTTTGGTGGGAATGG	534	36
CTXM-15	F- AATCACTGCGCCAGTTCACGCT R- GAACGTTTCGTCTCCCAGCTGT	479	35
OXA-11	F- AACTATGATTGGGGATTGAG R- TCAACAAATCGCCAGAGAAG	276	37
NDM-1	F-GGTTTGGCGATCTGGTTTTC R- CGGAATGGCTCATCACGATC	621	38
VIM-1	F-GATGGTGTTTGGTCGCATA R- CGAATGCGCAGCACCAG	390	39
IMP- 1	F- GGAATAGAGTGGCTTAAYTC R- CCAAACYACTASGTTATCT	188	39
KPC-1	F-CTTGCTGCCGCTGTGCTG R- GCAGGTTCCGGTTTTGTCTC	489	40
KPC-2	F- GCTACACCTAGCTCCACCTTC R- GCATGGATTACCAACCACTGT	989	41

F: Sense Primer; R: Antisense Primer

Results

Morphological and biochemical characterization

All of the clinical isolates obtained from various clinical specimens identified as *A. baumannii* based on their morphological and biochemical characterization.

Screening of ESBLs and MBLs

Out of the 250 clinical isolates of *A. baumannii,* 209 (83.6%) were found to be ESBLs positive with ceftazidime/clavulanate and PCR, and 167 isolates were MBL positive as evident through imipenem/EDTA and PCR study. Five clinical isolates (2.3%) which were positive for MBLs on disc diffusion test but negative in PCR showed MBL activity by spectrophotometric assay indicating involvement of other genes which were not included in the study. The overall prevalence of ESBLs and MBLs in *A. baumannii* isolates varied from sample to sample.

Diversity of ESBLs and MBLs

Table 2 shows the prevalence of ESBLs and MBLs in different clinical isolates. TEM-type ESBLs (bla_{TEM-1}, bla_{TEM-2}, and bla_{TEM-50}) were varied from 82 to 87% in all of the isolates obtained from pus, blood, sputum and urine. SHV-type ESBLs (bla_{SHV-1} and bla_{SHV-10}) ranged from 67 to 78%, CTX-M type ESBLs (CTX-M-9 and CTX-M-15) were ranged 60 to 67 and OXA type ESBLs (bla_{OXA11}) varied from 51 to 56%. Among the MBLs, NDM-1 varied from 40 to 49%, IMP-1 varied from 51 to 55%, VIM-1 varied from 55 to 59% and KPC (KPC-1 and KPC-2) ranged from 47 to 55% in all of the isolates.

Antimicrobial susceptibilities of clinical isolates

A significant difference in resistant and susceptibility pattern was observed with the drugs used in the study against the clinical isolates. An antibiogram of the isolates was presented in table 3. Of the 206 isolates of *A. baumannii* tested for their antibiogram, more than 90-93% isolates have shown susceptibility to ceftriaxone plus EDTA plus sulbactam and 7-10% were resistant. Amoxicillin plus clavulanic acid showed the highest percentage of resistance varying from 65 to 76% in various clinical specimens such as, pus, blood, sputum and urine followed by piperacillin plus tazobactam (46-62%), cefoperazone plus sulbactam (51-60%), imipenem (54-58%) and meropenem (46-50%).

Discussion

In recent years, the increasing resistance to antibiotics has alarmed the whole world. Production of β-lactamases has been one of the important resistance mechanism of many bacterial species particularly in the *Enterobacteriacae* [10,17-19]; high prevalence of ESBLs and MBLs producing *A. baumannii* strains has been documented by various groups [3,5,6,10]. In the present study, the percentage of ESBLs and MBLs in *A. baumannii* clinical isolates ranged from 50% to 85% and 40% to 59%, respectively. Previous studies have reported ESBL production varying from 68%-75% [11,20,21], where as MBL producing organism ranged from 28 to 42% [10,19]. Very recently, Jaggi et al. [22] reported that the *A. baumannii* obtained from entire hospital showed 89.6% carbapenem resistance, this resistance increased to 93.2% in ICU clinical samples. Antibiotic resistance in *A. baumannii* is increasing at an alarming rate leading to increased morbidity, mortality at ICU settings as revealed by surveillance studies from Europe, Asia pacific region, Latin America and North America over the last 3-5 years [23].

In the present study, the highest percentage of ESBL producers were observed in isolates obtained from blood 88.42% (84 isolates) followed by sputum 81% (55 isolates), pus 83.33% (35 isolates) and urine 77.77% (35 isolates). Similarly, the highest percentage of occurrence of MBL was reported in blood 74.73% (71 isolates) followed by sputum 61.76% (42 isolates), pus 64.28% (27 isolates) and urine 60% (27 isolates) (Table 3). Roy et al. [24] from India isolated twelve isolates of *A. baumannii* from blood culture of septic neonates during 2007 and 2008 and reported 33.33% isolates were carbapenem resistant.

Table 2: Distribution of ESBLs and MBLs genes among clinical samples of *A. baumannii*.

ESBL positive isolates (n=209)										MBL positive isolates (n=167)				
	TEM-1	TEM-2	TEM-50	SHV-1	SHV-10	CTX-M-9	CTX-M-15	OXA-11		NDM-1	IMP-1	VIM-1	KPC-1	KPC-2
Pus	11	10	8	16	10	11	10	18	Pus	11	15	16	8	7
(n=35)									(n=27)					
Blood	27	28	12	35	31	31	26	44	Blood	35	39	41	16	18
(n=84)									(n=71)					
Sputum	22	18	08	19	18	18	15	31	Sputum	18	23	25	11	12
(n=55)									(n=42)					
Urine	14	13	03	13	11	10	13	19	Urine	13	14	15	6	8
(35)									(27)					

Table 3: Antibiogram of clinical isolates of *A. baumannii.*

S. No	Name of antibiotic	Clinical isolates from pus ((n= 42))				Clinical isolates from blood (n=95)				Clinical isolates from sputum (n=68)				Clinical isolates from urine (n=45)			
		Susceptibility		Resistant		Susceptibility		Resistant		Susceptibility		Resistant		Susceptibility		Resistant	
		No of isolates	%	No of isolates	%	No of isolates	%	No of isolates	%	No of isolates	%	No of isolates	%	No of isolates	%	No of isolates	%
1	Ceftriaxone +EDTA + sulbactam	38	90.47	4	9.5	88	92.63	7	7.36	62	91.11	6	8.82	42	93.33	3	6.66
2	Piperacillin + Tazobactam	16	38.05	24	62	41	43.15	54	56.84	27	39.7	41	60.29	19	42.2	26	57.77
3	Amoxycillin + Clavulanic acid	12	28.57	30	71.42	33	31.57	62	65.26	16	23.52	52	76.47	14	31.11	31	68.88
4	Cefoperazone + Sulbactam	17	40.47	25	59.52	38	40	57	60	29	42.64	39	57.35	18	40.0	27	60.0
5	Imipenem	19	45.23	23	54.76	40	42.10	55	57.89	31	45.58	37	54.41	20	44.44	25	55.55
6	Meropenem	21	50	21	50	47	47.47	48	50.52	37	54.41	31	45.58	24	53.33	21	46.66

The antibiogram clearly showed an increasing resistance of *A. baumannii* to various antibiotics. However, ceftriaxone plus EDTA plus sulbactam was active more than 90% of *A. baumannii* clinical isolates obtained from pus, blood, sputum and urine. Amoxicillin plus clavulanic acid showed the highest percentage of resistance varying from 65 to 76% followed by piperacillin plus tazobactam (46-62%), cefoperazone plus sulbactam (51-60%), imipenem (54-58%) and meropenem (46-50%). Several authors have documented on the reduced susceptibility to imipenem and meropenem among *A. baumannii* isolates [3,10,24-26]. Prakasam et al. [27] demonstrated that *A. baumannii* showed 80.4% resistance against piperacillin plus tazobactam and 63% resistance to meropenem. Another study conducted by Kaul et al. [28] reported the increased carbapenem resistance in gram negative bacilli. Similarly, Srinivasa Rao et al. [29] has reported high level of resistance (> 75%) to both carbapenem and other antibiotic routinely used for the treatment of gram negative bacilli.

Interestingly, ceftriaxone plus EDTA plus sulbactam showed intermediate to resistant response to those strains which were positive with higher classes of TEM and OXA including TEM-50, OXA-11, whereas piperacillin plus tazobactam, amoxicillin plus clavulanic acid, cefoperazone plus sulbactam, imipenem and meropenem were resistant to those isolates were positive with MBL gene including NDM-1, VIM-1, KPC-1, KPC-2, IMP-1 and ESBL genes such as TEM-50 and OXA-11 [21]. However, ceftriaxone plus EDTA plus sulbactam appeared to be highly susceptible to MBL positive genes including NDM-1, VIM-1, KPC-2, IMP-1.

Multi-drug resistant *A. baumannii* bacteria remain an important cause of infection around the world. Ceftriaxone plus EDTA plus sulbactam is active against *A. baumannii* isolates of pus, blood, sputum and urine origin, even when susceptibility to other drugs has been lost. Thus, in case of infection with multidrug-resistant *A. baumannii*, ceftriaxone plus EDTA plus sulbactam can be drug of choice.

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