Original Article

Polyclonal spread of *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ in *Acinetobacter baumannii* isolates from Argentina

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Abstract

Background: In order to study the enzymatic carbapenem resistance mechanisms in *Acinetobacter baumannii* isolates from Argentina, we performed molecular characterization on 41 epidemiologically unrelated strains isolated from 1995 to 2006 with diminished susceptibilities to imipenem and meropenem.

Methodology: *Acinetobacter baumannii* isolates were identified with the ARDRA technique. The total genomic DNA was used to detect each carbapenem β-lactamase gene described so far in this species and those insertion sequences usually associated to carbapenem β-lactamase genes (IS*Aba1*, *2*, *3*, *4* and IS*18*) by the PCR technique with specific primers.

Results: 26 out of 41 *Acinetobacter baumannii* isolates with diminished susceptibilities to carbapenems harboured the *bla*$_{OXA-23}$ gene. The *bla*$_{OXA-58}$ was detected in 13 out of 41 isolates. IS*Aba1* was always located upstream *bla*$_{OXA-23}$. All isolates containing the *bla*$_{OXA-58}$ gene showed IS*Aba3* downstream of the carbapenemase, while 4 isolates had a second copy of the IS*Aba3* upstream of the gene.

Conclusion: Enzymatic carbapenem resistance in *Acinetobacter baumannii* was found in 88% of 41 non-epidemiologically-related strains mediated by the polyclonal spread of the *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ genes. The genetic structures surrounding the oxacillinase genes found in our bacterial isolates revealed a particular epidemiology in our geographical region. This data suggests the need of local molecular surveillance to help control multirresistance *Acinetobacter baumannii* infections.

Key Words: *Acinetobacter baumanni*, carbapenemases, insertion sequence.

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Introduction

Acinetobacter baumannii (AB) is an opportunistic pathogen that causes a wide variety of serious infections that frequently occur in severely ill intensive care unit (ICU) patients with chronic illnesses or prolonged hospitalizations [1]. As a distinctive feature, it is a multidrug resistant species with the ability to acquire determinants of resistance to several antimicrobial agents, including β-lactam mechanisms [1]. Infections are often difficult to treat since carbapenems are now almost always the drug of choice for the treatment of *Acinetobacter* infections.

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) strains were first isolated from clinical samples in South America during the 1990s [2]. Nosocomial infection outbreaks caused by CRAB isolates are now documented worldwide [3]. Recent reports also showed that the spread of the β-lactamase-mediated carbapenem resistance is the most common mechanism found in CRAB isolates carried out by the class B (MBL) (IMP-like, VIM-like, and SIM-1 enzymes) or carbapenem-hydrolyzing class D β-lactamases (CHDLs) (OXA-23, -24, -51 and -58 -related families) [3].

The widespread dissemination of *bla*$_{OXA-23}$ among different clones of *A. baumannii* in

Colombia and Brazil has been documented [4,5]; previous studies from our laboratory also showed that the *bla*$_{OXA-51}$-type genes are ubiquitous in the AB genome [6]. Moreover, we found that the *bla*$_{OXA-51}$-type alleles varied within a strain and were found in different PFGE clones, either susceptible or resistant to carbapenems [6]. Recently, it has been observed that the insertion of IS*Aba1* upstream of both genes may provide a promoter to enhance gene expression, potentially contributing to increase the levels of resistance to carbapenemes [1,7]. The goal of the present study was to survey the presence of enzymatic mechanisms in 41 epidemiologically unrelated AB isolates with diminished susceptibilities to carbapenems.

Materials & Methods

Bacterial strains and growth conditions

A total of 41 epidemiologically unrelated AB isolates were collected from 1995 to 2006 in 3 hospitals in Buenos Aires City, Argentina (Table 1). Most of them were obtained from respiratoty secretions (*n* = 16) and blood (*n* = 15) samples from ICUs, but also from catheters (*n* = 5), urine (*n* = 3) and others (*n* = 2). The isolates were identified at species level by phenotypic scheme [8] and amplified ribosomal DNA restriction analysis (ARDRA) that consists of amplification of the 16S rRNA-gene followed by restriction fragment length polymorfism [9]. Isolates were stored at -70º C in Brain Heart Infusion (BHI) (Difco Laboratories, Detroit, USA) and supplemented with 10% glycerol until used.

Antimicrobial susceptibility testing

The MIC to imipenem and meropenem was performed by agar dilution method according to CLSI recommendations (Table 1) [10].

DNA techniques

Total DNA extraction and PCR reactions were performed as described by Sambrook *et al*. [11]. Specific primers used for detecting the carbapenem β-lactamase genes and the insertion sequence (IS) elements are listed in Table 2. The *Taq* polimerase enzyme (Invitrogen, Carlsbad, CA) was used to amplify the different *bla* genes. PCR DNA products were analysed by conventional agarose gel electrophoresis. PCR products were purified using the QIAquick kit according to the manufacturer´s instructions (Qiagen Inc., Studio City, Calif.).

Table 1. Characteristics of *Acinetobacter baumannii* isolates.

Strains	Hospital	Genotype[1]	Year of isolation	*bla*$_{OXA-23}$[2]	*bla*$_{OXA-58}$[3]	MIC of imipenem (µg/ml)[4]	MIC of meropenem (µg/ml)[5]	Masuda Test[6]
AB1	H1	IV	1999	-	+	16	8	+
AB14	H1	I	2000	-	-	16	8	-
AB16	H1	III	2000	-	+	16	2	+
AB21	H1	I	2000	-	-	16	16	-
AB49	H1	III	2000	-	+	8	4	+
AB1400	H1	IV	2000	-	+	8	4	+
AB1420	H1	IV	2000	-	+	16	8	+
AB1504	H1	III	2000	-	-	8	8	-
AB1525	H1	IV	2000	-	+	16	16	+
AB2856	H1	IV	2000	-	+	8	2	+
AB3	H2	IV	2001	+	-	8	4	+
AB4	H2	IV	2001	-	-	8	8	-
AB5	H2	IV	2001	+	-	8	8	+
AB305	H2	I	2002	+	-	64	8	+
AB311	H2	I	2002	+	+	16	2	+
AB315	H2	I	2002	+	-	16	8	+
AB316	H2	I	2002	+	-	16	8	+
AB318	H2	I	2002	+	-	16	8	+
AB320	H2	I	2002	+	-	64	8	+
AB321	H2	I	2002	+	-	64	8	+
AB323	H2	I	2002	+	-	16	8	+
AB341	H2	I	2002	+	-	8	2	+
AB342	H2	I	2002	+	-	8	2	+
AB343	H2	I	2002	+	-	8	2	+
AB344	H2	I	2002	+	-	32	16	+
AB394	H2	I	2002	+	+	32	16	+
AB395	H2	III	2002	+	-	32	16	+
AB396	H2	I	2002	+	-	32	32	+
A123	H3	IV	1995	-	+	8	8	+
A134	H3	IV	1997	-	+	8	8	+
AB171	H3	IV	2005	+	-	16	16	+
AB172	H3	IV	2005	+	-	16	16	+
AB173	H3	I	2005	+	-	16	16	+
AB174	H3	IV	2005	+	-	32	16	+
AB175	H3	I	2005	+	-	32	16	+
AB176	H3	IV	2005	+	-	8	8	+
AB178	H3	IV	2006	+	-	32	16	+
AB179	H3	IV	2006	+	-	32	16	+
AB181	H3	IV	2006	-	+	8	2	+
AB183	H3	I	2006	-	+	8	4	+
AB185	H3	I	2006	+	-	32	16	+

[1]Genotype was determined by PFGE. The clones belonged to clones previously described in hospitals from Argentina, clones I, III, and IV [12]. [2] -, +, negative or positive, respectively, for the presence of *bla*$_{OXA-23}$ by PCR reaction with specific primers. [3] -, +, negative or positive,

respectively, for the presence of bla_{OXA-58} by PCR reaction with specific primers. [4] The MIC was evaluated for imipenem in µg/ml. [5] The MIC was evaluated for meropenem in µg/ml. [6] -, +, negative or positive, respectively, for the microbiological disk assay performed with the method described by Bou *et al*. [13] that is a modification of Masuda method.

Table 2. Primers used in this study.

Primer	Sequence (5′-3′)	Target	Reference
OXA24F	GTACTAATCAAAGTTGTGAA	$bla_{OXA-24,25,26,40}$	Merkier *et al.* [6]
OXA24R	GGAACTGCTGACAATGC		
IMP F	AACCAGTTTTGCCTTACCAT	$bla_{IMP-1,4,5,6,7,9,10,16,18,21}$	Merkier *et al.* [6]
IMP R	CTACCGCAGCAGAGTCTTTG		
IMPA	ATGAAGAAATTATTTGTTTTATG	$bla_{IMP-2,8,10,13,19,20,22,24}$	Riccio *et al.* [21]
IMPB	TTAGTTACTTGGTGATGATG		
VIM-A	TGGGCCATTCAGCCAGATC	$bla_{VIM-1,2,3,4,5,11,8}$	Poirel *et al.* [22]
VIM-B	ATGGTGTTTGGTAGCATATC		
SIM1-F	TACAAGGGATTCGGCATCG	bla_{SIM-1}	Lee *et al.* [23]
SIM1-R	TAATGGCCTGTTCCCATGTG		
GIMF	AGAACCTTGACCGAACGCAG	bla_{GIM-1}	Castanheira *et al.* [19]
GIMR	ACTCATGACTCCTCACGAGG		
OXA51F	ATGAACATTAAAACACTCTTACT	bla_{OXA-51}-type-genes	Merkier *et al.* [6]
OXA51R	TATAAAATACCTAATTGTTC		
OXA23F	CCCGAGTCAGATTGTTC	$bla_{OXA-23,27,49}$	Merkier *et al.* [6]
OXA23R	TCCATCTGGCTGCTCAA		
OXA-58A	CGATCAGAATGTTCAAGCGC	bla_{OXA-58}	Poirel *et al.* [15]
OXA-58B	CGATCAGAATGTTCAAGCGC		
SPM-1A	CTGCTTGGATTCATGGGCGC	bla_{SPM-1}	Poirel *et al.* [24]
SPM-1B	CCTTTTCCGCGACCTTGATC		
ISAba1F	GTTATATCTTATCTTAAACA	IS*Aba1*	This study
ISAba1R	GCTCACCGATAAACTCTCT		
IS 18A	CACCCAACTTTCTCAAGATG	IS*18*	Poirel *et al.* [15]
IS 18B	ACCAGCCATAACTTCACTCG		
ISAba2A	AATCCGAGATAGAGCGGTTC	IS*Aba2*	Poirel *et al* [15]
ISAba2B	TGACACATAACCTAGTGCAC		
ISAba3A	CAATCAAATGTCCAACCTGC	IS*Aba3*	Poirel *et al* [15]
ISAba3B	CGTTTACCCCAAACATAAGC		
IS Aba4 A	ATTTGAACCCATCTATTGGC	IS*Aba4*	Corvec *et al.* [17]
IS Aba4 B	ACTCTCATATTTTTTCTTGG		

Sequencing of all positive reactions was performed on both DNA strands using the ABI Prism 3100 BioAnalyzer equipment. The nucleotide sequences were analyzed using the Genetics Computer Group (GCG) software and the NCBI/NLM Blast V2.0 software (URL: http: //www.ncbi.nlm.nih.gov/BLAST/).

Genotypes were defined by macrorestriction. Genomic DNAs embedded in agarose plugs were obtained as previously described [12]. DNA was digested with 20 U *Apa*I (Promega Corporation, Madison, WI, USA), and digests were separated by PFGE (CHEF-DR III system, Bio-Rad, Richmond, CA, USA). Running conditions were 24 hours at 6 V/cm and 13°C, with pulse time from 1 second to 30 seconds.

Microbiological test for hydrolytic activity

The microbiological disk assay was performed with the method described by Bou *et al*., [13] which is a modification of the Masuda method.

Results

Antimicrobial susceptibility pattern of A. baumannii *isolates.*

Isolates with imipenem or meropenem MIC of 4-8 µg/ml were considered to be low-level resistant in agreement with Afzal-Shah *et al*. [14]. The obtained range of the MIC for imipenem was 8-64 µg/ml and for meropenem was 2-32 µg/ml. The MIC90 for imipenem was 32 µg/ml, and for meropenem was 16 µg/ml.

Study of clonal relationships

Three different clones were delineated in the 41 AB isolates by the PFGE method and they were identified as I, III, and IV [12] (Table 1).

During 1996, the clone IV was recovered with a susceptible imipenem MIC90 of 0.5 µg/ml from H1 [12]. In the present study, the same clone IV was found in some isolates of hospital H3 with an MIC for imipenem of 32 µg/ml (Table 1), and from hospital H1 with an MIC for imipenem of 16 µg/ml (Table 1).

The study of the enzymatic inactivation of imipenem and/or meropenem exhibited 4 non-hydrolizing strains over 41 AB isolates, performed

by a modification of the Masuda method [13] (Table 1).

Detection of carbapenemases by PCR and sequencing

We searched for the presence of carbapenemase genes within the 41 AB isolates by PCR reactions with specific primers. CHDLs genes were detected in all isolates that were positive for the Masuda test (Table 1). PCR results only indicate the presence of particular genetic determinants but not their expression. Therefore, it must be considered that some of the *bla*$_{OXA}$ genes found in this study could not be expressed, yielding unexpected values of MIC. We found the presence of *bla*$_{OXA-23}$ gene in 26 out of 41 AB isolates (Table 1). The *bla*$_{OXA-58}$, recently characterized [3,15], was also detected in 13 out of 41 isolates. No other carbapenemase gene was found except for the *bla*$_{OXA-51}$-like genes harboured by all AB isolates. Although both genes, *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$, were found in the AB394 and AB311 isolates that belong to clone I, the MIC values were not increased (Table 1). In this regard, multicopy *bla*$_{OXA-58}$ gene was found as a source of high-level resistance to carbapenems in AB isolates from Italy [16]; we found isolates with a MIC value of $\leq$ 16 µg/ml in our AB collection (n=11), which is in agreement with the presence of only one copy of *bla*$_{OXA-58}$ in these strains [16].

The highest MICs observed in this study (imipenem 64 µg/ml) corresponded to three clone I isolates (AB305, AB320 and AB321) that carry the *bla*$_{OXA-23}$ gene alone. In addition, a MIC of 32 µg/ml for imipenem was obtained for some clones, also carrying the *bla*$_{OXA-23}$ gene. Whereas this may suggest that the *bla*$_{OXA-23}$ gene yields elevated MIC values, we found several AB isolates, also harbouring the *bla*$_{OXA-23}$ gene, showing a MIC value of 8 µg/ml. On the other hand, we found four AB isolates with MIC values of 8-16 µg/ml, which did not exhibit any hydrolyzing activity (Table 1). Our data strongly suggest that the presence of other factors, such as porin enzymatic deficiency and/or efflux mechanism may contribute in a multifactorial way to the carbapenem resistance.

Genetic location of the bla$_{OXA-23}$ *and bla*$_{OXA-58}$ *genes*

Previous studies showed that the CHDLs genes were found in several genetic contexts surrounded by different IS that are responsible for their spreading and regulation [3,15,17]. We searched for the presence of IS*Aba1*, *2*, *3*, *4* and IS*18* upstream and downstream of every CHDL gene identified in our isolates. PCR amplification with specific primers and sequence analysis revealed that IS*Aba1* was located upstream of all *bla*$_{OXA-23}$ genes and upstream of a *bla*$_{OXA-51}$-type gene (AB316), the *bla*$_{OXA-66}$ allele (GenBank nº EF051061). The contribution of IS*Aba1* upstream of the *bla*$_{OXA-66}$ gene to the imipenem/meropenem resistance (CIM values of 16/8 µg/ml respectively) could not be evaluated since the AB316 isolate also harbours the *bla*$_{OXA-23}$ gene. IS*Aba1* was previously described upstream of *bla*$_{OXA-58}$ in one isolate from Turkey [3]. Conversely, we did not find in any case IS*Aba1* upstream *bla*$_{OXA-58}$ in our isolates. All isolates containing the *bla*$_{OXA-58}$ gene showed IS*Aba3* downstream of the carbapenemase (*n*=14), and only 4 isolates (AB1400, AB1420, AB1525, and AB311) had a second copy of the IS*Aba3* upstream of the gene (GenBank nº DQ987830). Previous reports showed that the *bla*$_{OXA-58}$ gene could be found embedded in various genetic platforms [3,15]. Because none of the *bla*$_{OXA}$ genes were found adjacent to IS*Aba2*, IS*18* or IS*Aba4* in the AB isolates from our study, it can be presumed that other unknown structures might be in the boundaries of these CHDLs.

Discussion

AB is one of the most frequently gram-negative bacilli isolated from nosocomial infections in our country [18]. Nowadays, over 80% of AB isolates are resistant to extended-spectrum cephalosporins and the imipenem resistance increased from 5% to 54% in the period 2000-2004 [18]. In this regard, the differences observed in the molecular epidemiology of CHDLs in AB isolates detected in this study when compared to other reports [3,15,17] reveal the need of continuous molecular surveillance in order to prevent a higher dissemination of CRAB epidemic multiresistant clones emphasizing control of barrier precaution measures and antibiotic overuse. Moreover, the data provided at epidemiological and phenotypical level of AB isolates from our country [18], previous molecular studies [2,4,12,15,619,13,16,20] as well as the findings of the present work, suggest that treatment options should be redefined according to local epidemiology.

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