Development of phenotypic and genotypic methods for the detection of carbapenemases in clinical isolates of *A. baumannii* resistant to imipenem

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Abstract

Objectives The aim of this study was to develop different technics to detect and identify the presence of carbapenemases in clinical isolates of *Acinetobacter baumannii* resistant to imipenem.

Methods The study included 58 resistant isolates obtained at Hospital de Santa Marina (Bilbao, Northern Spain) during 2002 belonging to four clones: clone I (19 isolates), II (13), III (3) and IV (1). Phenotypic detection was done using the following methods: Hodge test, Hodge test plus zinc sulphate, EDTA test, and two variants of DDST test (one disk containing EDTA+SMA and double disk EDTA/SMA). Genetic experiments to detect *bla*-OXA 40, *bla*-IMP and *bla*-VIM genes were performed by DNA amplification (primers in table 1).

Results The 91.4% of the isolates bore *bla*-OXA 40 gene; The correlated between the phenotypic and genotypic tests for carbapenemases (Hodge test positive or Hodge plus SO_4Zn test positive) was of 96%.

The 14% of the isolates tested showed positive results for all the phenotypic tests (Hodge test , Hodge plus SO_4Zn test and Double disk synergy test - DDST), but the genotypic tests to detect metallo-ß-lactamases were all negatives. Four isolates showed the presence of VIM-2 gene being all DDST negative.

Conclusions Phenotypic tests were easy to perform but, specially with metallo-ß-lactamase tests the interpretation was rather difficult. The majority of isolates showed carbapenemase activity which correlated with the detection of the OXA-40 gene. Some metallo-ß-lactamase tests were positive but no genetic correspondence was observed; moreover, the four VIM-2 positive isolates found were all negative in the phenotypic tests. This is the first time we detect VIM 2 enzymes in our envirorment.

Introduction

Acinetobacter baumannii and its close relatives are important opportunistic pathogens which play an important role in hospital intensive care units (ICUs), causing a wide spectrum of nosocomial infections, and others, including bacteremia, wound infection, urinary tract infection and meningitis, especially in immunocompromised patients.

The widespread resistance found in these bacteria to the major groups of antibiotics means that such infections are difficult to treat successfully, even with combination therapy. This is caused by multiple mechanisms of resistance that this bacteria presents. Resistance to carbapenems is one of most reliable problem of hospitalized patients.

The number of bacteria with acquired carbapenemases and metallo-ß-lactamases emerged and spread during the early 1990s, and the detection of a considerable number of OXA, VIM and IMP type carbapenemases has been reported in many countries. The fact that most of these enzymes could reside on transmisible elements should increase our concern regending their spread.

The aim of this study was to detect the presence of carbapenemases and metallo-ß-lactameses such as OXA, VIM and IMP types, either by phenotypic and genotypic methods.

Materials and Methods

Clinical isolates 58 clinical isolates of *Acinetobacter baumannii* resistant collected from the Hospital of Santa Marina, Bilbao, Spain during 2002. This hospital is a 200-bed general institution with a great number of elederly people who are recovering from illnesses and with a medium- large stay.

Isolates were previously identified by RAPD-PCR with primers M13 and ERIC2, as clone I (19), clone II (13), clone III (3) and clone IV (1).

Phenotipic tests for carbapenemases The phenotypic characterization of carbapenemases in imipenem-resistant isolates was done by using the **Hodge test**. The suface of Mueller-Hinton agar plate was inoculated with a suspension of *E. coli* 25922 adjusted to one-tenth turbidity of McFarland 0.5. An imipenem disk was placed at the centre of the plate, an imipenem-resistant test strains from the overnight culture plates were streaked heavily from the edge of the disk to the periphery of the plate. The presence of a distorted inhibition zone after overnight incubation was interpreted as modified Hodge test positive.

The experiment was repeated adding zinc sulfate to the Mueller-Hinton agar plate to a final concentration of 70µg/ml in order to measure the metallo-ß-lactamase activity. The distorted area in the inhibition zone was intensified in the presence of a metallobetalactamase producing strain (Fig 2.).

To detect metallo-ß-lactamases producing isolates **imipenem-EDTA disk method** was performed. An overnight culture of the test strain was suspended to the turbidity of a McFarland 0.5 and used to swab inoculate a Mueller Hinton agar plate. Two 10 µg-imipenem disks and a blank filter paper disk were placed on the plate. 10 µl of 0.5 M EDTA was applied to one of the imipenem disk and to the blank disk. After overnight incubation, the presence of an enlarged inhibition zone in the imipenem-EDTA disk, compared with the EDTA disk, was interpreted as positive.

Metallo-ß-lactamase activity was also detected by the **imipenem-EDTA double disk synergy test (DDST)**. The surface of an agar plate was inoculated with 100 µl of an overnight solution of the strain to be tested adjusted to a turbidity of 0.5 McFarland units. Two different disks were placed at the corresponding distance (Fig 1.): one 10µg/imipenem disk, and a blank filter paper disk. A 10 µl of the 6V SMA (300mg/ml) + 4 V EDTA 0.5 M solution were added to this last disk. After overnight incubation the presence of a large synergistic inhibition zone between both disks was interpreted as a positive result. A variant of this method was also done, placing two 10µg/imipenem disks and a blank filter paper disk on the plate. 10 µl of 0.5 M EDTA was applied to one of the imipenem disk and to the blank disk. After overnight incubation, the presence of an enlarged inhibition zone in the imipenem-EDTA disk, compared with the EDTA disk, was interpreted as positive.

Genotypic detection of carbapenemases Isolates were examined by PCR for the presence of carbapenemases using the primers shown bellow (table 1).

PRIMERS AND PROBES	SEQUENCE	REFERENCE
P2 (oxa-40 for)	TTCCCCTAACATGAATTTGT	Bou G. et al (Antimicrob Ag Chemother, 2000)
P1 (oxa-40 rev)	GTACTAATCAAAGTTGTGAA	Bou G. et al (Antimicrob Ag Chemother, 2000)
IMP-F	CTACCGCAGCAGAGTCTTTG	Senda K. et al (J Clin Microbiol, 1996)
IMP-R	AACCGATTTTGCCTTACCAT	Senda K. et al (J Clin Microbiol, 1996)
VIM-Diaf	CAGATTGCCGATGGTGTTTGG	Lombardi G. et al (J Clin Microbiol, 2002)
VIM-Diar	AGGTGGGCCATTCAGCCAGA	Lombardi G. et al (J Clin Microbiol, 2002)
VIM-1upv	GTCGCAAGTCCGTTAGCCCAT	Sevillano E. et al (14th ECCMID Praga, 2002)
VIM-2upv	GATTCTAGCGGTGAGTATCCG	Sevillano E. et al (14th ECCMID Praga, 2002)
OXA-40Leu probe	GGACTGGCCTAGAGCTA	Sevillano E. et al (14th ECCMID Praga, 2002)

TABLE 1.

Results

The results obtained with differents tests are shown in the following table:

	Phenotypic tests				Genotypic tests			
CLON	H1	H2	DDST	TOTAL	OXA	VIM1	VIM2	IMP
I	+	+	+	6	5	0	0	0
	+	+	-	21	21	0	0	0
	+	-	nt	9	9	0	0	0
	-	+	-	2	2	0	0	0
	-	-	nt	3	2	0	1	0
II	+	+	+	1	1	0	0	0
	+	+	-	11	11	0	3	0
	+	-	nt	1	1	0	0	0
III	+	+	+	1	1	0	0	0
	-	-	nt	2	0	0	0	0
IV	-	-	1	1	0	0	0	0

H1: Hodge test **H2**: Hodge test plus SO_4Zn

DDST: double disk synergy test **nt**:no tested

The 91.4% of the isolates were OXA-40 positive; the 96% of the OXA-40 positive result correlated with the phenotypic tests for carbapenemases (H1 or H2 positive).

The 14% of the isolates tested showed positive result for all the phenotypic tests (H1, H2 and DDST), but the genotypic tests to detect metallo-ß-lactamases were all negatives. The four isolates that obtained VIM-2 positive result, were DDST negative.

The 13.8% of the isolates were imipenem-EDTA disk test positive, but no genetic correspondence was observed

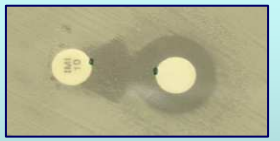

Fig 1. DDST (EDTA plus SMA) positive result

Fig 2. Hodge test plus SO_4Zn positive result

Conclusions

-The majority of isolates that showed carbapenemase activity with the Hodge test, correlated with the detection of the OXA-40 gene by PCR. The 91.4% of *Acinetobacter baumannii* isolates bored *bla*-OXA 40, this gene are widely extended in clinical isolates of *Acinetobacter baumannii*.

-Phenotypic methods were easy to perform but, with metallo-ß-lactamases tests the interpretation was rather difficult. Some metallo-ß-lactamases tests were positive but no genetic correspondence was observed (the four VIM-2 positive isolates found were all negative in the metallo-ß-lactamases test). These results could indicate the presence of a new metallobetalactamase gene, or could be false positive results by phenotypic tests.

-Genetic experiments showed the presence of VIM-2 gene in four isolates of *A. baumannii* resistant to imipenem. This is the first time we detect VIM enzymes in our envirorment.