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Ia Kusradze, Seydina M. Diene, Marina Goderdzishvili, Jean-Marc Rolain. Molecular detection of OXA carbapenemase genes in multidrug-resistant isolates from Iraq and Georgia. *International Journal of Antimicrobial Agents*, 2011, 38 (2), pp.164. 10.1016/j.ijantimicag.2011.03.021 . hal-00711304

**HAL Id: hal-00711304**

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Submitted on 23 Jun 2012

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## Accepted Manuscript

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PII: S0924-8579(11)00179-8  
DOI: doi:10.1016/j.ijantimicag.2011.03.021  
Reference: ANTAGE 3604

To appear in: *International Journal of Antimicrobial Agents*

Received date: 2-2-2011  
Revised date: 22-3-2011  
Accepted date: 28-3-2011

Please cite this article as: Kusradze I, Diene SM, Goderdzishvili M, Rolain J-M, Molecular detection of OXA carbapenemase genes in multidrug-resistant *Acinetobacter baumannii* isolates from Iraq and Georgia, *International Journal of Antimicrobial Agents* (2010), doi:10.1016/j.ijantimicag.2011.03.021

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## Molecular detection of OXA carbapenemase genes in multidrug-resistant *Acinetobacter baumannii* isolates from Iraq and Georgia

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### ARTICLE INFO

#### *Article history:*

Received 2 February 2011

Accepted 28 March 2011

#### *Keywords:*

*Acinetobacter baumannii*

Carbapenem-resistant

OXA carbapenemase genes

NDM-1

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## ABSTRACT

The aim of this study was to determine the susceptibility to imipenem (IPM) of *Acinetobacter baumannii* isolates from different countries and to characterise the carbapenemase-encoding genes in IPM-resistant isolates. A total of 12 *A. baumannii* strains collected in Belgium ( $n = 2$ ), Iraq ( $n = 8$ ) and Georgia ( $n = 2$ ) were included in the study. Identification of the isolates was confirmed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Antibiotic susceptibility testing was performed by the disk diffusion method, and Etest was used to determine the IPM minimum inhibitory concentrations (MICs) of resistant isolates. The presence of carbapenemase-encoding genes was investigated by polymerase chain reaction (PCR). All *A. baumannii* isolates were eventually identified by MALDI-TOF MS with high score values. Among the 12 strains, 6 were found to be resistant to IPM (MICs  $\geq 16 \mu\text{g/mL}$ ), comprising clinical isolates from wound infections of soldiers who were injured either during the Iraq war in 2007 (5 isolates) or during the Georgian–Russian war in 2008 (1 isolate from Georgia). All isolates contained IS*Aba1* and *bla*<sub>OXA-51-like</sub>, but isolates from Iraq contained the *bla*<sub>OXA-23</sub> gene located on a plasmid whereas the isolate from Georgia contained the *bla*<sub>OXA-24</sub> gene located on the chromosome. None of the IPM-resistant isolates contain the *bla*<sub>OXA-58</sub> or *bla*<sub>NDM-1</sub>-encoding genes. In conclusion, these results re-emphasise the worldwide dissemination of OXA carbapenemase genes in multidrug-resistant clinical isolates of *A. baumannii* and, to the best of our knowledge, reports the first IPM-resistant *A. baumannii* strain isolated from a patient during the Georgian–Russian war with the *bla*<sub>OXA-24</sub> gene located on the chromosome.

## 1. Introduction

Over the last decade, *Acinetobacter baumannii* has become a serious and emerging nosocomial pathogen worldwide [1]. There is a wide variety of clinical manifestations of *A. baumannii* infections, including hospital-acquired pneumonia, bloodstream infection, urinary tract infection, meningitis and wound infections (especially in burn units and in traumatic battlefield wounds) [1]. *Acinetobacter baumannii* has an extraordinary ability to become resistant to almost all antibiotics, leading to multidrug-resistant bacteria, through the acquisition of antibiotic resistance-encoding genes by lateral gene transfer [2]. However, until recently most *A. baumannii* isolates remained susceptible to carbapenems, although resistance to these compounds was reported since the early 1990s [1]. Nevertheless, the rapid and worldwide emergence of isolates resistant to carbapenems is now considered as a significant health problem because of limited options for antibiotic treatment [1].

Carbapenemases found in *Acinetobacter* may belong to class B (metalloenzymes: IMP, VIM, SIM) or to class D (OXA enzymes) carbapenemases, the latter being the most frequently encountered worldwide [1]. The OXA enzymes may be divided into four main subgroups, the acquired OXA-23-like, OXA-24-like and OXA-58-like and the chromosomally located intrinsic OXA-51-like associated with IS*Aba1* upstream [1]. Other OXA enzymes recently reported included OXA-143 in *A. baumannii* [3,4] and OXA-134 in *Acinetobacter lwoffii* [5]. Nosocomial outbreaks of imipenem (IPM)-resistant *A. baumannii* producing these OXA enzymes have been reported worldwide: OXA-24-like (OXA-24, OXA-25, OXA-26 and OXA-40) were found in Spain, Belgium, Portugal, Czech Republic, France and the USA; OXA-23-like (OXA-23, OXA-27 and OXA-49) were identified from Europe, Singapore, China, Brazil,

Australia, USA, Algeria, Egypt, Libya, South Africa, Thailand, Tunisia, South Korea, Colombia, Iraq and French Polynesia; and OXA-58-like were identified in France, Spain, Belgium, Turkey, Italy, Austria, Greece, UK, Argentina, Australia, USA, Kuwait and Pakistan [1,6,7]. The *bla*<sub>OXA-51-like</sub> gene was originally found on the chromosome but recently it has been reported that this gene can be located on plasmids and in this case *ISAba1-bla*<sub>OXA-51-like</sub> was enough to confer high-level carbapenem resistance [8]. Finally, the coexistence of *bla*<sub>OXA-23</sub> along with the new *bla*<sub>NDM-1</sub> metallo- $\beta$ -lactamase has been recently demonstrated in clinical isolates of *A. baumannii* from India [9].

The aim of this study was to determine the susceptibility to IPM of *A. baumannii* isolates from different countries and to characterise the carbapenemase-encoding genes in IPM-resistant isolates.

## 2. Materials and methods

### 2.1. Bacterial strains, identification and susceptibility testing

A total of 12 clinical isolates were used in this study. These strains were from the collection of the G. Eliava Institute of Bacteriophages, Microbiology and Virology (Tbilisi, Georgia) isolated from patients with wound infections, including 8 strains from soldiers injured during the Iraq war in 2007, 2 strains from Georgia from soldiers injured during the Georgian–Russian War in 2008 and 2 strains from Belgium also isolated from soldiers. Bacterial strains were grown at 37 °C in brain–heart infusion broth and agar. Bacterial identification to species level was confirmed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

(MALDI-TOF MS) (Autoflex™; Bruker Daltonics, Bremen, Germany) with the Flex control software (Bruker Daltonics) [10]. A score value >1.9 was considered for identification at species level as previously reported [10]. The profiles obtained for each strain were compared and analysed by MALDI Biotyper 2.0 software (Bruker Daltonics) and finally a dendrogram was created based on cross-wise minimum spanning tree matching using the standard settings of the MALDI Biotyper 2.0 software.

Antibiotic susceptibility testing was performed by the disk diffusion method using a panel of 14 different antibiotics, including rifampicin, amikacin, piperacillin/tazobactam, gentamicin, piperacillin, colistin, tobramycin, trimethoprim/sulfamethoxazole, ceftazidime, ticarcillin, ciprofloxacin, norfloxacin, fosfomycin and IPM. Susceptibility breakpoints used were those recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). For IPM, according to EUCAST breakpoints an isolate should be considered as resistant to IPM if the inhibition zone diameter is <17 mm and susceptible if the diameter is ≥22 mm; for isolates with a diameter <17 mm, IPM minimum inhibitory concentrations (MICs) were determined by Etest (AB BIODISK, Solna, Sweden) and resistance was defined as isolates with an MIC for IPM >8 µg/mL.

## 2.2. Molecular detection of carbapenemase-encoding genes and ISAb<sub>1</sub>

Primers used for amplification and sequencing of *bla*<sub>OXA-51-like</sub>, *bla*<sub>OXA-23</sub>, *bla*<sub>OXA-24</sub>, *bla*<sub>OXA-58</sub> and ISAb<sub>1</sub> are given in Table 1. Positive polymerase chain reaction (PCR) products were purified using a QIAquick PCR Purification Kit (QIAGEN, Courtaboeuf, France) and were sequenced using an ABI 3100 Automated Sequencer (Applied



Biosystems, Foster City, CA). Results of sequencing were analysed with software available on the Internet (<http://www.ncbi.nlm.nih.gov>). Real-time PCR was performed to identify the *bla*<sub>NDM-1</sub> gene using primers and TaqMan probe (Table 1).

### 2.3. Plasmid transfer by transformation

Putative plasmid DNA was purified from *bla*<sub>OXA-23</sub><sup>-</sup> and *bla*<sub>OXA-24</sub>-positive *A. baumannii* strains using a QIAprep Spin Miniprep Kit (QIAGEN). Absence of contamination with other nucleic acids, including chromosomal DNA, was verified after elution by agarose gel analysis of eluted plasmid DNA. Purified plasmids were then used to transform IPM-sensitive *A. baumannii* strains using the following steps: 50 µL of recipient strain with 5 µL of plasmid was left in ice for 30 min and was then incubated at 42 °C for 30 s, left for 2 min in ice again, and then 450 µL of Luria–Bertani broth was added and incubated at 37 °C for 1 h. After centrifugation, transconjugants were plated on IPM-containing plates (8 µg/mL) for 24 h at 37 °C for selection of transformants. Colonies growing on IPM-containing plates were checked for the presence of OXA-encoding genes by PCR using specific primers as described above (Table 1).

## 3. Results

### 3.1. Phenotypic properties of the strains

All 12 isolates were eventually identified as *A. baumannii* using MALDI-TOF MS, with score values above 2.2 for all strains. A dendrogram showing the relationship between the isolates is given in Fig. 1, showing that strains could be separated using

this method into at least two clusters (distance level at 500, clusters A and B).

Cluster A contains only one isolate from Georgia (isolate G7), whereas cluster B contains the other strains.

Results of antibiotic susceptibility testing for the strains are summarised in Table 2.

Among the 12 strains, 6 were found to be resistant to IPM (MICs  $\geq 16$   $\mu\text{g/mL}$  confirmed using Etest) (Table 2; Fig. 1) and were clinical isolates from wound infections of soldiers who were injured either during the Iraq war in 2007 (5 isolates) or during the Georgian–Russian war in 2008 (1 isolate from Georgia). These six isolates were also intermediate or fully resistant to the other antibiotics tested, except colistin (Table 2).

### 3.2. Molecular characterisation of imipenem-resistant strains

All 12 strains were checked for the presence of carbapenemase-encoding genes using the PCR methods described above. All of them were positive for *bla*<sub>OXA-51-like</sub> and *ISAbal* genes. The five IPM-resistant strains from Iraq contained the *bla*<sub>OXA-23</sub> gene, whereas the IPM-resistant isolate from Georgia contained the *bla*<sub>OXA-24</sub> gene. Genes *bla*<sub>OXA-23</sub> and *bla*<sub>OXA-24</sub> from these strains were sequenced and the protein sequence was compared with GenBank database using the BLAST tool and were 100% identical to that of GenBank accession nos. HQ700358 and HQ219688 for *bla*<sub>OXA-23</sub> and *bla*<sub>OXA-24</sub>, respectively. All of the remaining susceptible strains did not contain *bla*<sub>OXA-23</sub> or *bla*<sub>OXA-24</sub>-encoding genes. Finally, none of the 12 strains contained either the *bla*<sub>OXA-58</sub> or the *bla*<sub>NDM-1</sub>-encoding genes.

Finally, plasmids from these 12 strains were extracted and purified and the same PCR methods as above were performed, showing that the *bla*<sub>OXA-51-like</sub> gene was present in all plasmids whereas the *bla*<sub>OXA-23</sub> gene was present in five plasmids corresponding to the five IPM-resistant strains from Iraq (Fig. 1). Conversely, PCR for the *bla*<sub>OXA-24</sub> gene was negative for all strains, suggesting that the *bla*<sub>OXA-24</sub>-encoding gene detected in the IPM-resistant strain from Georgia (isolate G7) was chromosomally encoded. Finally, PCR for the *ISAba1* gene was positive for nine plasmids (Fig. 1).

The six plasmids isolated from IPM-resistant strains were used to transform two IPM-sensitive *A. baumannii* isolates (isolate 2 and 280) and were successfully cultured on IPM-containing plates (8 µg/mL).

#### 4. Discussion

Since the first description of the first acquired OXA carbapenemase in *A. baumannii* in 1993, the emergence and spread of acquired OXA enzymes has been well documented worldwide [1]. In this study, we have described six IPM-resistant *A. baumannii* clinical isolates recovered from 12 soldiers injured either during the Iraq war or the Georgian–Russian War in 2008. These six strains were resistant to almost all antibiotics tested except colistin. Interestingly, all isolates were correctly identified by MALDI-TOF, confirming the accuracy of this technique for routine bacterial identification [10]. To the best of our knowledge, this was not previously reported for *A. baumannii* [10]. In the present study, all isolates contained an intrinsic *bla*<sub>OXA-51-like</sub> gene, confirming that detection of this carbapenemase-encoding gene was a reliable means of identification of *A. baumannii* at species level [11]. This carbapenemase is

known to have only weak carbapenem-hydrolysing activity. However, overexpression of this carbapenemase by promoters located in upstream insertion sequences such as *ISAb<sub>1</sub>* may be associated with carbapenem resistance in *A. baumannii* [8]. It has recently been reported that isolates from Taiwan with a plasmid bearing the *ISAb<sub>1</sub>-bla<sub>OXA-51-like</sub>* gene that did not contain other OXA-encoding genes had higher rates of resistance to IPM compared with isolates with a chromosomally encoded *ISAb<sub>1</sub>-bla<sub>OXA-51-like</sub>* gene [13]. In the present study, five of the strains containing a plasmid bearing both *ISAb<sub>1</sub>* and *bla<sub>OXA-51-like</sub>* genes but that did not contain other OXA-encoding genes were still susceptible to IPM (isolates G3, 2, 1, 257 and 10) (Fig. 1). Such discrepancy may be explained by a synergistic effect from other determinants located on the plasmids for the isolates previously reported in Taiwan [13] or by another location of *ISAb<sub>1</sub>* in the plasmids.

In this study, the five isolates recovered from soldiers during the Iraq conflict contained a *bla<sub>OXA-23</sub>* gene that was located on a plasmid. It is well known that the *bla<sub>OXA-23</sub>* gene is one of the most prevalent carbapenemase-encoding genes worldwide, which can be located on the chromosome or plasmids in different genetic structures [1,6]. IPM-resistant isolates recovered from American and British soldiers repatriated from the Iraq conflict have been reported to be associated either with OXA-23- or OXA-58-encoding genes [14]. However, to the best of our knowledge, we report the first clinical isolate of IPM-resistant *A. baumannii* with an OXA-24-encoding gene chromosomally encoded in a soldier who was injured during the Georgian–Russian war in 2008 who was treated in the Military Hospital of Gori, Georgia. The isolate was recovered in January 2009 from sputum during his stay in the Intensive Care Unit and was believed to be of nosocomial origin. The *bla<sub>OXA-24</sub>*

gene may be located either on the chromosome or on plasmids [1]. Interestingly, the isolate in this study contains both a plasmid bearing the *ISAba1* and *bla*<sub>OXA-51-like</sub> genes and a *bla*<sub>OXA-24</sub> gene chromosomally encoded and, interestingly, *A. baumannii* transformants bearing this plasmid only were resistant to IPM as with isolates from Taiwan [13]. The NDM-1 metallo- $\beta$ -lactamase detected recently in Enterobacteriaceae and also in *A. baumannii*, especially in patients from India and Pakistan [15], as well as *bla*<sub>OXA-58</sub> were not detected in any isolates in this study.

These results re-emphasise the worldwide dissemination of OXA carbapenemase genes in multidrug-resistant clinical isolates of *A. baumannii* and, to the best of our knowledge, we report the first IPM-resistant *A. baumannii* strain isolated in a patient during the Georgian–Russian war with the *bla*<sub>OXA-24</sub> gene located on the chromosome.

### **Acknowledgment**

The authors thank Linda Hadjadj for technical assistance.

### **Funding**

This work was partly funded by the Centre National de la Recherche Scientifique (CNRS) (France).

### **Competing interests**

None declared.

### **Ethical approval**

Not required.

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**Fig. 1.** Cross-wise minimum spanning tree (MSP) dendrogram representing the relationship between *Acinetobacter baumannii* strains isolated in different countries. R, resistant (inhibition zone diameter <17 mm); S, sensitive (inhibition zone diameter  $\geq$ 22 mm). Minimum inhibitory concentrations (MICs) determined by the Etest method. <sup>a</sup> Genes detected on a plasmid: +, positive; –, negative.

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**Table 1**

Primers and probe used in the study

Target	Primer name	Primer sequence	Amplicon size (bp)	Reference/source
<i>bla</i> <sub>OXA-51-like</sub>	OXA51 <sub>like</sub> -F	TAATGCTTTGATCGGCCTTG	353	[11]
	OXA51 <sub>like</sub> -R	TGGATTGCACTTCATCTTGG		
<i>bla</i> <sub>OXA-23</sub>	OXA23-F	GATCGGATTGGAGAACCAGA	501	[11]
	OXA23-R	ATTTCTGACCGCATTTCAT		
<i>bla</i> <sub>OXA-24</sub>	OXA24-F	ATGAAAAATTTATACTTCCTATATTCAGC	825	[12]
	OXA24-R	TTAAATGATTCCAAGATTTTCTAGC		
<i>bla</i> <sub>OXA-58</sub>	OXA58-F	AGTATTGGGGCTTGTGCT	453	[12]
	OXA58-R	AACTTCCGTGCCTATTTG		
ISA <sub>ba1</sub>	ISA <sub>ba</sub> -F	CATTGGCATTAAACTGAGGAGAAA	451	[12]
	ISA <sub>ba</sub> -R	TTGGAAATGGGGAAAACGAA		
<i>bla</i> <sub>NDM-1</sub>	NDM1-F	GCGCAACACAGCCTGACTTT	155	This study
	NDM1-R	CAGCCACCAAAGCGATGTC		
	NDM-1 probe	Fam-CAACCGCGCCCAACTTTGGC-TAMRA		

**Table 2**Antibiotic susceptibility of the 12 *Acinetobacter baumannii* clinical isolates

Antibiotic (concentration)	Susceptibility [(inhibition zone diameter (mm))]											
	1 (Iraq)	2 (Iraq)	3 (Iraq)	4 (Iraq)	5 (Iraq)	6 (Iraq)	9 (Iraq)	10 (Iraq)	257 (Belgium)	280 (Belgium)	G3 (Georgia)	G7 (Georgia)
RIF (30 mg/L)	I (17)	I (15)	I (15)	I (15)	I (16)	I (15)	I (16)	I (14)	I (16)	I (16)	I (14)	I (14)
CAZ (30 mg/L)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (8)	R (6)
PIP (75 mg/L)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	I (14)	I (13)	R (8)	R (6)
TZP (85 mg/L)	I (18)	I (15)	R (6)	R (6)	R (8)	R (8)	R (8)	I (16)	I (17)	I (15)	R (10)	R (10)
CIP (5 mg/L)	R (6)	R (14)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (10)
NOR (5 mg/L)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)
FOS (50 mg/L)	R (6)	R (6)	R (6)	R (6)	R (6)	R (7)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)
COL (50 mg/L)	S (16)	S (16)	S (16)	S (16)	S (16)	S (17)	S (16)	S (16)	S (15)	S (15)	S (15)	S (16)
SXT (25 mg/L)	R (6)	I (15)	R (7)	R (7)	R (6)	R (6)	R (8)	R (6)	R (6)	I (10)	R (6)	R (6)
GEN (15 mg/L)	R (7)	S (22)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	S (18)	I (17)	S (20)	R (6)
AMI (30 mg/L)	R (10)	S (17)	R (13)	R (12)	R (10)	R (12)	R (14)	R (10)	R (10)	R (11)	R (10)	R (10)

TOB (10 mg/L)	R (6)	S (18)	R (8)	R (8)	R (6)	R (7)	R (9)	R (6)	S (18)	S (18)	S (18)	R (6)
TIC (75 mg/L)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	R (10)	R (6)	R (14)	R (6)
IPM (10 mg/L)	S (30)	S (32)	R (14)	R (15)	R (14)	R (12)	R (12)	S (25)	S (30)	S (25)	S (26)	R (6)
IPM Etest MIC ( $\mu\text{g/mL}$ )	0.38	0.38	16	32	16	32	16	0.75	1	0.75	0.5	32

RIF, rifampicin; CAZ, ceftazidime; PIP, piperacillin; TZP, piperacillin/tazobactam; CIP, ciprofloxacin; NOR, norfloxacin; FOS, fosfomycin; COL, colistin; SXT, sulfamethoxazole/trimethoprim; GEN, gentamicin; AMI, amikacin; TOB, tobramycin; TIC, ticarcillin; IPM, imipenem; MIC, minimum inhibitory concentration; I, intermediate; R, resistant; S, susceptible.

