



Enfermedades Infecciosas y Microbiología Clínica

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Original article

First identification of *bla*_{NDM-1} carbapenemase in *bla*_{OXA-94}-producing *Acinetobacter baumannii* ST85 in Spain



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ARTICLE INFO

Article history:

Received 26 November 2018

Accepted 8 March 2019

Keywords:

A. baumannii
NDM-1
OXA-94
ST85

ABSTRACT

Introduction: NDM-1 carbapenemase is spreading rapidly all over the world, but this metallo-beta-lactamase has just been detected for the first time in an *Acinetobacter baumannii* (Ab) isolate of the ST85 clone in Spain. The aim of this study was to characterize a NDM-1-producing carbapenem-resistant *A. baumannii* (CR-Ab) isolate submitted to the Andalusian PIRASOA [infection prevention program] referral laboratory.

Methods: Carbapenemases were detected by PCR and Sanger DNA sequencing. Whole genome sequencing was performed by NGS (Miseq, Illumina). Resistance genes were identified with RESfinder, while MLSTfinder was used for sequence typing (ST). The genetic location of *bla*_{NDM-1} was determined by nuclease S-1/PFGE/hybridization with specific probe.

Results: The isolate was susceptible to amikacin and tigecycline and belonged to the ST85 clone. *bla*_{OXA-94} and *bla*_{NDM-1} were identified by PCR and Sanger DNA sequencing, respectively. The resistance genes *aadB*, *bla*_{ADC-25}, *bla*_{NDM-1}, *bla*_{OXA-94}, *msr*(E), *mph*(E) and *floR*, *sul2* were identified by NGS. The chromosome of the isolate contained a defective Tn125 transposon with *bla*_{NDM-1} flanked by the insertion sequences ISAbA125 and ISAbA14. The *bla*_{NDM-1} gene was only detected in the chromosomal DNA.

Conclusion: This is the first time that *bla*_{NDM-1} has been detected and characterized in a *bla*_{OXA-94}-producing CR-Ab isolate belonging to the ST85 clone in Spain.

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Detección por primera vez en España de la carbapenemasa *bla*_{NDM-1} en *Acinetobacter baumannii* ST85 productor de *bla*_{OXA-94}

RESUMEN

Introducción: La carbapenemasa NDM-1 se está diseminando rápidamente por todo el mundo, pero esta metalo-beta-lactamasa se detecta por primera vez en un aislado de *A. baumannii* del clon ST85 procedente de España. El objetivo de este estudio es caracterizar un aislado de *A. baumannii* resistente a carbapenémicos productor de NDM-1 remitido al laboratorio de referencia PIRASOA de Andalucía.

Métodos: La detección de carbapenemasas se realizó mediante PCR y secuenciación de ADN Sanger. La secuenciación del genoma completo se realizó mediante NGS (MiSeq, Illumina). La detección de genes de resistencia y el secuenciotipo (ST) se obtuvo mediante ResFinder y MLSTfinder, respectivamente. La localización de *bla*_{NDM-1} se determinó utilizando el método de la nucleasa S1/PFGE/hibridación con sonda específica.

Palabras clave:

A. baumannii
NDM-1
OXA-94
ST85

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Resultados: El aislado era sensible a amikacina y tigeciclina, y pertenecía al clon ST85. Se identificaron las variantes *bla*_{OXA-94} y *bla*_{NDM-1}, respectivamente, mediante PCR y secuenciación Sanger. Mediante secuenciación masiva se detectaron los genes de resistencia *aadB*, *bla*_{ADC-25}, *bla*_{NDM-1}, *bla*_{OXA-94}, *msr*(E), *mph*(E), *floR* y *sul2*. El aislado contenía en su cromosoma un transposón defectivo de tipo Tn125 con *bla*_{NDM-1} flanqueado por las secuencias de inserción ISAbA125 y ISAbA14. El gen *bla*_{NDM-1} solo se detectó en el ADN cromosómico.

Conclusión: En este estudio se detecta y se caracteriza por primera vez en España *bla*_{NDM-1} en un aislado de *A. baumannii* resistente a carbapenémicos productor de *bla*_{OXA-94} y perteneciente al clon ST85.

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Introduction

Acinetobacter baumannii is a successful pathogen characterized by its ability to acquire multidrug resistance (MDR) and to cause nosocomial outbreaks. Resistance to carbapenems in *A. baumannii* has increased in recent years and has been related to the production of carbapenemases, alterations in porins, overexpression of efflux pumps and altered penicillin-binding proteins.¹ The main mechanism of resistance to carbapenems in *A. baumannii* is the acquisition of genes coding for carbapenem-hydrolyzing class D beta-lactamase (CHDL), also known as oxacillinases (*bla*_{OXA-23}, *bla*_{OXA-24/40}, *bla*_{OXA-58}, *bla*_{OXA-143} and *bla*_{OXA-235}).² The role of the naturally occurring chromosomal *bla*_{OXA-51}-like oxacillinases (i.e. *bla*_{OXA-51}, *bla*_{OXA-66}, *bla*_{OXA-69}, *bla*_{OXA-71}, *bla*_{OXA-94}) in the resistance to carbapenems is unclear. Class B beta-lactamases (metallo-beta-lactamases; MBL) (i.e. *bla*_{VIM}-like) are generally much less frequent than oxacillinases in carbapenem-resistant *A. baumannii*. Nevertheless, MBLs tend to hydrolyze carbapenems more efficiently than oxacillinases and have a broader spectrum of hydrolytic activity (including expanded-spectrum cephalosporins and aztreonam).^{2,3} Some of these MBLs, like the New Delhi metallo-β-lactamase-1 (NDM-1), are rapidly spreading worldwide in gram-negative bacteria, which represent a serious problem from a clinical and an epidemiological point of view.^{4,5} In *A. baumannii*, the gene encoding NDM-1 has been detected on the chromosome and, less frequently, in plasmids from isolates from different countries around the world.⁶ Since this is the first time that NDM-1-producing *A. baumannii* has been detected in Spain, we decided to (i) characterize this isolate; (ii) determine the genetic localization (chromosomal or plasmidic) of *bla*_{NDM-1} and (iii) analyze its genetic environment.

Material and methods

Bacterial isolate

A carbapenem-resistant *A. baumannii* isolate obtained from a rectal swab (Table 1 shows the most relevant clinical features) from a patient in April 18, 2017 was submitted to the Andalusian reference laboratory for multidrug-resistant nosocomial pathogens (PIRASOA program, Seville, Spain) for characterization (see below).

Bacterial identification, antimicrobial susceptibility testing and molecular typing

Identification of genomic species of *Acinetobacter* was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MALDI-TOF MS (MALDI Biotyper CA system; Bruker Daltonics, Madrid, Spain). Detection of *bla*_{OXA-51} was performed by PCR,⁷ whole genome sequencing (WGS) and the SpeciesFinder 1.2 tool (<https://cge.cbs.dtu.dk/services/SpeciesFinder/>).

Antimicrobial susceptibility testing was performed with the Microscan Neg MIC Panel, Type 44 (Beckman Coulter, Inc, Madrid, Spain). Imipenem and meropenem susceptibility testing was performed by Etest (LioChem Inc, Madrid, Spain) and disk diffusion (Oxoid, Madrid, Spain) in Mueller-Hinton agar plates (Oxoid). Susceptibility to colistin was tested by microdilution using the UMIC kit (Biocentric; Bandol, France).

The isolate was genotyped by PFGE using the restriction enzyme *Apal*,⁸ multilocus sequence typing (MLST) was performed according to the Institut Pasteur scheme (<https://pubmlst.org/abaumannii/>) and using WGS (see below) and the MLST 1.8 tool (<https://cge.cbs.dtu.dk/services/MLST/>).

Detection of antimicrobial resistance genes

Phenotypic detection of carbapenemases was performed with the combination disk test (Rosco, Madrid, Spain) according to EUCAST guidelines.⁹

Detection of genes coding for carbapenemases was carried out by PCR using specific primers for class A (*bla*_{KPC}), B (*bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}) and D (*bla*_{OXA-23}, *bla*_{OXA-24/40}, *bla*_{OXA-58}, *bla*_{OXA-48}) carbapenemases.^{7,10} Allelic variants positive by PCR were determined by Sanger sequencing (Macrogen, Madrid, Spain). Other genes associated with resistance to antimicrobials were also identified using WGS (see below) and the ResFinder 3.0 tool (<https://cge.cbs.dtu.dk/services/ResFinder/>).

Conjugation and transformation assays

Conjugation experiments were carried out in Luria-Bertani broth (LB, Oxoid) with sodium azide-resistant *Escherichia coli* J53 and *Acinetobacter baylyi* (MIC of rifampicin 16 mg/L) as the recipients. Transconjugants were selected by plating onto LB agar plates (Oxoid) supplemented with 100 mg/L of sodium azide and 0.125 mg/L of ertapenem (Sigma, Madrid, Spain) using *E. coli* J53 as recipient, and 8 mg/L of rifampicin (Sigma, Madrid, Spain) and ertapenem, respectively, using *A. baylyi* as recipient.

Plasmid DNA was extracted by the Kieser method, electroporated into *E. coli* DH10B and plated on MacConkey agar (Becton Dickinson, Madrid, Spain) supplemented with ertapenem at 0.125 mg/L.¹¹

Whole genome sequencing

The genome of the isolate was sequenced with next-generation sequencing (NGS) using the Illumina MiSeq system. The sample library was prepared with the Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). DNA sequencing was carried out with the MiSeq Reagent Kit V3 (600 cycles) and the Illumina MiSeq sequencer (2×300 paired end reads). The reads were quality filtered. De novo assembly and gene annotation was performed using the CLC Genomics Workbench v10 (Qiagen) and

Table 1
Relevant clinical features.

Age/gender	Nationality	Admission			Type of infection	Antimicrobial therapy ^a	Antimicrobial therapy ^b
		Date	Hospital	Service		Start: February 09, 2017 Finish: February 17, 2017	Start: February 23, 2017 Finish: February 27, 2017
64/F	Spanish	January 21, 2017	Hospital Puerta del Mar (Cádiz, Spain)	ICU	Bilateral bronchopneumonia	Colistin plus amikacin	Amoxicillin-clavulanic acid

^a Inhaled colistin (200 million of IU every 12 h); IV amikacin (1 g every 24 h).^b Amoxicillin-clavulanic acid (1 g every 8 h).

the RAST server (<http://rast.nmpdr.org/>), respectively. Characterization of the resistome was carried out using the ResFinder server (<http://cge.cbs.dtu.dk/services/ResFinder-3.0>).

Plasmid analysis and Southern hybridization

Plasmid size was determined by electrophoresis using a 0.7% agarose gel and *E. coli* 50192 harboring 154, 66, 48 and 7 kb plasmids as size markers. The genetic localization of *bla*_{NDM-1} was determined by Southern blotting. Genomic DNA was digested with nuclease S1 (Roche, Madrid, Spain), separated by PFGE electrophoresis, transferred to a positively charged nylon membrane (Amersham Hybond-N+, Madrid, Spain) and hybridized with a digoxigenin-labeled *bla*_{NDM-1} probe.

Results

The isolate (Ab-NDM-1) was identified by MALDI-TOF MS and the SpeciesFinder 1.2 tool as *A. baumannii*, and was positive for *bla*_{OXA-51} by PCR. MICs obtained by microdilution using Type 44 panels are shown in Table 2. Etest MICs were >32 mg/L for imipenem and meropenem, and 1 mg/L for tigecycline. The isolate was susceptible only to amikacin according to EUCAST and CLSI breakpoints of 2018.^{12,13} The MIC of colistin was >64 mg/L using the UMIC kit.

The isolate was assigned to ST85 by MLST and the MLSTFinder 1.8 tool, and was clonally unrelated (more than 5 band differences) to previous pulsotypes of carbapenem-resistant *A. baumannii* in the

Andalusian database of the PIRASOA program reference laboratory. Synergy was observed between the meropenem disks and the dipicolinic acid disks. *bla*_{OXA-51-type} and *bla*_{NDM-type} genes were detected by endpoint PCR, and were identified as *bla*_{OXA-94} and *bla*_{NDM-1}, respectively, by Sanger sequencing.

After trimming, 578,845 reads were obtained by WGS. The average length of these reads was 260.3 bp and GC content was 39.5%. After assembly, the draft genome consisted of 316 contigs, with mean length of 22,375 bp and mean sequencing coverage of 53, yielding a total read length of 3,950,783 bp. After genome annotation using Rapid Annotations using Subsystems Technology (RAST) server (<http://rast.theseed.org/FIG/rast.cgi>), a total of 3703 coding sequences and 78 RNAs were obtained.

The acquired antimicrobial resistance genes identified in the assembled contigs with the ResFinder 1.2 tool were associated with resistance to aminoglycosides (*aadB*, which confers resistance to gentamicin and tobramycin), beta-lactams (chromosomal cephalosporinase *bla*_{ADC-25}, class B carbapenemase *bla*_{NDM-1} and the OXA-51-type CHDL, *bla*_{OXA-94}), macrolides, lincosamides and streptogramins [*msr*(E), *mph*(E)], phenicols (*floR*) and sulfonamide (*sul2*). The resistome for the most relevant group of antimicrobials is shown in Table 2. WGS revealed that *bla*_{NDM-1} was located on a defective transposon (Δ Tn125) flanked by the insertion sequences ISAb125 and ISAb14 inserted in the upstream and downstream regions, respectively (Fig. 1).

With respect to chromosomal mechanisms related to carbapenem resistance, mutations in the porin genes, *carO* and *oprD-like*, were not detected, whereas several point mutations (V90I, H127K, S168G, T173S, D183N, S236T, S243E and V270L) were observed in the *omp33-36* gene. No mutations were detected in genes coding for the two-component regulatory system *adeR-adeS*, which regulates the expression of genes encoding the efflux pump *adeABC*.

With respect to colistin resistance, acquired resistance genes (*mcr-1*) or mutations in the chromosomal genes *pmrA*, *lpxA*, *lpxC* and *lpxD* were not detected. Table 2 shows the aminoacid changes detected in *pmrB* and *pmrC*.

The isolate carried three different plasmids, which were ~150 kb, ~37 kb and ~6 kb in size, respectively. No transconjugants or transformants producing NDM were obtained. The *bla*_{NDM-1} probe hybridized to chromosomal DNA, but not to any of the three plasmids observed.

Nucleotide sequence accession number

This draft genome project has been deposited registered at GenBank (accession number QBY00000000.1) with the BioProject ID PRJNA449628 (<http://www.ncbi.nlm.nih.gov/bioproject/449628>).

Table 2
MICs and resistome of the NDM-1 producing *A. baumannii* isolate.

Antimicrobial class	Antimicrobial agent	MIC (mg/L)	Resistance genes/mutations ^a
β-Lactam	Piperacillin/tazobactam	>64	<i>bla</i> _{ADC-25} <i>bla</i> _{OXA-94} <i>bla</i> _{NDM-1}
	Ampicillin/sulbactam	>16/8	
	Ceftazidime	>16	
	Cefepime	>16	
	Imipenem	>8	
	Meropenem	>8	
Aminoglycoside	Gentamicin	>8	<i>aadB</i>
	Tobramycin	>8	
	Amikacin	≤8	
Fluoroquinolone	Levofloxacin	>4	<i>gyrA</i> (S81L), <i>gyrB</i> (A265T) <i>parC</i> (S84L , G569S), <i>parE</i> (V237A)
	Ciprofloxacin	>2	
Polymyxin	Colistin	>4	<i>pmrC</i> (I115V, N284D, I326T) <i>pmrB</i> (T187P, A227V)

^a In bold are indicated the mutations described in other studies associated with colistin resistance.

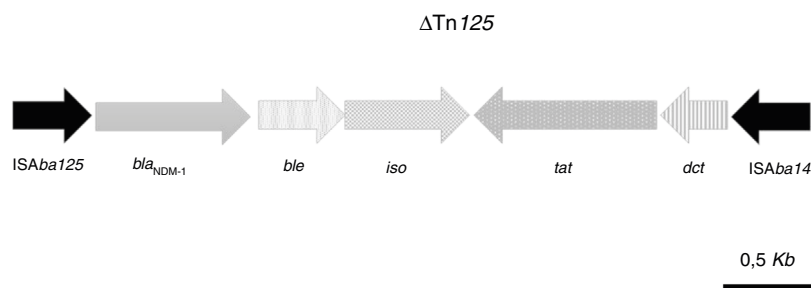


Fig. 1. Schematic representation of the genetic environment of *bla_{NDM-1}*: *ble* gene, encoding the bleomycin resistance protein; *iso* gene, encoding a putative phosphoribosyl-anthranilate isomerase; *tat* gene, encoding oxidoreductase DsbD superfamily protein; *dct* gene, encoding divalent cation tolerance protein.

Discussion

In countries all over the world, the acquired carbapenemase NDM-1 is spreading efficiently in several gramnegative microorganisms in nosocomial and community-acquired infections, which poses a major challenge for the treatment and control of healthcare-associated infections.^{4,5,14,15} In *A. baumannii*, this MBL has been detected in carbapenem-resistant isolates in different countries of Europe (Switzerland, Slovenia, Germany, France, Belgium, Czech Republic, Turkey), Latin America (Colombia), Africa (Algeria, Ethiopia, Syria,) and Asia (India).^{15–21} In Spain, NDM-1-producing *A. baumannii* has not been reported before.

Isolate Ab-NDM-1 showed cross-resistance with other antimicrobial families (quinolones and some aminoglycosides) consistent with other NDM-1-producing isolates in other countries. This reduces the therapeutic options for infection to certain toxic antimicrobials such as colistin.¹⁴ Although this isolate lacked some of the most frequently described CHDL genes in *A. baumannii* (OXA-23-like, OXA-24/40-like, OXA-58-like), carbapenem resistance is fully explained by the acquisition of *bla_{NDM-1}*. Nevertheless, additional carbapenem resistance mechanisms, probably related to the Omp 33–36 porin, may be implicated, as described in *A. baumannii* isolates in other studies.^{22–24} Although some of the amino acid changes observed in Omp 33–36 of Ab-NDM-1 could be associated with decreased susceptibility to carbapenems, additional studies with isogenic mutants are required to understand their role in carbapenem resistance.

The absence of mutations in the regulatory genes *adeS* and *adeR* suggests that the efflux pump *adeABC*, implicated in *A. baumannii* resistance to some antimicrobials and biocides, is not overexpressed in Ab-NDM-1, which is in agreement with the absence of resistance to tigecycline.^{25,26}

Isolate Ab-NDM-1 also showed resistance to colistin, which was associated with some aminoacid changes in *pmrC* and particularly in *pmrB* (i.e. A227V). The implication of other less frequent or undescribed mechanisms of colistin resistance related to the synthesis of lipid A cannot be ruled out.²⁷

In *Enterobacteriaceae*, *bla_{NDM-1}* is frequently plasmid encoded, whereas it is more frequently found on the chromosome in *A. baumannii*, although it has also been detected on plasmids.^{6,15,16} Similar genetic environments have been detected harboring *bla_{NDM-1}* in *Enterobacteriaceae* and *A. baumannii*, indicating horizontal transmission (plasmid transfer) of this carbapenemase, probably from *Enterobacteriaceae* to *A. baumannii*.²⁸

The carbapenemase *bla_{NDM-1}* was detected on the chromosome of Ab-NDM-1, but not in any of the three plasmids observed. This finding suggests that the clone must have been introduced into the Hospital Puerta del Mar by clonal dissemination, rather than by plasmid transfer from a NDM-1-producing gramnegative isolate not previously detected in this center. Another possible hypothesis

that was not investigated in this study is phage-mediated transfer of *bla_{NDM-1}*, as has been described among isolates of *A. baumannii*.⁶

Tn125-like transposons are the most frequent genetic platforms for mobilization of *bla_{NDM-1}*.¹⁵ In Ab-NDM-1 isolates, the *bla_{NDM-1}* carbapenemase is associated with a truncated transposon (ΔTn125) containing a genetic environment similar or identical to those previously described in other carbapenem-resistant NDM-1- and OXA-94-producing *A. baumannii* isolates, particularly those belonging to the ST85 clone previously detected in some countries (France, Algeria, Turkey, Syria, Tunisia) (19–22,30).^{18–21,29} In Spain, the *A. baumannii* ST85 clone has only been detected in OXA-23-producing carbapenem-resistant isolates causing nosocomial outbreaks.³⁰

Tn125-like transposons have also been detected in *A. baumannii* clones other than ST85, such as clone ST25 (in Slovenia, Germany, Lebanon), which is not related to CC92/CC2, but included in a different CC together with the ST6 clone belonging to international clonal lineage I (in Switzerland).^{18–21}

The patient with Ab-NDM-1 made no reference to any history of travel to other countries where NDM-1-producing bacteria are prevalent, so that the source of this Ab-NDM-1 isolate in the Hospital Puerta del Mar remains unclear. At the same time, contact between this patient and other patients or healthcare personnel colonized with NDM-1-producing gramnegative microorganisms cannot be ruled either.

This study has some limitations. One of them is the lack of epidemiological and clinical data about other patients admitted to the same center, which makes difficult to investigate potential reservoirs and transmission routes, or the country of origin of the Ab-NDM-1 isolate.

In conclusion, to the best of our knowledge, this is the first time that *bla_{NDM-1}* has been detected in *bla_{OXA-94}*-producing carbapenem-resistant *A. baumannii* belonging to the ST85 clone in Spain.

Funding

This work was partially funded by a grant (PI11/02046) from the Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad.

Conflict of interest

None to declare.

Acknowledgements

This work was presented in part at the 2018 ECCMID in Madrid (Poster 1410).

This work was supported by the Plan Nacional de I+D+I 2013–2016 and the Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI; RD16/0016/0001)-co-financed by European Development Regional Fund “A way to achieve Europe”, Operative program Intelligent Growth 2014–2020.

We would like also to thanks to María del Mar Tomás Carmona (Hospital de A Coruña, Spain) for her kindness in giving us a strain of *Acinetobacter baylyi*.

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