Emergence and spread of carbapenem-resistant *Acinetobacter baumannii* international clones II and III in Lima, Peru

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Abstract

Carbapenem-resistant *Acinetobacter baumannii* is the top-ranked pathogen in the World Health Organization priority list of antibiotic-resistant bacteria. It emerged as a global pathogen due to the successful expansion of a few epidemic lineages, or international clones (ICs), producing acquired class D carbapenemases (OXA-type). During the past decade, however, reports regarding IC-I isolates in Latin America are scarce and are non-existent for IC-II and IC-III isolates. This study evaluates the molecular mechanisms of carbapenem resistance and the epidemiology of 80 non-duplicate clinical samples of *A. baumannii* collected from February 2014 through April 2016 at two tertiary care hospitals in Lima. Almost all isolates were carbapenem-resistant (97.5%), and susceptibility only remained high for colistin (95%). Pulsed-field gel electrophoresis showed two main clusters spread between both hospitals: cluster D containing 51 isolates (63.8%) associated with sequence type 2 (ST2) and carrying OXA-72, and cluster F containing 13 isolates (16.3%) associated with ST79 and also carrying OXA-72. ST2 and ST79 were endemic in at least one of the hospitals. ST1 and ST3 OXA-23-producing isolates were also identified. They accounted for sporadic hospital isolates. Interestingly, two isolates carried the novel OXA-253 variant of OXA-143 together with an upstream novel insertion sequence (ISAba47). While the predominant *A. baumannii* lineages in Latin America are linked to ST79, ST25, ST15, and ST1 producing OXA-23 enzymes, we report the emergence of highly resistant ST2 (IC-II) isolates in Peru producing OXA-72 and the first identification of ST3 isolates (IC-III) in Latin America, both considered a serious threat to public health worldwide.

Introduction

*Acinetobacter baumannii* is an opportunistic nosocomial pathogen responsible for a broad range of nosocomial infections1, including ventilator-associated pneumonia and bacteremia (35–52% mortality)1,2, as well as skin and soft tissue infections, endocarditis, urinary tract infections, and meningitis1,3. Nosocomial isolates of this bacterium are often resistant to most currently available antibiotics. Carbapenem-resistant *A. baumannii* has recently been considered the most critical pathogen for public health, topping the global priority list of antibiotic-resistant bacteria published by the World Health Organization4. *Acinetobacter baumannii* strains can develop resistance to all the antibiotics available5. Outbreaks caused by multidrug-resistant (MDR), extensively drug-resistant (XDR), and even pan-drug-resistant (PDR) strains having been reported worldwide6–9. In the past decade, resistance rates have been rising, and recent reports show a steady increase in carbapenem resistance among *A. baumannii* strains8,10–13.

Resistance to carbapenems in *A. baumannii* is usually mediated by the expression of carbapenem-hydrolyzing
class D β-lactamases, also known as OXA-type carbapenemases, although the expression of different class B metallo-β-lactamases (MBLs), or even *Klebsiella pneumoniae* carbapenemase (KPC) enzymes, has also been reported. The OXA-type enzymes described in *A. baumannii* belong to six different families, namely the intrinsic OXA-51 oxacillinase family, which is usually chromosomally encoded but has rarely been reported in plasmids, and the acquired OXA-23, OXA-24, OXA-58, OXA-143, and OXA-235 families. Although the population structure of *A. baumannii* strains is quite diverse, there seems to be a clonal spread of a few epidemic lineages that predominate over the rest. In particular, the international clones I–III account for most *A. baumannii* infections worldwide and are usually associated with the production of OXA-23-like, OXA-24-like, or OXA-58-like enzymes.

Our knowledge of the epidemiology and antibacterial susceptibility profiles of *A. baumannii*, however, is still incomplete in many parts of the world, including many countries in Latin America. The present study was designed to evaluate the phenotypic resistance patterns, the presence of carbapenem resistance mechanisms, and the clonal relatedness of *A. baumannii* isolates circulating in Lima, Peru.

**Results**

**Bacterial isolates**

A total of 80 non-redundant *A. baumannii* isolates were recovered from blood (*n* = 59, 73.8%), bronchial aspirate (*n* = 15, 18.8%), cerebrospinal fluid (*n* = 2, 2.5%), and urine samples (*n* = 2, 2.5%) of different patients admitted to two tertiary care hospitals in Lima. Fifty-three isolates were recovered at the Instituto Nacional de Enfermedades Neoplásicas (INEN) from February 2014 through April 2016, and the remaining 27 isolates originated from inpatients at the Hospital Nacional Arzobispo Loayza (HNAL) from July through October 2015.

**Table 1** Antimicrobial susceptibility and molecular characterization of selected *A. baumannii* isolates from two tertiary hospitals in Lima, Peru

| Strain | Source | MIC (mg/L) | PT | ST | aOXA | iOXA |
|--------|--------|------------|----|----|------|------|
|        |        | IP | MP | CAZ | FEP | CTX | AK | GM | COL | TGC | CIP | LEV |
| 3      | INEN   | >32 | >32 | >256 | 192 | >32 | 48 | >256 | 0.5 | 4   | >32 | >32 | A   | 1   | 23  | 69 |
| 29     | INEN   | >32 | >32 | >256 | 256 | >32 | 66 | >256 | 1   | 4   | >32 | >32 | A   | 1   | 23  | 69 |
| 33     | INEN   | >32 | >32 | >256 | 48  | >32 | 32 | 2   | 0.5 | 4   | >32 | >32 | B   | 79  | 253 | 65 |
| 54     | HNAL   | 0.38 | 0.75 | 4   | 16  | >32 | 96 | 3   | 1   | 2   | >32 | >32 | C   | 79  | —   | 65 |
| 21     | INEN   | >32 | >32 | 128 | 16  | >32 | >256 | >256 | 2   | 2   | >32 | >32 | D   | 2   | 72  | 66 |
| 34     | INEN   | >32 | >32 | >256 | 24  | >32 | 3   | 2   | 0.5 | 3   | >32 | >32 | D   | 2   | 72  | 66 |
| 47     | INEN   | >32 | >32 | 192 | 24  | >32 | >256 | >256 | 0.5 | 3   | >32 | >32 | D   | 2   | 72  | 66 |
| 55     | HNAL   | >32 | >32 | 192 | 24  | >32 | >256 | >256 | 0.5 | 3   | >32 | >32 | D   | 2   | 72  | 66 |
| 69     | HNAL   | >32 | >32 | >256 | 24  | >32 | >256 | >256 | 0.5 | 2   | >32 | >32 | D   | 2   | 72  | 66 |
| 79     | HNAL   | >32 | >32 | >256 | 24  | >32 | >256 | >256 | 0.5 | 2   | >32 | >32 | D   | 2   | 72  | 66 |
| 42     | INEN   | >32 | >32 | >256 | 24  | >32 | >256 | >256 | 0.5 | 2   | >32 | >32 | E   | 2   | 72  | 66 |
| 4      | INEN   | >32 | >32 | >256 | 24  | >32 | 16  | >256 | 0.5 | 1.5 | >32 | >32 | F   | 79  | 72  | 65 |
| 32     | INEN   | 0.5 | 1   | >256 | 24  | >32 | 64 | 4   | 0.5 | 2   | >32 | >32 | F   | 79  | —   | 65 |
| 56     | HNAL   | >32 | >32 | 96  | 24  | >32 | 32  | 256 | 1   | 1.5 | >32 | >32 | F   | 79  | 72  | 65 |
| 37     | INEN   | >32 | >32 | 96  | 32  | >32 | 16  | 0.38 | 1   | 0.25 | >32 | >32 | G   | 3   | 23  | 71 |
| 60     | HNAL   | >32 | >32 | 96  | 64  | >32 | 32  | 0.38 | 1   | 0.5 | >32 | >32 | G   | 3   | 23  | 71 |
| 61     | HNAL   | >32 | >32 | >256 | 16  | >32 | 48  | >256 | 1   | 1   | >32 | >32 | H   | 108 | 72  | 132 |
| 53     | INEN   | >32 | >32 | >256 | 32  | >32 | 24  | 2   | 0.5 | 2   | >32 | >32 | I   | 79  | 253 | 65 |

MIC minimum inhibitory concentration, INEN Instituto Nacional de Enfermedades Neoplásicas, HNAL Hospital Nacional Arzobispo Loayza, IP imipenem, MP meropenem, CAZ ceftazidime, FEP cefepime, CTX cefotaxime, AK amikacin, GM gentamicin, COL colistin, TGC tigecycline, CIP ciprofloxacin, LEV levofloxacin, PT pulsotype, ST sequence type, aOXA acquired OXA-type, iOXA intrinsic OXA-type

*Colistin’s MIC was determined by broth microdilution*
Detection of carbapenem resistance genes

The presence of genes encoding both intrinsic and acquired class D carbapenemases, KPC, and MBLs was investigated by polymerase chain reaction (PCR). None of the isolates were positive for the genes encoding KPC or MBLs, but the intrinsic bla_{OXA-51}-like gene was detected in all the samples. In two isolates, this gene was the only carbapenemase detected, although it could not be associated with the presence of an upstream IS_{Aba1} element, in good agreement with the carbapenem-susceptible phenotype of these two isolates.

The bla_{OXA-24}-like gene, however, was predominant and present in 65 isolates (81.3%) collected from both hospitals, whereas 11 isolates (13.8%) carried the bla_{OXA-23}-like gene, and 2 isolates (2.5%) proved positive in the multiplex PCR for the bla_{OXA-143}-like gene. Overall, there were no substantial differences between the two hospitals regarding the proportion of isolates carrying different acquired class D carbapenemases (Table 2).

Pulsed-field gel electrophoresis

The clonal relatedness was initially studied by pulsed-field gel electrophoresis (PFGE), as described below, which allowed the classification of all the isolates into nine different clusters, or pulsotypes (A–I) (Fig. 1). Two major clusters disseminating between the two healthcare settings were identified: pulsotypes D and F, which contained 51 (63.8%) and 13 (16.3%) isolates, respectively. All the isolates in these two clusters were associated with the carriage of OXA-24-like carbapenemases, except for one isolate within pulsotype F with no acquired carbapenemases that was susceptible to carbapenems. OXA-23-like-producing A. baumannii were mostly grouped in pulsotype G (n = 6, 7.5%), containing isolates recovered from both hospitals, and pulsotype A, with only four isolates (5%) that were recovered from INEN. Pulsotype I contained only two isolates also recovered from INEN, but one isolate carried an OXA-23-like enzyme, while the other carried an OXA-143-like oxacillinase. Pulsotypes B, C, E, and H contained singletons (1.3%) producing OXA-24-like (pulsotypes E and H), OXA-143-like (pulsotype B), or no acquired OXA-like enzymes (pulsotype C), the latter isolate being susceptible to carbapenems. Figure 2 shows the temporal and spatial distributions of all the clones.

Multilocus sequence typing analysis and OXA sequencing of selected strains

Eighteen isolates were selected for further characterization on the grounds of their clustering in the PFGE dendrogram, susceptibility profiles, and carriage of acquired OXA-type carbapenemases (Table 1). Multilocus sequence typing (MLST) analysis using the Pasteur scheme of selected isolates showed full agreement among isolates clustered within the same pulsotype and ST designation (Table 1). Isolates from the major PFGE cluster D as well as the singleton from pulsotype E were assigned

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**Table 2** Number and percentages of A. baumannii isolates from each participating center carrying different acquired bla_{OXA} genes

| OXA-type (acquired) | INEN | HNAL | Total |
|---------------------|------|------|-------|
|                     | N    | %    | N     | %    | N | % |
| bla_{OXA-24}        | 43   | 81.1 | 22    | 81.5 | 65 | 81.3 |
| bla_{OXA-23}        | 7    | 13.2 | 4     | 14.8 | 11 | 13.8 |
| bla_{OXA-143}       | 2    | 3.8  | 0     | 0    | 2  | 2.5 |
| None                | 1    | 1.9  | 1     | 3.7  | 2  | 2.5 |
| Total               | 53   | 27   | 80    |      |    |    |

N number of isolates, % percentage of isolates, INEN Instituto Nacional de Enfermedades Neoplásicas, HNAL Hospital Nacional Arzobispo Loayza
Fig. 1 (See legend on next page.)
to ST2 (ST2), which belonged to clonal complex 2 (CC2) and to international clone II. Isolates from the PFGE cluster F, both isolates from pulsotype I and the singletons B and C were assigned to ST79, which was included in CC79, international clone V17. Isolates from pulsotype A were assigned to ST1, belonging to CC1 and international clone I, and those of pulsotype G to ST3, belonging to CC3, international clone III. The H singleton belonged to ST108, which was not assigned to any CC or international clone (Fig. 3).

Sequencing of the acquired class D oxacillinase genes in all the selected isolates revealed the presence of the OXA-72 variant in ST2, ST79, and ST108 isolates bearing a blaOXA-24-like gene as well as the presence of the novel OXA-253 variant in the two isolates with a blaOXA-143-like allele. Likewise, sequencing of the intrinsic class D oxacillinase gene identified specific OXA-51-like variants associated with each ST: ST1 isolates presented the blaOXA-69 gene, ST2 was associated with blaOXA-66, ST3 isolates carried the blaOXA-71 gene, ST79 was linked with blaOXA-65, and ST108 had the blaOXA-132 variant (Table 1).

Of note, sequence analysis of the genetic structures surrounding the blaOXA alleles identified the presence of the insertion sequence ISAba1 in reverse orientation upstream from the blaOXA-23 gene in all ST1 and ST3 isolates, although we were not able to detect the presence of a downstream ISAba1 copy, suggesting a Tn2008-like structure in these isolates18. Additionally, a novel insertion sequence was identified in forward orientation upstream of blaOXA-253 in the two strains carrying this OXA enzyme. To provide an attribution number to the new IS, its nucleotide sequence was submitted to ISFinder, the reference center for bacterial insertion sequences (http://www-is.biotoul.fr)19, and it was designated as ISAba47. This novel mobile element showed 82.5% identity at the nucleotide level with ISAba9 and was related to the IS982 family. The downstream region flanking blaOXA-253, however, resembled that previously described by Girlich et al.20 A 2334 bp sequence containing the blaOXA-253 gene together with upstream and downstream flanking regions (which included ISAba47) from strain 33 was also submitted to GenBank and can be retrieved under accession number MH347317.

Discussion

In recent decades, we have witnessed the emergence of MDR A. baumannii isolates worldwide, which has been associated with the rapid spread of a few carbapenem-resistant epidemic lineages producing acquired OXA-type carbapenemases1,15. To date, however, there are only a few studies regarding the epidemiology and carbapenem susceptibility of A. baumannii in Latin America, and this is the first study providing such data from Peru. Interestingly, the predominant A. baumannii lineages reported in Latin America so far are different from those reported in other parts of the world, as international clones II and III are currently absent in this region. International clone II was reported in Brazil in the past, when ST2 isolates producing OXA-23 were described from 1999 through 2003, but it disappeared in 200421,22. Instead, predominant clones are linked to ST79 (international clone V), ST25 (international clone VII), ST15, and, to a lesser extent, ST1 (international clone I), all of which are
mainly associated with the production of OXA-23 enzymes\textsuperscript{17,23–30}. Nevertheless, there are a few reports describing OXA-72-producing \textit{A. baumannii} isolates in Latin America, and overall, these isolates are considered less prevalent\textsuperscript{30–33}, or at least they were until recently. In 2017, Pagano et al.\textsuperscript{34} described the emergence of CC79 and CC15 \textit{A. baumannii} isolates carrying \textit{bla}_{OXA-72} in Brazil and warned of the potential dissemination of these epidemic lineages. Likewise, Nunez Quezada et al.\textsuperscript{35} reported an outbreak in Ecuador in 2017 caused by OXA-72-producing \textit{A. baumannii}\textsuperscript{35}, although the ST of these isolates was not investigated.

The data presented in this study show extremely high resistance rates (>97%) and MIC levels (>32 mg/L) of imipenem and meropenem as well as the predominance of the MDR phenotype among \textit{A. baumannii} isolates recovered from two tertiary hospitals in the capital city of Peru. Fortunately, the majority (95%) of isolates remain susceptible to colistin, as opposed to several studies reporting increasing rates of colistin-resistant \textit{A. baumannii} isolates in different countries\textsuperscript{8}. During the study period, carbapenem resistance in both settings was linked to the widespread dissemination of a major clone producing OXA-72 and belonging to ST2, the founder ST of the epidemic international clone II that has spread globally (Fig. 3)\textsuperscript{15}. A second group of highly clonal strains producing OXA-72 and belonging to ST79, international clone V, was also identified in both hospitals. In addition, we report the presence of a few sporadic clones producing OXA-23 and linked to international clones I and III (ST1 and ST3, respectively).

International clonal lineages have traditionally been associated with the carriage of specific genetic variants of the intrinsic \textit{bla}_{OXA-51} gene\textsuperscript{24,28,29}. This correlation is also shown in our study and supports the genetic relatedness of these isolates with epidemic international lineages. The \textit{bla}_{OXA-65}, \textit{bla}_{OXA-66}, \textit{bla}_{OXA-69}, and \textit{bla}_{OXA-71} genes were detected in isolates belonging to ST79, ST2, ST1, and ST3, respectively. We also identified the \textit{bla}_{OXA-132} variant in an ST108 isolate that did not belong to any known CC. Interestingly, a single ST108 isolate carrying \textit{bla}_{OXA-132} was reported in Lebanon in 2014, and
**Materials and methods**

**Samples**

This study included 80 consecutive clinical isolates of *A. baumannii* collected from different inpatients at two tertiary care hospitals (HNAL and INEN) from February 2014 through April 2016 in Lima, Peru. Only the first isolate from each patient was included in the study. The samples were obtained from blood, bronchial aspirate, soft tissues, cerebrospinal fluid, and urine, all of which were initially identified as *Acinetobacter* spp. by the BD Phoenix™ Automated Microbiology System (BD Biosciences, USA) and later identified to the species level by matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker, Germany) as described previously.

**Antimicrobial susceptibility testing**

Antimicrobial susceptibility was assessed by disc diffusion on Mueller–Hinton agar plates in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines for the following antimicrobials: ampicillin-sulbactam, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, gentamicin, amikacin, levofloxacin, doxycycline, tetracycline, meropenem, imipenem, and trimethoprimsulfamethoxazole. Susceptibility to colistin was assessed by broth microdilution as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group. The MIC values of selected strains were also determined by gradient diffusion (E test, bioMérieux, Sweden) for the following antimicrobials: imipenem, meropenem, cefotaxime, ceftazidime, cefepime, gentamicin, amikacin, tigecycline, ciprofloxacin, and levofloxacin. The MICs were interpreted according to CLSI clinical breakpoints and expert rules for *Acinetobacter* except for tigecycline, which was interpreted using the EUCAST breakpoints and rules for Enterobacteriaceae (Version 8.0, January 2018). *Escherichia coli* ATCC 25922 and *A. baumannii* ATCC 19606 were used as quality control strains.

All the isolates were categorized as MDR, XDR, or PDR according to the following ad hoc definitions; MDR, non-susceptible to at least one antimicrobial agent from three classes tested; XDR, non-susceptible to all antimicrobial agents tested but two or fewer; PDR, resistant to all the antimicrobial agents tested.

**Detection of carbapenem resistance genes**

The presence of the following carbapenemase-encoding genes was screened by PCR: *blaKPC*, for serine class A carbapenemases; *blaNDM*, *blaIMP*, *blaVIM*, *blaOXA-23*, *blaSPM*, and *blaSIM* for class B MBLs; and *blaOXA-51*-like, *blaOXA-23*-like, *blaOXA-58*-like, *blaOXA-143*-like, and *blaOXA-253*-like, for class D oxacillinas. Amplification products were purified from agarose gels (SpinPrep™ Gel DNA Kit, San Diego, CA, USA) and sent for Sanger sequencing (Macrogen, Korea) whenever necessary. The genetic identity of *blaOXA* was determined upon pairwise sequence alignment with reference sequences retrieved from http://www.lahey.org/Studies/.
The presence of IS sequences flanking the blaOXA genes was studied by PCR using specific primers as well as by inverse PCR and primer walking whenever needed. IS was studied by PCR using species specific primers as well as by inverse PCR and primer walking whenever needed.

Molecular typing
PFGE was performed as described previously, using genomic digestions with the Apal restriction enzyme and a CHEF-DRIII system (Bio-Rad Laboratories). Molecular patterns were analyzed with InfoQuest™ FP v.5.4 software (Bio-Rad Laboratories) and the unweighted pair group method with arithmetic mean to create dendrograms based on Dice’s similarity coefficient. Using bandwidth tolerance and optimization values set at 1.5 and 1%, respectively, isolates were considered to belong to the same PFGE cluster (pulsotype) if their Dice similarity index was ≥85%.

MLST was performed using the Pasteur scheme for A. baumannii. The allele sequences and STs of selected strains were identified and retrieved from the PubMLST A. baumannii MLST database (http://pubmlst.org/abbaumannii/). The population structure of STs was evaluated using the goeBURST software (http://www.phyloviz.net/goeburst/).

Acknowledgements
This study was supported by Cienciactiva de CONCYTEC, contract no. 164-2016-FONDECYT, Planes Nacionales de I+D+i 2006-2011/2013-2016, Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD12/0015/0013 and REIPI RD16/0016/0010), the 2017 call for Strategic Action on Health (PI17/01932), co-financed by European Development Regional Fund “A way to achieve Europe” and operating program Intelligent Growth 2014-2020; and grant 2014 SGR 0653 financed by Generalitat de Catalunya, grant SLT002/16/00349. Part of these data have been presented as a poster communication at the 18th International Congress on Infectious Diseases, 3–4 March, 2018, Buenos Aires, Argentina, and at the XXVIII-European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Madrid (Spain), 21–24 April, 2018.

Authors' contributions
Collected the samples: S.L.-B., S.P.-R., V.V.-T., J.V.-P. Conceived and designed the experiments: I.R., L.M., JdV-M., J.V. Performed the experiments: S.L.-B., I.R., S.P.-R., L.M., JdV-M., M.J.P. Analyzed the data: S.L.-B., I.R., S.P.-R., V.V.-T., J.V.-P., J.M.-M., JdV-M., J.V. Wrote the paper: S.L.-B., I.R., J.d.V.-M., J.V. All authors critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

Conflict of interest
The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare that they have no conflict of interest.

Ethics approval
This study was performed on clinical laboratory isolates. The authors had no contact or interaction with the patients. Personal information of the patients was not collected, to guarantee anonymity and confidentiality. Ethics approval was obtained from the Committee of the Instituto de Investigación Nutricional (IIN), Lima, Peru.

Received: 23 February 2018 Revised: 19 May 2018 Accepted: 4 June 2018
Published online: 04 July 2018

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