Journal of Antimicrobial Chemotherapy (2004) **54**, 255–258
DOI: 10.1093/jac/dkh269
Advance Access publication 9 June 2004

JAC

Long-term dissemination of an OXA-40 carbapenemase-producing *Acinetobacter baumannii* clone in the Iberian Peninsula

G. J. Da Silva[1]*, S. Quinteira[2,3], E. Bértolo[4], J. C. Sousa[2], L. Gallego[5], A. Duarte[4] and L. Peixe[2] on behalf of the Portuguese Resistance Study Group†

[1]Laboratório de Microbiologia, Faculdade de Farmácia, Universidade de Coimbra and Centro de Estudos Farmacêuticos, Couraça dos Apóstolos, 51 r/c Esq., 3000-432 Coimbra; [2]Faculdade da Farmácia, Universidade do Porto; [3]Escola Superior de Saúde do Vale do Ave, VN Famalicão; [4]Faculdade da Farmácia, Universidade de Lisboa, Portugal; [5]Departamento de Imunologia, Microbiologia and Parasitologia, Faculdade de Medicina, Universidade do Pais Vasco, Bilbao, Spain

Received 5 February 2004; returned 22 February 2004; revised 1 April 2004; accepted 10 April 2004

***Objective*: The main objectives of this study were to assess the clonal relatedness of *Acinetobacter baumannii* carbapenem-resistant isolates recovered from the Iberian Peninsula and to investigate the production of carbapenemases.**

***Methods*: One hundred and sixty-two imipenem-resistant *A. baumannii* isolates were collected from 1998 to 2003 in three Portuguese university hospitals. An imipenem-resistant isolate (988FFP strain) recovered in 1995 from a smaller hospital unit, was also included, as well as an OXA-40-producing *A. baumannii* Spanish strain (SM28). Susceptibility tests were carried out by disc diffusion and Etest methods. DNA fingerprints were obtained by PFGE of *Apa*I-digested chromosomal DNA. Carbapenemase activity was determined by a bioassay and spectrophotometry. The detection of the bla_{OXA-40} gene was conducted through PCR analysis, cloning and nucleotide sequencing.**

***Results*: All the isolates presented a similar multi-resistance pattern, including imipenem (MIC >32 mg/L). The Iberian isolates showed an identical PFGE pattern with minor band variations, including isolate 988FFP collected in 1995. PCR results revealed a bla_{OXA}-type gene in 65 isolates and nucleotide sequence analysis revealed the presence of the bla_{OXA-40} gene in seven representative Portuguese isolates from the various geographically dispersed hospitals.**

***Conclusions*: Our results indicate that a multi-resistant epidemic clone of *A. baumannii*, carrying bla_{OXA-40}, is disseminated in the Iberian Peninsula, persisting in Portugal since 1995.**

Keywords: *Acinetobacter* spp., epidemic clones, oxacillinases, β-lactamases

Introduction

Oxacillinases (Ambler class D) with carbapenemase activity, namely OXA-23, OXA-24, OXA-25, OXA-26, OXA-27 and OXA-40, have been found mainly in *Acinetobacter baumannii* isolates from the UK, Spain, Belgium and Singapore.[1–4] *A. baumannii* is recognized as an important cause of nosocomial infections mainly observed in intensive care units. Numerous outbreaks of *Acinetobacter* spp. infections have been reported, often associated with the spread of multi-resistant strains.[5,6]

Some outbreaks due to imipenem-resistant *A. baumannii* isolates occurred between 1998 and 2003 in various Portuguese hospitals located in distinct cities. These isolates were resistant to most of the alternative antimicrobials. Clonal outbreaks of infection caused by carbapenem-resistant strains of *A. baumannii* have also been reported in Spain.[4–6] The purposes of this study were to assess the clonal relatedness of *A. baumannii* imipenem-resistant isolates collected from Iberian Peninsula hospitals and to investigate the production of carbapenemases.

Materials and methods

Bacterial isolates

One hundred and sixty-two multi-resistant *A. baumannii* isolates were collected between 1998 and 2003, from different patients

*Corresponding author. Tel: +351-239-852567; Fax: +351-239-852569; E-mail: gjsilva@ci.uc.pt
†Members of the Portuguese Resistance Study Group are listed in the Acknowledgements.

hospitalized at intensive care units, surgery and medicine wards in three university teaching hospitals each with more than 600 beds. One hundred and eight isolates were recovered between October 1998 and September 2000 from Hospital de Santa Maria in Lisboa (HSM). During the period of January 1999 to October 2000, 23 isolates were collected in Hospitais da Universidade de Coimbra (HUC) located in the centre of Portugal. The remaining 31 isolates were collected in Hospital Santo António in Porto (HSA), located in the north, during 2001–2003. A representative strain of the Spanish isolates from Hospital Santa Marina de Bilbao (SM), the SM28 isolate,[4] was included in this study, as well as a strain from the laboratory bacterial collection of Faculdade de Farmácia da Universidade of Porto, *A. baumannii* 988FFP, isolated in 1995 from an inpatient of Hospital Padre Américo (HPAP), a small hospital unit located near Porto. This strain presented an antimicrobial profile identical to the isolates recovered recently.

Bacterial identification and susceptibility tests

All isolates were identified by the API 20NE system (bioMérieux, Marcy l'Étoile, France) and the basic properties of the genus *Acinetobacter*. Genospecies identification was carried out by amplified ribosomal DNA restriction analysis (ARDRA).[7]

Susceptibility testing was carried out by the disc diffusion method, according to NCCLS guidelines, using amoxicillin, co-amoxiclav, ticarcillin, ticarcillin/clavulanic acid, piperacillin–tazobactam, cefoxitin, cefuroxime, cefotaxime, ceftazidime, ceftriaxone, cefpirome, cefepime, aztreonam, imipenem, amikacin, gentamicin, netilmicin, tobramycin, nalidixic acid, norfloxacin, ciprofloxacin and polymyxin B discs (Oxoid). MICs of β-lactam antibiotics were determined by the Etest method (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions. The following β-lactam antibiotics were used: amoxicillin, amoxicillin/-clavulanic acid (2:1), piperacillin, piperacillin/tazobactam (4 mg/L), ticarcillin, ticarcillin/clavulanic acid (2 mg/L), ampicillin/sulbactam (2:1), cefoxitin, ceftriaxone, ceftazidime, cefepime, cefpirome, aztreonam and imipenem. Resistance was defined according to the NCCLS guidelines.

Pulsed-field gel electrophoresis

Genomic DNA patterns were analysed by pulsed-field gel electrophoresis (PFGE). Chromosomal DNA was digested with *Apa*I (New England Biolabs, Izasa, Portugal). The restriction fragments were separated by electrophoresis in a 1% agarose gel in 0.5 × TBE buffer, pH 8.0, at 200 V and 15°C for 24 h, with pulse time ramp from 5 to 15 s, with a CHEF DRII (Bio-Rad).

Determination of carbapenemase activity

Detection of carbapenemase activity was carried out in crude extracts by a microbiological assay using imipenem discs (10 μg) impregnated with enzymic extracts and EDTA solution. Inactivation of imipenem was shown by growth of the *Escherichia coli* ATCC 25922 strain within the expected inhibition zone. Specific β-lactamase activity was evaluated in extracts by spectrophotometry in the presence of zinc or EDTA solutions, as described elsewhere,[8] using 65FFC strain (IMP-5 producer) and ATCC 19606 *A. baumannii* as controls.

PCR amplification, cloning and sequencing of bla_{OXA} gene

The presence of bla_{OXA} genes was determined using the $bla_{OXA\text{-}24}$ primers.[5] The amplification reaction conditions were: an initial denaturation step of 3 min/94°C, followed by 30 cycles of denaturation 1 min/94°C, annealing 1 min/50°C and elongation 2 min/72°C, with a final extension of 8 min/72°C. PCR products were cloned into the pPCR-Script Cam SK(+) vector according to the manufacturer's instructions for the PCR-Script Cam Cloning Kit (Stratagene, Cambridge, UK). *E. coli* XL10-Gold transformants were selected with chloramphenicol (30 mg/L). Nucleotide sequences were determined and compared with published sequences using CLUSTAL W software.

Results

Bacterial identification and susceptibility tests

The isolates were classified in the complex *Acinetobacter calcoaceticus–A. baumannii* and representative isolates from each hospital identified by ARDRA as *Acinetobacter* genospecies 2, known as *A. baumannii*. All the isolates showed an identical β-lactam resistance profile, including resistance to amoxicillin and its association with clavulanic acid, ureidopenicillins and their associations, cefoxitin, ceftriaxone, ceftazidime, cefepime, cefpirome, aztreonam (MICs between 128 and >256 mg/L), ampicillin/sulbactam (MIC of 64 mg/L) and high resistance to imipenem (MIC >32 mg/L). Isolates were resistant to ciprofloxacin whereas the susceptibility profile to aminoglycosides was variable.

DNA fingerprinting

DNA profiles obtained by PFGE of representative isolates from different Portuguese hospitals, recovered at different times, differed by only one or two bands (Figure 1), and are closely related, according to the criteria of Tenover *et al.*[9] Moreover, the SM28 Spanish strain showed the same overall pattern of Portuguese isolates.

Carbapenemase activity

The results of the microbiological assay carried out for 54 isolates suggested the production of a carbapenemase not inhibited by EDTA, except in two HUC isolates (141FFC and 150FFC) and in one HSA isolate that apparently did not hydrolyse imipenem. The specific β-lactamase activity was determined in strain 988FFP and in 141FFC and 150FFC extracts, for confirmation. The 988FFP extract hydrolysed imipenem (5.11 nmol/min per mg of protein) and similar values were obtained when the carbapenemase activity was evaluated in the presence of zinc and EDTA (5.07 and 5.13 nmol/min per mg of protein, respectively), suggesting the production of a serine-β-lactamase with carbapenemase activity. The specific activity values of 141FFC and 150FFC extracts (0.12 and 0.41 nmol/min per mg of protein) were similar to the β-lactamase activity obtained with the imipenem-susceptible *A. baumannii* ATCC 19606.

Nucleotide sequence of bla_{OXA} gene

PCR with OXA-24 primers amplified an ~980 bp fragment in 65 isolates tested. Seven isolates, two from each hospital (including 141FFC isolate) and the 988FFP strain were selected for cloning. The nucleotide sequence of the inserts revealed an open reading frame (ORF) of 825 bp, with 100% homology with $bla_{OXA\text{-}40}$.

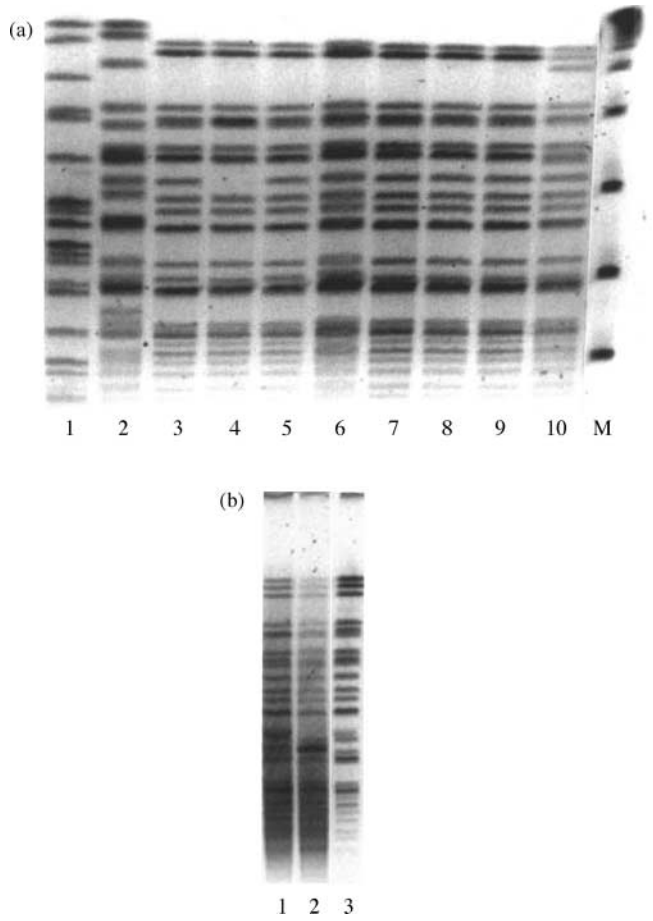

Figure 1. PFGE of genomic DNA from representative *A. baumannii* isolates, recovered from different Portuguese university hospitals, after digestion with *Apa*I (inverse image). (a) and (b) are from different gels. (a) Lane 1, ATCC 19606 strain; lane 2, imipenem-susceptible isolate; lanes 3–5 and 7–9, isolates from HUC (Coimbra), including 141FFc and 150FFC (lanes 3 and 5, respectively); lane 6, isolate from HSA (Porto); lane 10, isolate from HSM (Lisboa); lane M, lambda DNA concatemers. (b) lanes 1 and 2, isolates from HSM; lane 3, isolate SM28.

Discussion

A. baumannii is an emergent nosocomial pathogen, in part due to the capability of acquiring resistance to multiple antimicrobial agents. Clonal outbreaks due to imipenem-resistant strains of *A. baumannii* have been reported in particular hospitals.[3,5,6]

Since 1998, a high imipenem resistance incidence has been observed among nosocomial *A. baumannii* isolates in Portugal. Therefore, it was our purpose to ascertain if the resistance was due to the dissemination of an imipenem resistance determinant and/or to the spread of one *A. baumannii* clone.

Microbiological and spectrophotometric assays showed that imipenem was hydrolysed by enzymic extracts. An alteration of carbapenemase activity was not observed in the presence of zinc and EDTA, suggesting the production of a non-metallo-β-lactamase. OXA Class D carbapenemases have been identified mainly in *A. baumannii* strains collected in Europe.[1–4] OXA-24, OXA-25 and OXA-40 β-lactamases have been found in isolates from Spain associated with outbreaks. Hérétier *et al.*[10] have recently characterized biochemically and genetically the OXA-40 carbapenemase in a single *A. baumannii* isolate, recovered from a Portuguese patient who was transferred directly to France. This study shows that *A. baumannii* isolates collected from the three main Portuguese university hospitals between 1998 and 2003 and the 988FFP strain, isolated in 1995, carried the $bla_{\text{OXA-40}}$ gene, just as the Spanish isolate SM28.[4] Among the isolates tested, three isolates did not significantly hydrolyse imipenem, although they carried the $bla_{\text{OXA-40}}$ gene, as shown by PCR, and in 141FFC strain, confirmed by cloning and sequencing results. The data indicate that detection of $bla_{\text{OXA-40}}$ by PCR does not always correlate with carbapenemase production and may over-report the frequency of OXA carbapenemase producers. Other resistance mechanisms, like reduced permeability of the outer membrane and alteration in PBPs, may also contribute to carbapenem resistance.[5]

The clonal relatedness of *A. baumannii* Portuguese isolates and SM28 strain was evaluated by antibiotyping and PFGE. All the isolates presented an identical antibiotic resistance profile, only variable for aminoglycosides, and a very similar *Apa*I pattern (Figure 1), differing by no more than three fragments. Such a correspondence of phenotypic and genotypic characteristics can be explained by a common clonal origin. They are likely to represent genotypic variants of the same clone, which, over time, suffered minor rearrangements. Curiously, the Spanish strain SM28 exhibited an identical DNA profile to that of the Portuguese isolates. This observation suggests that the OXA-40-producing strain might be dispersed in hospitals throughout the Iberian Peninsula. Moreover, the results reveal that the spread of this clone dates to at least 1995.

Geographical spread, at a national or international scale, may be an important feature in the epidemiology of *A. baumannii*. Increased resistance among *A. baumannii* isolates has been generally observed in the last decades. However, because epidemiological relatedness of strains was not assessed in most of these studies, susceptibility data may have been influenced by the unrecognized inclusion of epidemic strains that are often multidrug resistant. Our results indicate that the observed carbapenem resistance among Portuguese *A. baumannii* isolates is due to the emergence of a resistant strain under antimicrobial selective pressure and the spread of an OXA-40-producing epidemic clone. This observation emphasizes the importance of having effective infection control measures in our hospitals, such as early detection of colonized patients, isolation procedures and a judicious use of antibiotics.

In summary, the present work reports for the first time the dissemination among Portuguese hospitals of an *A. baumannii* strain carrying $bla_{\text{OXA-40}}$, a clone endemic for several years in this country, and genetically related to the SM28 strain from Spain.

Acknowledgements

We thank FCT, POCTI (FEDER), Centro de Estudos Farmacêuticos, Faculdade de Farmácia da Universidade de Coimbra and ADEIM (Associação para o Desenvolvimento do Ensino e Investigação da Microbiologia) for financial support.

Members of the Portuguese Resistance Study Group are: Maria José Salgado, Melo Cristino e Luís Lito (Hospital de Sta Maria, Lisboa), Graça Ribeiro (Hospitais da Universidade de Coimbra) and Helena Ramos (Hospital de Santo António, Porto).

References

1. Donald, H. M., Scaife, W., Amyes, S. G. B. *et al.* (2000). Sequence analysis of ARI-1, a novel OXA β-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92. *Antimicrobial Agents and Chemotherapy* **44**, 196–9.

2. Bou, G., Oliver, A. & Martínez-Beltrán, J. (2000). OXA-24, a novel class D β-lactamase with carbapenemase activity in an *Acinetobacter baumannii* clinical strain. *Antimicrobial Agents and Chemotherapy* **44**, 1556–61.

3. Afzal-Shah, M., Woodford, N. & Livermore, D. M. (2001). Characterization of OXA-25, OXA-26, and OXA-27, molecular class D β-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy* **45**, 583–8.

4. Lopez-Otsoa, F., Gallego, L., Towner, K. J. *et al.* (2002). Endemic carbapenem resistance associated with OXA-40 carbapenemase among *Acinetobacter baumannii* isolates from Northern Spain. *Journal of Clinical Microbiology* **40**, 4741–3.

5. Bou, G., Cerveró, G., Domínguez, M. *et al.* (2000). Characterization of a nosocomial outbreak caused by a multiresistant *Acinetobacter baumannii* strain with a carbapenem-hydrolyzing enzyme: high-level carbapenem resistance in *A. baumannii* is not due solely to the presence of β-lactamases. *Journal of Clinical Microbiology* **38**, 3299–305.

6. Corbella, X., Montero, A., Pujol, M. *et al.* (2000). Emergence and rapid spread of carbapenem resistance during a large and sustained hospital outbreak of multiresistant *Acinetobacter baumannii*. *Journal of Clinical Microbiology* **38**, 4086–95.

7. Grundmann, H. J., Towner, K. J., Dijkshoorn, L. *et al.* (1997). Multicenter study using standardized protocols and reagents for evaluation of reproducibility of PCR-based fingerprinting of *Acinetobacter* spp. *Journal of Clinical Microbiology* **35**, 3071–7.

8. Da Silva, G. J., Correia, M., Vital, C. *et al.* (2002). Molecular characterization of bla_{IMP-5}, a new integron-borne metallo-β-lactamase gene from an *Acinetobacter baumannii* nosocomial isolate in Portugal. *FEMS Microbiology Letters* **215**, 33–9.

9. Tenover, F. C., Arbeit, R. D., Goering, R. V. *et al.* (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* **33**, 2233–9.

10. Hérétier, C., Poirel, L., Aubert, D. *et al.* (2003). Genetic and functional analysis of the chromosome-encoded carbapenem-hydrolyzing oxacillinase OXA-40 of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy* **47**, 268–73.