

Endemic Carbapenem Resistance Associated with OXA-40 Carbapenemase among *Acinetobacter baumannii* Isolates from a Hospital in Northern Spain

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Eighty-two carbapenem-resistant isolates of *Acinetobacter baumannii* from a single hospital in Bilbao were typed into two major clusters and several subclusters. Disk synergy tests and PCR indicated the production of a zinc-independent OXA-class carbapenemase. Sequencing identified this enzyme, OXA-40, as a variant of the OXA-24–OXA-25–OXA-26 cluster.

Acinetobacter baumannii and its close relatives are important agents of nosocomial pneumonia in intensive care units (2, 20) and also cause a wide range of other nosocomial infections, especially in immunocompromised patients (2, 20). Many nosocomial acinetobacters are multiply resistant, meaning that such infections are difficult to treat, even with combination therapy. Moreover, acinetobacters often cause outbreaks and survive for long periods in the hospital environment, thereby posing a difficult challenge for infection control (20).

Carbapenems remain active against acinetobacter infections in many centers (2), but reports of carbapenem resistance have accumulated worldwide (1). Carbapenem-resistant strains of *A. baumannii* have been mostly sporadic in northern Europe but are endemic in some southern European countries, including parts of Spain (3, 7). Some reports have associated carbapenem resistance in *Acinetobacter* spp. with altered penicillin-binding or outer-membrane proteins (6, 12, 22), but *Acinetobacter* isolates producing carbapenem-hydrolyzing β -lactamases (carbapenemases) have been reported from at least 12 countries (1). Some of these carbapenemases are IMP- or VIM-class metallo- β -lactamases (5, 8, 18, 21, 25), but most acinetobacters produce zinc-independent members of molecular class D (1). Sequenced carbapenemases of this latter class from acinetobacters include OXA-23 (ARI-1) (9), OXA-24 (4), OXA-25, OXA-26, and OXA-27 (1).

Carbapenem resistance in *Acinetobacter* spp. has been endemic in Bilbao hospitals for several years (11). This study retrospectively investigated 82 isolates of *Acinetobacter* spp. isolated between June 1998 and April 1999 from 79 chronic bronchiectasis patients (three patients yielded two different isolates) attending the Hospital de Santa Marina, Bilbao,

Spain. This is a 200-bed institution specializing in respiratory illness. The isolates were obtained from sputa (62 isolates), surgical wound swabs (4 isolates), skin ulcer swabs (4 isolates), urines (4 isolates), blood cultures (3 isolates), transtracheal aspirates (3 isolates), feces (1 isolate), and bronchoalveolar lavage specimens (1 isolate). Isolates were initially identified with the API 32 system (Biomérieux, Marcy l'Etoile, France) and stored at -80°C in nutrient broth containing 50% glycerol (vol/vol). As part of the present study, all isolates were re-identified as members of the *A. baumannii* complex (either *A. baumannii* or the closely related genomic species 13TU) by the technique of transfer DNA fingerprinting, in which PCR is used to amplify the species-specific spacer regions found between clusters of tRNA genes (10).

MICs for the isolates were determined by the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method (16). Based on the NCCLS resistance breakpoint of ≥ 16 $\mu\text{g/ml}$ for bacteria that grow aerobically, 49 (60%) and 45 (55%) of the 82 isolates were resistant to imipenem and meropenem, respectively. Isolates that were resistant to meropenem were also resistant to imipenem. Among the 49 imipenem-resistant isolates, resistance or intermediate resistance was also observed to ticarcillin (92%), piperacillin (100%), piperacillin plus tazobactam (61%), cefotaxime (94%), ceftazidime (90%), aztreonam (98%), gentamicin (90%), amikacin (69%), and ciprofloxacin (88%).

DNA was extracted from imipenem-resistant isolates and used with DAF-4, ERIC-2, and M13 core primers for the generation of randomly amplified polymorphic DNA (RAPD) fingerprints (11). RAPD fingerprints were also generated for 51 *A. baumannii* isolates associated with outbreaks of nosocomial infection in 18 different countries. Among these were carbapenem-resistant *A. baumannii* isolates from Madrid (strain RYC 52763/97) (3) and Barcelona (clones D and E; strains 228620 and 224220, respectively) (7). Fingerprint profiles were clustered with BioNumerics version 2.0 software (Applied Maths, Kortrijk, Belgium) by using the Dice coeffi-

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TABLE 1. RAPD typing relationships amongst 49 imipenem-resistant isolates of *A. baumannii* from Bilbao

DAF-4 cluster	M13 cluster	ERIC-2 cluster [no. of isolates]	Total no. of isolates	Representative isolate
I	1	A [2]	2	SM10
	2	B [1], E [1], L [1], T [1], M [3]	7	SM76
	3	N [1], P [7], C [2], R [4]	14	SM28
II	4	Q [26]	26	SM80

cient and the UPGMA method. Isolates that clustered at a similarity coefficient (S_{AB}) of >0.70 with a particular primer were considered to be related (13, 24).

Two major clusters (I and II) were identified among the Bilbao isolates with primer DAF4. Cluster I could be divided into three subclusters using M13 primer and ten subclusters using ERIC-2 primer (Table 1). Isolates from each of these subclusters showed carbapenemase activity in the modified Hodge test (14), but there was no evidence for metallo- β -lactamase activity in EDTA-disk synergy tests (14). By using DAF-4 (the least discriminatory primer), representative isolates of M13 clusters 1 (isolate SM10; imipenem MIC, >128 $\mu\text{g/ml}$), 2 (isolate SM76; imipenem MIC, >128 $\mu\text{g/ml}$), 3 (isolate SM28; imipenem MIC, >128 $\mu\text{g/ml}$), and 4 (isolate SM80; imipenem MIC, >128 $\mu\text{g/ml}$) were directly compared with the collection of 51 *A. baumannii* isolates from other countries and regions. SM28 (M13 cluster 3) was related at an S_{AB} of 88% to two carbapenem-susceptible isolates from Barcelona, Spain, and Trieste, Italy, respectively. SM28 was unrelated to a Madrid isolate known to produce OXA-24 (3) but was closely related to one of two epidemic clones (clone D; strain 228620) responsible for a large and sustained outbreak of *A. baumannii*

infection in Barcelona (7). Isolates SM10, SM76, and SM80 were unrelated ($S_{AB} < 70\%$) to all isolates from other locations.

Isolates SM28 and SM80 (DAF-4 clusters I and II, respectively) were investigated by PCR for the presence of *bla*_{IMP} (19), *bla*_{VIM} (17), or OXA-type (1) carbapenemase genes. PCR with OXA-24 primers (1) amplified a ca. 1,030-bp fragment with both SM28 and SM80. No PCR evidence was obtained for the presence of *bla*_{IMP}- or *bla*_{VIM}-type genes. The ca. 1,030-bp fragment was sequenced on both strands by using a dye-labeled dideoxynucleoside triphosphate terminator cycle sequencing Quickstart kit (Beckman Coulter UK Ltd., High Wycombe, United Kingdom) and a CEQ 2000 automated sequencer (Beckman). Sequence data were analyzed with the GeneBuilder component of BioNumerics. The sequences obtained from SM28 and SM80 were identical and were 1,023 bp in size, including a reading frame of 825 bp. Figure 1 shows the relationship between the amino acid sequence coded by the gene from SM28 and SM80 and those of the OXA-24 (found in Madrid), OXA-25 (found in Murcia, Spain), and OXA-26 (found in Ghent, Belgium) enzymes, all of which share 98% similarity (1). The Bilbao enzyme had a closely related amino acid sequence, with two amino acids different from OXA-24 and OXA-25 and one amino acid different from OXA-26. The enzyme from Bilbao has been allocated the designation OXA-40 (K. Bush, personal communication).

The spread of specific acinetobacter clones has been demonstrated previously between hospitals in particular cities, such as Brooklyn, New York (15), or across whole countries, as seen with a single carbapenem-susceptible strain of *A. baumannii* that has been disseminated throughout Spain and the Canary Islands (23). Clonal outbreaks of infection caused by carbapenem-resistant strains of *A. baumannii* have been reported from Madrid (3) and Barcelona (7). The present study demon-

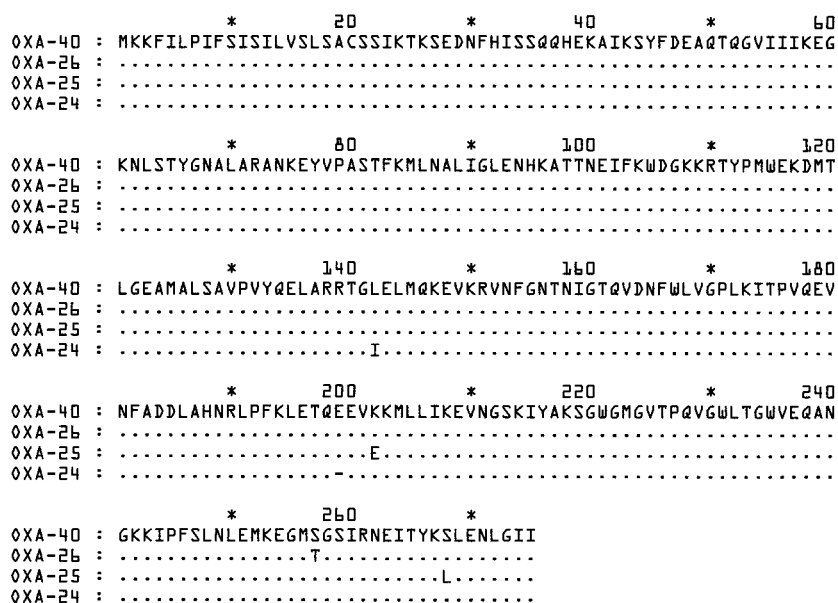


FIG. 1. Comparison of the amino acid sequence of OXA-40 from Bilbao (strains SM28 and SM80) with the sequences of OXA-24, OXA-25, and OXA-26. The sequences are identical except where differences are indicated.

strated that several different clusters of carbapenem-resistant *A. baumannii* isolates were endemic within a single hospital in Bilbao in 1998 and 1999 and produced a carbapenemase (designated OXA-40) that showed minor amino acid changes relative to OXA-24, OXA-25, and OXA-26.

The possible origins of class D carbapenemases have been considered previously and three hypotheses have been proposed, none of them mutually exclusive (1). One possibility is that an *Acinetobacter* strain (or strains) may have acquired a parental enzyme gene which has since diversified by mutation. In support of this hypothesis, it is notable that OXA-40 was closely related to the OXA-24 and OXA-25 enzymes reported from other parts of Spain but was more remote from the OXA-23–OXA-27 cluster, whose representatives have been found in the United Kingdom, Singapore, and Brazil (1). This suggests that minor diversification has occurred within the OXA-24–OXA-25–OXA-40 cluster in Spain rather than reported de novo evolution.

While this study was being prepared, a preliminary report appeared describing the characterization of an OXA-type enzyme with a sequence identical to that of OXA-40 (L. Poirel, C. Héritier, and P. Nordmann, Abstr. 12th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. O384, 2002) found in a single recent isolate of *A. baumannii* from a patient transferred directly to France from a hospital in Portugal. While the precise relationship of this recent Portuguese isolate to the strains endemic in Bilbao in 1998 and 1999 has not yet been investigated, it appears that the OXA-40 gene has spread across the Iberian peninsula. The OXA-40 gene has been assigned GenBank accession number AF509241.

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