# Analysis of plasmids as genetic support of the class D carbapenemase OXA-40 in multidrug-resistant *Acinetobacter baumannii* isolates

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The aim of this study was to analyse the presence of plamids in multidrug-resistant *A. baumanni* isolates and its relationship with the class D OXA-40 carbapenemase. Isolates belonging belonging to the European Clone I and European Clone II lineages, respectively, were selected from a collection of *A. baumannii* isolates obtained from a hospital in Northern Spain in 1999 - 2008. The representative isolates included both OXA-40-positive and -negative isolates. Clonal relatedness was confirmed by PCR-fingerprinting and PFGE with *Apa* I enzyme. To locate *bla* $_{\text{OXA-40}}$ Southern transfer hybridization experiments were carried out using a specific DNA probe labelled with digoxigenin. Calculation of the linear size of the most frequent plasmid was done by PFGE and S1 nuclease digestion. Isolates from both clones carried plasmids ranging from 2.5 to 125 kb in size, frequently associated in groups. Identical size plasmids were observed in isolates obtained throughout the period of study, indicating that they were stably inherited in the population. The predominant location of the $bla_{\text{OXA-40}}$ gene was on a 32 kb plasmid.

**Keywords** *Acinetobacter baumannii*; imipenem resistance, carbapenemase, *bla* $_{\text{OXA-40}}$, plasmid.

## 1. Introduction

*Acinetobacter baumannii* is a non-fermentative, gram-negative, non-motile, oxidase-negative bacillus. Although members of the genus *Acinetobacter* are widely distributed in the natural environment, *A. baumannii* is largely confined to the health care setting and its natural reservoir remains to be determined. Nevertheless, it is a very effective colonizer of patients and the hospital environment (1,2).

Although *A. baumannii* is generally non-pathogenic in healthy individuals these organisms have become an increasing cause of serious opportunistic infection, particularly ventilator-associated pneumonia and invasion of burns wounds. Over the last decade it is emerging as a nosocomial pathogen that frequently cause fatal infections in immunocompromised patients (1,3 ). The factors contributing to colonization, virulence, and invasion by *A. baumannii* are still being defined (4,5,6) but it is apparent that this opportunistic pathogen expresses a myriad of factors that could play a role in the pathogenesis of the infection it causes in humans. Among these factors are the attachment to and persistence on solid surfaces, the acquisition of essential nutrients such as iron, the adhesion to epithelial cells and their subsequent killing by apoptosis, and the production and/or secretion of enzymes and toxic products that damage host tissues. Its enhaced survival and prolonged viability under dry conditions is also facilitated by resistance to disinfectants (1,2,5).

Hospital outbreaks of *A. baumannii* have been frequently reported worldwide from many medical, surgical and neonatal intensive care and burn units (7,8,9,10).The emergence and rapid spread of multidrug-resistant isolates are of great concern since very few therapeutic options remain active against such isolates (11,12,13). Risk factors for adquisition of multidrug-resistant *A. baumannii* include admittance to an ICU or burns unit, large hospital size (>500 beds) and previous exposure to antibiotics, notably carbapenems. Immunosuppression, emergency admission, respiratory failure or mechanical ventilation, invasive procedures, urinary catheterisation and recent surgery are further risk factors (1,5).

Atributable mortality is hard to define, since *A. baumannii* tends to cause serious infection only in very vulnerable patients and its pathogenic properties may be clone-specific; nevertheless, mortality rates of up to 20% and 70% have been claimed (1,2,14).

## 2. Resistance to carbapenems and carbapenemases in *Acinetobacter baumannii*

For over the past years *A. baumannii* has adquired resistance to many types of antibiotics (15). Its capacity to acquire resistance to multiple antimicrobial agents has made this organism a significant challenge in many health care institutions (12,13,16).

The carbapenems, chiefly imipenem and meropenem, have become standard therapy for serious acinetobacter infections, but resistance is emerging to them too, mostly in strains that are already multi-resistant to other treatments (12). The mechanisms underlying resistance to carbapenem in *A. baumannii* are: a) loss of outer membrane proteins or porins that results in decreased permeability to antibiotics through the outer membrane, b) modification of penicillin-binding-proteins (PBPs) that prevent their action, c) the activity of efflux pumps that further decrease the concentration

 

of antibiotic within the bacterial cell, and d) the emergence of numerous inactivating enzymes that is the most problematic recent occurrence (10,13,15).

The first reports of plasmid-mediated imipenem resistance in *A. baumannii* started to emerged in the 90´decade with the first of a novel group of OXA-type carbapenemases being discovered in 1993 in an imipenem-resistant *A. baumannii* strain from a patient in the Edinburgh Royal Infirmary, Scotland (17,18).

Since then, different types of carbapenemases have been subsequently described with class D oxacillinases being the most widespread in *A baumannii*. These enzymes belong to four clusters represented by OXA-23, OXA-40, OXA-51 and OXA-58 enzymes (19,20,21). Along with these carbapenemases, some class B metallo-betalactamases (VIM-1, VIM-2, SPM-1, and SIM-1) have been also identified as a minor source of carbapenem resistance (22 ).

The OXA-23 group has been reported worldwide and is particularly prominent in certain geographical regions as has been recently reported in the United Kingdom, Brazil and China (20,22) The OXA-40 group can be either chromosomal or plasmid mediated and appears to be restricted to Europe and the United States (23,24). The third group, OXA-58 is genetically dissimilar and has been reported from many countries around the world (15). In addition to those oxacillinases, strains of *A. baumannii* have been shown to contain an intrinsic carbapenemase which is seldom expressed, named OXA-51 (25,26). Its expression is normally quiescent but mediated by a hybrid promoter caused by the insertion of IS*Aba1* upstream of the structural gene. The significant contribution of these class D enzymes to carbapenem resistance in *A. baumannii* has been enphasized, particularly when they are accompanied by IS *Aba1* and IS *Aba3* in the naturally occurring plasmid.

Until now, most of the OXA-type genes have been chromosomally located and there are not many reports concerning its location on plasmids, although plasmid containing *bla* $_{\text{OXA-23}}$-like, *bla* $_{\text{OXA-40}}$-like and *bla* $_{\text{OXA-58}}$ –like genes have been described (27,28).

Unlike the majority of oxacillinases in other genera, none of the Acinetobacter OXA genes from carbapenem-resistant strains have been found on integrons (15,29).

## 3. Genetic basis of antibiotic resistance

*Acinetobacter* spp. appear to be well suited for genetic exchange and are among a unique class of gram-negative bacteria that are described as “naturally transformable “ (1,2,3 ). It is widely demonstrated that strains of *A. baylyi* displays a remarkable capacity for natural competence. To date, it is unknown whether other species of *Acinetobacter* are naturally competent or whether environmental conditions can be altered to facilitate pathogenicity or antibiotic resistance gene acquisition (13,15,30).

All three of the major modes of chromosomal gen transfer have been demonstrated in Acinetobacter spp.: transformation, transduccion and conjugation. Plasmids also play an important role in the biology of this genera encoding a large and useful range of phenotypic properties, including antibiotic resistance. It is demonstrated that *Acinetobacter* spp. can acquire antimicrobial resistance factors through conjugation of plasmids (1,2). Currently, transposons and integrons (predominantly class 1) are known to be important in the dissemination of genetic determinants of resistance in *Acinetobacter* spp. The description of insertion sequences that promote gene expression has also played an important role in explaining the regulation of resistance (17,18).

Reviews on plasmids in acinetobacters have noted that indigenous plasmids have been found in the majority of *Acinetobacter* isolates examined (31,32,33). Many plasmids had unidentified functions, while others had been associated with antibiotic and heavy metal resistance, aromatic hydrocarbon degradation, conjugation and restriction/modicification functions (1,2). The first descriptions of specific functions were an imipenem-resistance coding plasmid of 45 Kb and the sequence of pMAC a 9,5 kb plasmid harboring genes involved in organic peroxide resistance (34,35)

Recent studies (36,37) identified an 86-kb region called the AbaR1 resistance island in a MDR isolate, named AYE strain, that contained a cluster of 45 resistance genes. It was also composed of mobile genetic elements and other genes previously identified in *Pseudomonas* spp., *Salmonella* spp., and *E. coli*..

Recently, novel insights into *A. baumannii* virulence have been gained from whole-genome analysis of strain ATCC 17978 genome. A significant fraction (nearly 17% of its 4-Mb genome)) of the open reading frames were located in 28 putative alien islands, indicating that the genome acquired a large amount of foreign DNA by horizontal gen transfer. A large number of genes contained virulence islands involved in protein secretion, drug resistance, pilus formation, and cell wall biogenesis, suggesting that the organism devotes a considerable portion of its genome to pathogenesis. The largest virulence island contained genetic elements homologous to the type IV secretion systems of *Legionella* and *Coxiella burnetii*. It also posseses an aditional 74 potential drug resistance genes, including 32 efflux pump genes, 11 permease genes, and also 26 genes encoding resistance to heavy metals (38).

The complete sequence of an epidemic strain named *A. baumannii* ACICU showed the presence of an antibiotic island AbaR2, that had plausibly evolved by evolution from the AbaR1 island previously described in multiresistant strain *A. baumannii* AYE. Moreover, 36 putative alien islands (pAs) were detected, 24 of these had previously been described in the ATCC 17978 genome. Two plasmids, pACICU1 and pACICU2, of 28,279 and 64,366 bp respectively, were also detected (39).

## 4. Epidemiological profile of multidrug-resistant isolates

The population structure among clinical isolates is highly clonal, with single strains commonly affecting multiple patients in a unit or hospital. Indeed, a few lineages of *A. baumannii* have achieved the epidemic status as nosocomial pathogens, spreadind to cause outbreaks of infections in many hospitals and countries. Many of these outbreaks have involved multidrug-resistant strains, including to carbapenems and amikacin, with colistin and tigecycline representing the only remaining therapeutic options (40,41,42).

The emergence of two pan-European epidemic clones, referred to as European clones I and II, was reported in northwestern Europe in the 1980s and since then these clones have been documented in many regions of Europe associated with outbreaks of hospital infections (43,44,45,46,47,48).

Plasmid electrophoresis profiles can be used for epidemiological typing of strains (2,31,32) although plasmid typing has to be interpreted with caution due to the fact that can easily be lost or transferred among strains. Some plasmids can be considered markers for some epidemic clones such as pAN1, a particular cryptic plasmid of 8.7 kb found almost exclusively in strains of European clone I (43). This plasmid had been firstly described in 1999 in a study of a group of 103 isolates from the Czech Republic, where those structures were found in 92% of the isolates showing a high variability in the corresponding profiles (33). More recently whole-genome pyrosequencing of an epidemic multidrug-resistant *A. baumannii* strain belonging to the European clone II group showed the presence of two plasmids, pACICU1 (location of the *bla* $_{\text{OXA-58}}$ gene) and pACICU2.

Sets of strains representative of currently recognized clones and widespread genotypes thereof will be useful to validate new characterization methods and rapid identification tools. Early recognition of strains belonging to clones with high transmissibility potential would be beneficial by leading to the implementation of infection control measures in order to prevent further spread in hospitals. Study of the biological properties of widespread clones will be important to understand their evolutionary success and to determine whether they share special properties such as enhaced virulence or an increased capacity to survive in the hospital environment, or if their success is merely due to the fact that they acquired and express antimicrobial resistance genes (43,44,47,48,49)

## 5. Analysis of plasmids as genetic support of the carbapenemase OXA-40 in multidrug-resistant *Acinetobacter baumannii* isolates from Northern Spain

In the Iberian Peninsula clonal outbreaks of OXA-40-producing *A. baumannii* isolates have been identified mainly in hospitals from Northern Spain and Portugal (23,50). Although it had been first described as chromosomally coded (51)), the precise genetic location of the OXA-40 gene is still unknown. Reports from other countries, its detection in genetically unrelated iberian isolates and the identification of different plasmids harbouring the OXA-40 gene, support the contribution of these mobile elements (23,27,52).

Studies on Iberian clone I and II isolates from Northern Spain showed a 32 kb plasmid where *bla* $_{\text{OXA-40}}$ gene was located (28) whereas Quinteira et al. identified the OXA-40 gene on plasmids of 180 and 30 kb in an *A. haemolitycus* strain from Portugal (27). Recently, two isolates grouped into Europen clone II from Italy and producing the OXA-24/40 enzyme were investigated, identifying a plasmid named pABVA01 of 8,963 bp long (52) whose genetic organization was overall very similar to that of p2ABAYE and pAB0057, two small *Acinetobacter* plasmids previously detected in French and American clinical isolates, respectively (52). However, despite its interest as a reservoir of antibiotic resistance genes, many of the information that have been reported about plasmids in *A. baumannii* resulted from indirect methods due to proven difficulties found to isolate and characterize plasmids from clinical isolates. According to these difficulties, very little is known about plasmids in *A. baumannii* and as a consequence, its contribution towards carbapenem resistance.

The objective of the study was to investigate sequential isolates of two different multidrug-resistant *A. baumannii* clones producing the OXA-40 carbapenemase obtained from the years 1999 to 2008 in a hospital from Northern Spain . The main interest was to investigate the presence of plasmids and elucidate its role as the genetic support of the OXA-40 carbapenemase and to identify different genetic markers among clonally related and unrelated isolates including detection of the presence of other OXA-like carbapenemases and sequence diversity in the *csuE*, *ompA* and *bla*$_{\text{OXA-51-like}}$ genes.

## 6. Materials and Methods

### 6.1 Background

In total 102, 82, 30 and 51 *A. baumannii* isolates were obtained from patients attending a hospital of Osakidetza (Bilbao, Northern Spain) by the Microbiology Laboratory during the years 1999, 2002, 2005 and 2008, respectively. The hospital is a 240-bed respiratory illness-specialized institution with a prevalence of elderly patients which are

 

hospitalized for long periods of time (median, 1 month). Previous identification included tDNA fingerprinting to identify isolates at the species level and clonal relatedness investigation by RAPD-PCR fingerprinting with primers M13 (5'-GAGGGTGGCGGTTCT-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') . Digital images of the gels were analysed with *Molecular Analyst/ Macintosh Fingerprinting* program (Image Analysis System, Bio-Rad Laboratories) which identifies the positions and intensities of the bands in each lane of a gel and then calculates a similarity coefficient ($S_{AB}$) for every pair of strains.Those isolates with a $S_{AB}$ value of >0.72 were clustered together.

Susceptibility assays: Determination of the level of resistance to a wide range of antibiotics used for treatment of *A. baumannii* infections was done by the disk-diffusion method according to National Committee for Clinical Laboratory Standards document. In addition to previous analysis, Minimun Inhibitory Concentration of selected antimicrobial agents [cefotaxime (Hoescht Marion Roussel), ceftazidime (Glaxo Wellcome), imipenem (Merk Sharp & Dohme), meropenem (Zeneca Pharmaceutical), amikacin (Bristol Myers Squibb) and gentamicin (Schering Plough)] were also determined by the agar dilution method according to the guidelines described below. *Pseudomonas aeruginosa* ATCC 27853 was used as a control strain.

Previous investigation of carbapenemases using conventional phenotypic analysis and single PCR technique, had shown the presence of $bla_{\text{OXA-40}}$ gene but no metallo-beta-lactamases $bla_{\text{VIM}}$ $bla_{\text{IMP}}$ $bla_{\text{SPM}}$ $bla_{\text{GIM}}$ types were detected. In addition, multiplex PCRs wer performed to search for $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-51-like}}$, and $bla_{\text{OXA-40-like}}$ and $bla_{\text{OXA-58-like}}$ genes following the methodology described by Woodford et al (53).

### 6. 2 Clinical isolates

As two multidrug-resistant OXA-40 carbapenemase-producing clones, named I and II, were predominant over time their evolution was further analyzed in terms of changes of pathogenic factors and other genetic events by making a retrospective selection of 15 *A. baumannii* isolates as a representative group for the period 1999-2005 including $bla_{\text{OXA-40}}$ positive and negative isolates per year and clone. During 2008 only isolates belonging to clone I were recovered so it was decided to include 15 of them in the study. In order to assure isolates clonal relation we developed pulsed-field gel electrophoresis (PFGE) with *Apa* I enzyme following the methodology described by Turton et al (54). Analysis of DNA profiles was done using BioNumerics (Applied Maths, Kortrijk, Belgium) with the percentage similarity of profiles being calculated using the Dice coefficient.

Control strains for OXA-40/24 carbapenemase assays were *A. baumannii* SM28 and RYC 52763/97 (OXA-40 and OXA-24,respectively). For establishement of clonal relatedness with European clones I, II and III we used *A. baumannii* NIPH 527, NIPH 528 and NIPH 1669, respectively, kindly sent by Dr. Alexander Nemec (Cezch Republic).

### 6.3 Multiplex-PCR for the definition of *A. baumannii* sequence group

A PCR designed to selectively amplify the *ompA*, (an outer membrane protein that has also been found to induce apoptosis of epithelial cells) *csuE* (part of a pilus assembly system, thought to be essential for biofilm formation) and $bla_{\text{OXA-51-like}}$ (intrinsic carbapenemase gene in *A baumannii)* and sequencing of these genes was carried out on representative isolates following the methodology described by Turton et al. (54) PCR reactions were carried out in 25 μl volumes with 3 μl of extracted DNA, 12.5 pmole of each primer and 1.5 U of *Taq* DNA polymerase in 1x PCR buffer containing 1.5 mM $MgCl_2$ and 200 μM of each dNTP.

### 6.4 Class 1 integrons

To determine the presence of class 1 integrons, PCR was performed with primers 5´CS (5'-GGCATCCAAGCAGCAAG- 3') and 3´CS (5 '-AAAGCAGACTTGACCTGA- 3') To identify the presence of the $bla_{\text{OXA-40}}$ gene within the integron PCR reactions were developed combining 5´CS with the corresponding carbapenemase reverse primers under the same conditions employed for amplification of individual genes. Cycling conditions were as follows: 1X 94°C (5 min); 35X 94°C (1 min), 55°C (1 min), 72°C (1 min) and 1X 72°C (6 min)

### 6.5 Plasmid analysis and endonuclease mapping

Plasmid DNA was extracted using a commercial plasmid extraction kit (Plasmid Midi Kit, Qiagen) following the indications of the manufacturer. Plasmid content was analyzed by electrophoresis on 0.7% agarose gels and plasmid size was determined by comparison to plasmid DNA extracted from the standard strains *E.coli* NCTC 50193 (CECT 678) and NCTC 59192 (CECT679) carrying plasmids ranging in size from 2 kb to 163.3 kb .

About 1 μg of plasmid DNA was used for digestions with restriction enzymes *Eco*RI, *Pst*I and *Hind*III endonucleases (invitrogen) following the indications of the manufacturer.

### 6.6 Plasmid linearization with S1 enzyme

DNA was prepared following the same protocol used for *Apa* I digestion and was then incubated with 10 units of S1 enzyme per experiment at 37ºC for 45 minutes. The resulting fragments were visualised by *Pulsed Field Gel Electrophoresis* using ramping of 5-20 sec at 14ºC for 20 hours.

### 6.7 Hybridization experiments to locate *bla* $_{OXA-40}$ gene

Plasmid DNA and the corresponding digestions on electrophoresis gels were transferred to a nylon membrane by the Southern technique. The DNA fragments were then UV cross-linked for 2 min and hybridized with an OXA-40 probe, made of a PCR-generated 1023-bp internal fragment labelled with dUTP-digoxigenin included in the PCR mixture. Hybridization was carried out overnight at 42ºC in the presence of 50% formamide. Detection of hybrids was done by colorimetric detection using an anti-digoxigenin antibody coupled to alkaline phosphatase according to the manufacturers recommendations (Roche).

## 7 Results and discussion

### 7.1. General overview of predominant clones

Multidrug-resistant *Acinetobacter baumannii* has increasingly been recognized as an important nosocomial pathogen causing infections with a high rate of mortality (14). Frequently associated with hospital outbreaks, its biological properties favour situations of endemicity that have been described worldwide (7, 23, 31, 43, 50). During the period of study the total number of isolates recovered at the Microbiology Laboratory per year decreased, probably due to the control measures developed in the hospital following the initition of DNA-*fingerprinting* methodology in 1999 to control the situation. Although many sporadic clones were identified, mainly in 1999, the majority of the isolates grouped into two endemic genotypes named clone I and clone II whose prevalence changed over time. Clone II was predominant in 1999 but has decreased in importance by 2005 and had disappeared in 2008. When the age of the patients carrying these prevalent clones was analysed, it was clear that the majority were elderly (>65 years). In contrast, sporadic clones were more frequently detected in young patients.

The distribution over time of isolates belonging to the main genotypes changed during the period of study. Therefore, although clone II was predominant in 1999 representing a 49% of isolates , in 2002 and 2005 the majority of isolates belonged to clone I with a percentage of 59.5 % and 80%, respectively. By the end of the study, in 2008, the percentage of isolates grouped into clone I was the 100%.

The global evolution of resistance among isolates from clone I showed significant and important increases for several antibiotics from 1999 to 2005, with changes in resistance to amikacin, tobramicin, imipenem and meropenem. All isolates from 2008 were almost completely resistant (> 90%) to cefotaxime, aztreonam, imipenem, meropenem, ciprofloxacin, cefepime and piperacillin-tazobactam. Resistance to ampicillin/sulbactam increased from 0% in 1999 to 57% in 2008. All isolates were susceptible to colistin.

In contrast, isolates belonging to clone II showed a decrease in resistance to several antibiotics including imipenem, meropenem, amikacin and tobramicin and data obtained in 2005 indicated that all isolates were susceptible to colistin, amikacin and tobramicin. The other clones showed higher levels of susceptibility to most antibiotics.

Another important change over time concerned the presence of the *bla*$_{OXA-40}$ gene among the predominant clones. Whereas all isolates belonging to clone II were positive for OXA-40 at the beginning of the study, the percentage of isolates from clone I that were positive for OXA-40 increased from 22% in 1999 to 96% in 2005 and, finally to 100% in 2008.

All isolates were PCR positive for *bla*$_{OXA-51-like}$ gene but no isolates were positive for *bla* $_{OXA-23-like}$ or *bla* $_{OXA-58-like}$ genes.

Other additional data can be seen in previous publications (23,45,46,55)

### 7.2. Study of representative isolates from clones I and II.

To analyze if there were genetic variations among the clones as well as the relation with the adquisition of the OXA-40 carbapenemase gene we studied a representative group of 15 isolates obtained from 1999 to 2005, and another 15 isolated in 2008 were studied in detail. MICs of these representative isolates for imipenem, meropenem, cefotaxime, ceftazidime, amikacin and gentamicin showed a similar multidrug-resistant profile for all antibiotics tested compared to those obtained using the disc diffusion method.

PFGE fingeprinting profiles showed differences between clone I isolates that were indistinguishable by PCR experiments performed previously. The clone II pulsotype showed the same profile over time, but clone I isolates showed variations in their profile indicating new genetic events, such as extra bands of 207, 166 and 128 kb combined with an absence of 389 or 381 kb fragments. Although we can not known the exact meaning of these changes in the prevalence of the genotype it is clear that they mean evolutional events that could be important to succeed. The stability

 

of *A. baumannii* genotypes over time has not been properly investigated and it is clear that additional approaches including sequence-based methods, will be needed to better delineate clones and follow their diversification over time (36,44,48,56).

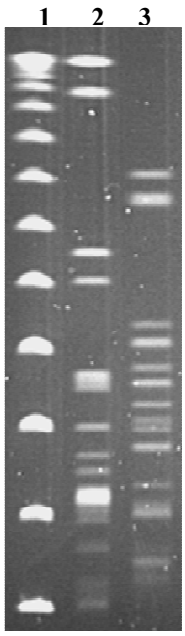


**Fig. 1.** PFGE profiles of *A. baumannii* isolates digested with *Apa* I enzyme. Lane 1, Molecular Weight Marker Lane 2, clone I ;Lane 3, clone II.

To deeply study some genetic markers, multiplex-PCR experiments were performed to search for other OXA-type enzymes and virulence factors as *csuE ompA* and *int*1genes.

A PCR designed to selectively amplify the *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$ alleles of a previously identified clonal complex designated Group 1 was carried out on all isolates, and sequencing of these genes was performed for representative isolates. Multiplex PCRs for the *csuE* and *ompA* genes showed that both clones harboured distinct alleles and sequence typing revealed that clones I and II belonged to two highly distinct lineages identified in the United Kingdom (sequence type groups 3 and 1 respectively) although these Iberian isolates lacked a $bla_{\text{OXA-23-like}}$ gene.

Comparison of representative isolates with control strains belonging to the major European lineages revealed a high similarity of our clone I and II with the EC I and II lineages, respectively. These results support the general conclusion that multidrug-resistant clones are spreading among European hospitals (7,43,44,47).

Once the genetic divergence of both OXA-40 producing clones had been clearly demonstrated, it was of interest to investigate the mobile structure responsible for dissemination of the OXA-40 carbapenemase gene as, except for the intrinsic OXA-51 enzyme, no other carbapenemases were detected.

Many clinical isolates carry class 1 integrons and their detection is used both as a resistance marker and as a typing method (2). Previous studies based on amplification from the conserved regions of the integrons had shown that all of the isolates in the present study carried class 1 structures (55). Isolates belonging to clone I yielded a 760 bp amplicon plus a 1500 bp from isolates obtained in 2008. All clone II isolates yielded a 550 bp and a 1200 bp amplicons. These integrons were not associated with the OXA-40 carbapenemase gene as no amplification product was obtained when primers corresponding for the integron structures and the OXA-40 gene were combined. This finding is in agreement with previous results from other authors revealing that the genes usually found on acinetobacter integrons are those coding resistance to aminoglycosides (15,38,55).

As preliminary findings indicated that plasmids could be the mobile element responsible for dissemination of the OXA-40 gene among the different clones, a more detailed study of these structures was performed. Plasmids of different sizes ranging from 2.5 to 125 kb were observed in all isolates belonging to both clones, with the most frequent plasmids being those of 2.5, 8 and 32 kb in size. The results showed that the same plasmids were present in both clones and that some structures were always present in groups. Hybridization experiments with an OXA-40 specific probe in order to locate the $bla_{\text{OXA-40}}$ gene showed different hybridization patterns although the most frequent signals corresponded to the 8 and 32 kb plasmids. In addition, many isolates also showed a positive hybridization signal with the chromosome. Endonuclease mapping of the 32 kb band and hybridization with the specific probe revealed that different fragments carrying the $bla_{\text{OXA-40}}$ gene could be detected. The 32kb plasmid coincided in size with the plasmid reported by Quinteira et al (27) in a Portuguese isolate, while the 8 kb plasmid was similar in size to a cryptic plasmid

described in clone II isolates (2). Digestion of plasmid DNA gave the same band profile as the control strain for OXA-40 carbapenemase indicating that it was the same plasmid previously identified in our hospital (28).

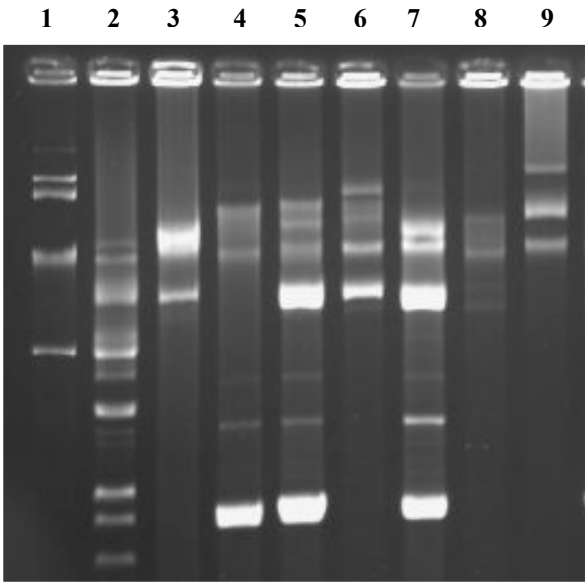


**Fig. 2** Different plasmid profiles obtained in the *A. baumannii* isolates. Lane 1, control strain CECT(679 (39R861); Lane 2, control strain CECT 678 (V517); Lanes 3 to 9, representative clinical isolates.

Clone I isolates from 2008 showed combinations of two/three plasmids as follows: a) 13 kb and 32/40 kb ; b) 10 kb, 13 kb and 40 kb.

As it was no clear if there were different plasmids or different forms of the same structure, S1 enzyme was used to linearize plasmid DNA. When S1 analysis was performed only one band was observed corresponding to the 32 kb plasmid in isolates from before 2008, and the 34/40 kb plasmid in isolates from 2008. These findings could be indicative of the acquisition of additional genes by the original plasmid, but further experiments are needed to elucidate this aspect.

Although there have been many publications about plasmids in previous years, until now, very little is known about those structures in *Acinetobacter baumannii*. This may be caused, in part, by the difficulty of plasmid isolation from this species (18, 31, 51, 56). In our experience, lysis often had to be repeated using different methods in order to obtain a useful result.

In conclusion previous results during the period of study, starting in 1999, had shown the systematic prevalence of two endemic OXA-40-producing clusters but, by 2008, only isolates belonging to clone I were isolated. These clones showed a high degree of homology with European clones I and II lineages. The *csuE* and *omp*A genes were present in all isolates, but each clone bore a different allele combination, indicating two clearly distinct genotypes. Sequence typing confirmed that clones I and II belong to two highly distinct lineages and comparison with clones from the United Kingdom showed its homology with sequence type groups 2 and 1 respectively. Multiplex PCR showed the presence of $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-40}}$ gene in all isolates.

In addition, the results indicated that all isolates contained plasmids of consistent sizes during the period of study mainly plasmids of 8 and 32 kb in isolates before 2008, and plasmids of 10/13 kb and 34/ 40 kb in isolates from 2008. Despite the high diversity found, the S1 linearization and hybridization studies indicated that the 32 kb plasmid was the main structure associated with the $bla_{\text{OXA-40}}$ gene from 1999 to 2005, but that a 34/40 kb plasmid was predominantly associated with $bla_{\text{OXA-40}}$ in 2008.

Further study of the biological properties of these widespread clones will be important to understand their evolutionary success and to determine whether they share special properties such as enhaced virulence or an increased capacity to survive in the hospital environment, or whether their success is merely due to the fact that they have acquired and express particular antimicrobial resistance genes.

Overall, although plasmids seem to be highly prevalent in acinetobacters, their significance in environmental strains, as well as the genes associated with them, is still largely unknown.

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