

Molecular techniques for detection and control of nosocomial infections caused by *Acinetobacter baumannii*

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Nosocomial infections caused by gram negative rods such as *Acinetobacter baumannii* are of great concern as they are responsible for serious infections as pneumonia, septicaemia, urinary tract infections, etc. The severity of these infections is intensified by the presence of multidrug-resistant strains, that make the treatment very difficult and, in many cases, lead to the death of the patient. For this reason it is essential to develop several techniques in order to identify them the sooner as possible and be able to take measures such as patient isolation that could help to control the spread among patients of these multidrug-resistant strains. In this aspect molecular techniques have been developed and are very useful to reach the identification to species level, to analyse the clonal relatedness of the microorganisms or to detect virulence genes. In this chapter we are going to explain the most useful molecular techniques and we are going to give a practical example where these techniques are used in order to analyse clinical isolates of *Acinetobacter baumannii*.

Keywords *Acinetobacter baumannii*, nosocomial infection, genotyping, multidrug-resistance.

1. Introduction

Nosocomial infections are a major cause of morbidity and mortality in hospitals [1]. The importance of these infections stems from the impact they have at the hospital; they involve an enormous increase in the cost of hospital care, as it is often required to stay extra days at hospital, to use different antibiotics or drugs, to carry out more diagnostic techniques, etc. which mean extra charges.

They occur with a higher prevalence in intensive care units and surgical wards, and affect especially immunocompromised patients. The most common nosocomial infections are those from the urinary tract, lower respiratory tract and wounds. Different microorganisms have been involved with these infections and *Acinetobacter baumannii* is emerging as a nosocomial pathogen responsible for severe infections [2, 3].

2. *Acinetobacter baumannii*: Emerging pathogen in nosocomial infections

Acinetobacter baumannii is a Gram-negative coccobacilli, non-motile, catalase-positive, oxidase-negative, non-fermenter, strictly aerobic and with a GC content of 38-47 %.

Different species of the genus *Acinetobacter* can be found in different environments. The species found in hospitals are *A. baumannii*, genomic species 3, and genomic species 13 TU, together forming the *A. baumannii*-complex; some species colonize the skin and are present in food as *A. johnsonii*, *A. lwoffii* and *A. radioresistens*; and other species can be found in the natural environment as *A. calcoaceticus* and *A. johnsonii*. Sometimes rare infections caused by non-clinical species such as *A. calcoaceticus*, *A. haemolyticus*, *A. junii*, *A. johnsonii*, *A. lwoffii*, *A. radioresistens* and *A. ursingii* are detected in the hospital. One special feature that differentiates hospital species from the others is the antibiotic resistance; while hospital species show antibiotic resistance, species from the other environments remain susceptible to antibiotics [4, 5].

The problem of *Acinetobacter* at hospitals is that it is widely spread due to its ability to contaminate floors, sinks, tabletops, doors, patient's charts, telephone handless, mattresses, pillows, bed linen and curtains with a great persistence of the strains along time, as they are very difficult to eradicate. Its survival in the clinical environment is due to its great resistance to dry conditions, disinfectants and antibiotics, which is related to the biofilm formation, and its capacity of using different metabolic sources [5].

Its pathogenicity and virulence factors make them responsible for severe nosocomial infections and outbreaks that are usually very difficult to treat due to their resistance to antibiotics, compromising in some cases the patient's life [4, 6]. *Acinetobacter* colonizes the skin and the respiratory tract due to its capacity of adherence to the host cells and to its resistance to the immune responses of skin and mucosal surfaces; it grows forming a biofilm, regulated by "quorum sensing". It has different virulence factors to damage the host tissues and to evade the immune response: a great capacity of adhesion, the production of the polysaccharide (capsule), the production of enzymes that damage soft tissues, endotoxins, lipopolysaccharide of the cell envelope and lipid A, and the production of slime, that inhibit neutrophils migration and increase the virulence in mixed infections [5, 7, 8].

The factors that promote colonization and damage the host are the induction of the inflammatory response, cytotoxicity (*ompA* produces damages in epithelial cells), iron uptake and resistance to complement activity.

Pneumonia, chronic bronchitis, bacteremia, meningitis, urinary tract infections and endocarditis are the major infection *Acinetobacter* causes, and affect specially immunocompromised patients. Other risk factors to develop this type of infections are the age (elderly people), patients with pre-existing diseases, under broad-spectrum antibiotic treatment, with mechanical ventilation, hospitalized for extended periods and burn wounds [8].

Infections are treated with different group of antibiotics: aminoglycosides, fluoroquinolones, betalactams of different groups: penicillins, cephalosporins, monobactams, carbapenems and betalactams with betalactamase inhibitors.

The most common *Acinetobacter* infections response to conventional treatment, but sometimes a multidrug-resistant phenotype is observed and carbapenems (imipenem and meropenem) are the drugs of choice for treating these infections resistant to other antibiotics. Unfortunately strains resistant to carbapenems have been reported worldwide, compromising the treatment that in many cases would lack of therapeutic alternative, with the subsequent death of the patient [9-12].

For all these reasons, it is essential to develop several techniques in order to identify antibiotic resistant strains the sooner as possible and be able to take measures such as patient isolation that could help to control the spread among patients of these multi-drug resistant strains.

3. Molecular techniques to control infections caused by *Acinetobacter baumannii*

Up to now, several molecular techniques can be used at three different levels to control infections caused by *Acinetobacter baumannii*:

3.1 Identification to species level:

Since *A. baumannii* is clinically by far the most significant of the *Acinetobacter* species, the ability to distinguish it rapidly from other members of the genus is of great value. Different techniques have been developed:

3.1.1 Identification of tRNA genes

tDNA fingerprinting uses primers designed to amplify the spacer regions of tDNA clusters. Different amplification profiles are observed when analysing different species, so this technique allows a rapid, simple and easy identification to species level. However, this method has some disadvantages because it does not discriminate between some species as *A. baumannii* and sp.13TU [13, 14].

3.1.2 Detection of the OXA-51 carbapenemase gene

OXA-51-like enzymes belong to the same group of naturally occurring oxacillinases in *A. baumannii*. This fact has been used to develop a PCR experiment with primers specific for this gene, which can be used as a simple and reliable way of identifying *A. baumannii* [15]. It must be considered that a little percentage of isolates doesn't have this gene, and recently it has been also detected in a clinical isolate of *Acinetobacter* genomic species 13 TU [16].

3.1.3 Multiplex *gyrB*.

The clinically relevant species *Acinetobacter baumannii* and *Acinetobacter* genomic species 3 and 13TU are often grouped together with the environmental *A. calcoaceticus* species as the *A. calcoaceticus*-*A. baumannii* complex because they are genetically closely related and phenotypically very difficult to differentiate from each other. The commercial identification systems API 20NE, Vitek 2, Phoenix and MicroScan Walk-Away, identify the bacteria to species level, but sometimes they are not able to differentiate among the species grouped in the complex. To solve this problem a multiplex PCR method based on the heterogeneity observed in the *gyrB* gene has been developed. The different band pattern observed allows the distinction between these species [17].

Figure 1 shows some examples of the different techniques used in the identification to species level that are described above. The different band pattern obtained in the tRNA and multiplex *gyrB* techniques allows the identification of the different species. The presence of the band corresponding to the *bla*_{OXA-51} gene identifies the *Acinetobacter baumannii* species.

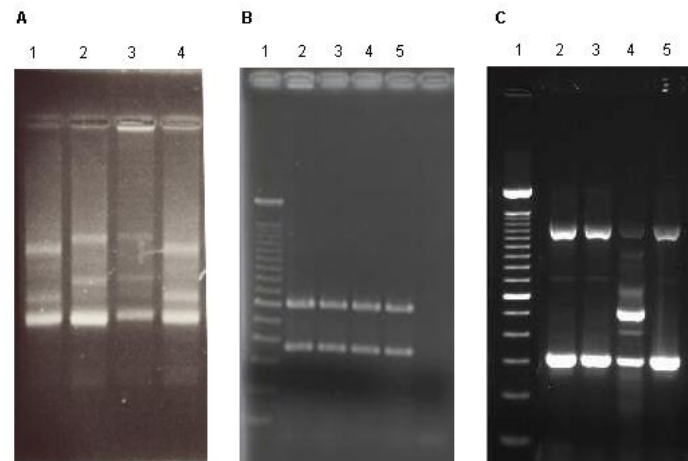


Fig.1. Identification to species level: **A:** Identification of tRNA genes. Lane 1: *A. baumannii*. Lane 2: *Acinetobacter* gs.2. Lane 3: *Acinetobacter* gs.3. Lane 4: *Acinetobacter* 13TU. **B:** Detection of the OXA-51 carbapenemase gene. Multiplex PCR to detect OXA-families. Lane 1: 100 bp DNA ladder. Lane 2-5: 353 bp band corresponding to the *bla*_{OXA-51}, and 599 bp band corresponding to the *bla*_{OXA-58}. **C:** Identification of heterogeneity in the *gyrB* gene. Lane 1: 100 bp DNA ladder. Lanes 2, 3 and 5: 300 bp and 1194 bp band pattern corresponding to *Acinetobacter* genomic species 13 TU. Lane 4: 300 bp and 500 bp band pattern corresponding to *Acinetobacter baumannii*.

3.2 Clonal relatedness

Typing techniques can be used to determine the origin and extension of an infectious outbreak, and to establish the mechanism of infection. They are very useful to study the evolution of the infection along time, and to evaluate the antibiotic treatment efficacy, as it can be influenced by the resistance levels of the microorganism and the patient's immune response.

In order to choose the most appropriate typing method some quality aspects must be taken into account; these include the typability of microorganisms, the reproducibility of the results, the discriminatory capacity and possibility of standardisation. Other important factor is the cost.

Some phenotypic techniques have been developed such as biotyping, antibiogram typing, serotyping, phage-typing or protein typing. These phenotypic methods have several disadvantages as they have a lower reproducibility and a lower discriminatory capacity; some isolates can't be typed as they depend on phenotypic expression.

Genotypic typing methods include some advantages over the phenotypic methods, and include the rapidity to get the results and a better sensitivity and specificity; in addition, results don't depend on phenotypic expression. Several genotypic typing methods have been developed; some of them are based on the use of restriction enzymes, other use the polymerase chain reaction, the analysis of plasmids or the DNA sequencing.

3.2.1 PCR-fingerprinting

The PCR has been widely used for genotyping. The different techniques developed are based on the use of different primers:

- PCR with primers corresponding to arbitrary or repetitive sequences of the bacterial genome: MAAP (multiple arbitrary amplicon profiling), acronym that include several techniques as AP-PCR (arbitrary primed-PCR), RAPD (random amplification of polymorphic DNA), and DAF-PCR (DNA amplification fingerprinting). For *A. baumannii* M13 or AP3 primers offer the best results. ERIC (enterobacterial repetitive intergenic consensus polymerase): for *A. baumannii* ERIC2 offers the best results. REP-PCR (repetitive extragenic palindrome sequence-based PCR) for *A. baumannii* the primer pair REP 1 and REP 2 has been used [18]. Figure 2 shows the band pattern obtained in a PCR-fingerprinting experiment with primer M13.

- Multiplex-PCR. Experiments in which more than one pair of primers are included allowing the detection of more than one gene in a single experiment [19].

- PCR experiments with subsequent restriction with enzymes and detection by hybridization with probes [20].

The advantages of these techniques are the rapidity, sensitivity, they allow the detection of fastidious organisms, there is no need of viable cells and a minimum sample is needed. However, they have a problem of false-positive/negative results, a lower reproducibility, they don't allow interlaboratory validation, and a clinical interpretation is needed.

3.2.2 Pulsed Field Gel Electrophoresis

This technique is the golden standard for DNA-fingerprinting. This method allows the separation of very large molecules of DNA by introducing an alternating voltage gradient that is periodically switched among three directions; one that runs through the central axis of the gel and two that run at an angle of 120 degrees either directions. A bacterial suspension is embedded in agarose plugs, and then treated with enzymes to digest the cell wall and proteins, leaving the naked DNA undamaged in the agarose. Then a low-frequency cutting enzyme is used to digest the DNA (*ApaI* restriction enzyme is used for *A. baumannii*), and then the plugs are loaded in the agarose gel and placed in the electrophoresis chamber with the following conditions: Ramps: 5-35 sec. for 30 hours. Related isolates have the same restriction patterns, and non related isolates different restriction patterns [21].

Figure 2 shows the band pattern obtained in a PFGE experiment with *ApaI* restriction enzyme.

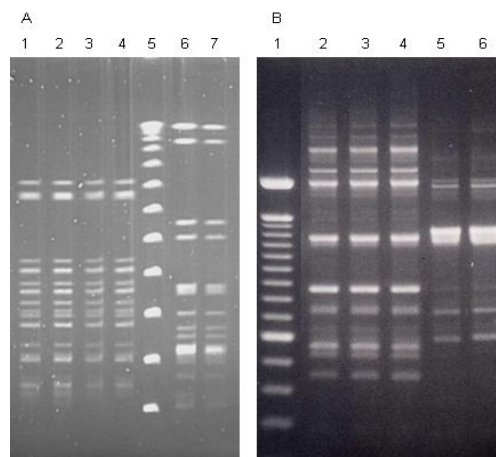


Fig.2. Examples of different techniques to analyse clonal relatedness. **A:** PFGE. Lanes 1-4: band pattern of isolates from clone II. Lane 5: λ DNA ladder. Lane 6,7: band pattern of isolates from clone I. **B:** PCR-fingerprinting with M13. Lane 1: 100 bp DNA ladder. Lanes 2-4: band pattern of isolates from clone I. Lanes 5, 6: band pattern of isolates from clone II.

3.2.3 Plasmid analysis

Plasmids are circular extra chromosomal elements that may encode a variety of supplementary genetic information, including the information of self-transfer to other cells, resistance to antibiotic, bacteriocin production, resistance to toxic metal ions, production of toxins and other virulence factors, reduced sensitivity to mutagens, and the ability to degrade complex organic molecules. They replicate independently of the chromosome, and are ubiquitous in bacteria.

In *Acinetobacter baumannii* clinical isolates plasmids seem to be highly prevalent. They carry genes encoding antibiotic resistance and transfer mechanisms, but due to the difficulty of analysing plasmids in *A. baumannii* little information is available concerning the presence of other genes associated with these plasmids.

Structures of different sizes have been detected; some of them appear with a higher prevalence as the 8 kb, 2 kb or 32 kb plasmids, which have been detected in unrelated isolates from different studies [22, 23].

Plasmid analysis can be carried out by conventional lysis methods (by alkali, SDS, proteinase K...), that yield good results in other bacteria, but sometimes not as good in clinical isolates of *A. baumannii*. Some commercial kits have been developed, that in some cases could yield better results. Both conventional and commercial methods can be also combined for a better result. These conventional techniques have several limitations, for instance when determining the exact size of the plasmid, as plasmid conformation affects electrophoretic mobility. In addition, megaplasmids are not visible and they break easily.

To avoid these inconvenients PFGE/S1 nuclease digestion technique has been developed. With this technique intact plasmidic DNA is obtained, and digestion with S1 nuclease linearizes the plasmid allowing the determination of the size. This technique makes megaplasmids visible [24].

3.2.4 DNA sequencing

Single-locus sequence Typing (SLST) is based on individual nucleotide differences in genes coding for virulence, pathogenicity, antibiotic resistance, etc. It identifies polymorphisms of a single nucleotide or repetitive-sequence areas.

Multilocus sequence typing (MLST) is based on the analysis of a major portion of genome comparing regions of 400-500 bp corresponding to "housekeeping" genes (>7). Polymorphisms of sequences are considered alleles, and isolates are defined by its allele profiles corresponding to sequenced locus.

3.3 Detection of virulence genes

For the detection of virulence genes different techniques such as hybridization with probes, nucleic acid amplification... have been developed. In the last few years the multiplex-PCR has acquired a great relevance due to its rapidity, specificity, and because it allows the amplification of different genes at the same time.

3.3.1 Antibiotic resistance

Several multiplex-PCR techniques have been developed for detecting genes that confer resistance to the different classes of antibiotics:

Resistance to carbapenems: multiplex-PCR for the main OXA-type carbapenemase families detected in *Acinetobacter baumannii*: OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like and OXA-143-like; or for the main metallo- β -lactamase families: VIM, IMP, SPM, GIM and SIM. [21, 25-29]

Resistance to aminoglycosides: multiplex-PCR to identify DNA sequences encoding the aminoglycoside acetyltransferase (AAC), adenyltransferase (ANT), and phosphotransferase (APH) enzymes. (assay1: *aac(3)-Ia*, *aac(3)-IIa*, and *aac(6)-Ih*, assay 2: *aph(3)-VI*, *ant(2)-Ia*, and *rrn*; assay 3: *aph(3)-Ia* and *aac(6)-Ib*) [30].

3.3.2 Mobile genetic elements

The virulence genes may be integrated in some mobile genetic elements as integrons, transposon or plasmids. This is of great concern as these structures can be transferred between isolates contributing to the spread of the virulence genes. Integrons can be detected by PCR using primers for detecting the different classes of integrases, or by using primers corresponding to the conserved sequences. Different methods have been developed to analyse plasmids (see detection in section 3.2.3). Other structures as the insertion sequences are frequently related with the virulence genes and can be detected by PCR using the corresponding primers. [22, 31, 32].

3.3.3 Virulence genes

Among the most important virulence genes we can find the *ompA* (major outer membrane protein gene) and *csuE* gene (involved in attachment and biofilm formation). They can be easily detected in one single experiment by multiplex-PCR including the corresponding primers [19].

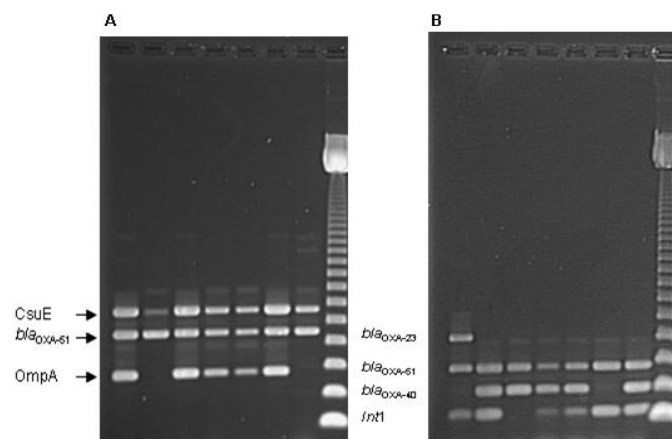


Fig.3. Detection of virulence genes. A: Multiplex-PCR to detect *csuE*, *ompA* and *bla_{OXA-51}* genes. B: Multiplex-PCR to detect some *bla_{OXA-like}* genes, and the integrase gene *Int1*.

For a better understanding of the usefulness of these techniques we describe a practical example based on our own experience analysing clinical isolates of multidrug-resistant *Acinetobacter baumannii* from 1999 to 2008 from a hospital of the Public Health Service in Northern Spain.

4. Molecular techniques applied to the detection and control of emerging *Acinetobacter baumannii* infections

4.1 Objectives:

The objectives of this study were:

To determine the level of resistance in *A. baumannii* clinical isolates obtained from a hospital of our environment (Basque Public Health Service Hospital, Northern Spain).

To develop DNA fingerprinting techniques for rapid and specific clonal identification in the hospital environment and to determine the origin and transmission of resistant isolates.

To detect and characterize the presence of genetic structures as integrons and carbapenemases as a resistance mechanism.

To study the evolution of the resistant isolates and the factors involved in their spread.

4.2 Work plan:

To achieve these objectives we carried out the following analysis:

Determination of the susceptibility to antibiotics (MIC)

Clonal identification: RAPD-PCR fingerprinting (primers M13 and ERIC 2) and pulsed-field gel electrophoresis (PFGE) with *ApaI*.

Resistance mechanisms: Aminoglycoside-inactivating enzymes and carbapenemases.

Detection of genetic mobile structures related to carbapenem resistance genes: Class 1 integrons (PCR, *HinfI* mapping) and plasmids.

Analysis of the evolution of the predominant clones and genetic structures related to antibiotic resistance.

4.3 Materials and Methods

Bacterial isolates. We recovered all clinical isolates of *Acinetobacter baumannii* obtained during the years 1999, 2002, 2005 and 2008 in a hospital of OSAKIDETZA (Basque Health Service hospital, Bilbao, Northern Spain). This hospital has 240 beds and although it is a general hospital, the most of the patients are elderly people with extended period of hospitalization, from 1 to 3 months. A total of 102 isolates were recovered during 1999, 86 in 2002, 30 in 2005 and 50 in 2008.

Antibiotic susceptibility testing. Antibiotic susceptibility was analysed by disk diffusion method and by determining the MIC (Minimal Inhibitory Concentration) to the following antibiotics: amikacin, gentamicin, tobramycin, trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid, ampicillin, aztreonam, cefazolin, cefepime, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, colistin, imipenem, meropenem, piperacillin/ tazobactam, rifampicin, fosfomycin, nitrofurantoin, and ciprofloxacin, according to the current CLSI guidelines [33].

Clonal relatedness. Clonal relatedness of the isolates was investigated by RAPD-PCR-fingerprinting with the primers M13 and ERIC2, and by PFGE with the *ApaI* enzyme [21, 34, 35]. Digital images of the gels were analysed using the 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 (SAB) for every pair of strains. Those isolates with an SAB value of >0.72 were clustered together. For PFGE the isolates were grouped into different clones following the criteria of Tenover *et al.* [36].

Detection of carbapenemases as a resistant mechanism to imipenem. Detection of metallo-beta-lactamase genes: *bla_{VIM-1}*, *bla_{VIM-2}*, and *bla_{IMP}* was carried out using PCR with the corresponding primers. [37, 39] Detection of class D beta-lactamases was done using a multiplex PCR to detect the main families of OXA-type carbapenemases: OXA-23-like, OXA-40-like, OXA-51 like, OXA-58-like and OXA-143-like. [21, 26].

Detection of class 1 integrons. PCR was performed with primers corresponding to the conserved sequences 5'CS and 3'CS. [34, 37]. To identify the presence of carbapenemase genes within the integron, PCRs were developed combining 5'CS with the corresponding OXA-40, VIM and IMP reverse primers under the same conditions as those for amplification of individual genes.

Plasmid analysis. Plasmid extractions were obtained using the Qiagen Plasmid Midi kit, following the manufacturer's instructions. Plasmids were visualized on agarose gels separated by electrophoresis.

Hybridization experiments. Gels of PCR amplicons or total DNA were transferred to a nylon membrane and hybridized with the corresponding carbapenemase probes made from PCR-generated fragments and labelled with digoxigenin-dUTP [20]. Detection of hybrids was carried out using an anti-digoxigenin antibody following the manufacturer's instructions (Roche Diagnostics).

4.4 Results

Genetic typing. Distinct genotypes were recognized in 1999 but the majority of the isolates were grouped into two main genotypes named clone I (27,4 %) and clone II (49 %). These two major clones were also detected in 2002 and 2005 but the proportions differed: clone I accounted for 63,4% and 83,3 %, whereas clone II accounted for 16,6% and 16,6 % of the isolates in 2002 and 2005 respectively. In 2008 all isolates belonged to clone I. (See table 1)

Antibiotic resistance. From 1999 to 2005 there were important increases in resistance to aminoglycosides and carbapenems in isolates of clone I; in 2008 resistance rates slightly decreased for some antibiotics, but not for imipenem as resistant rates increased along the period of study from 32 % in 1999, to 100 % in 2008. Resistance rates among isolates from clone II also increased through the years for the most of the antibiotics, but resistant rates to imipenem decreased from 84 % in 1999 to 40% in 2005 (no isolates belonging to clone II were detected in 2008).

Since clone I became predominant at the same time that resistance rates increased, the main interest of this study was to analyse the mechanisms that could be involved, such as the presence of carbapenemases and the presence of genetic mobile elements such as class 1 integrons or plasmids.

Presence of carbapenemases. We detected the OXA-40 carbapenemase in our isolates in 1999; in addition, it was the first description of this enzyme. The presence of this *bla*_{OXA-40} gene was very high in isolates from clone II (98 %) and maintained this level through the years. In isolates from clone I the presence of this gene increased along the study period from 20 % in 1999 to 100 % in 2008, at the same time that this clone became predominant. No metallo- β -lactamases were detected.

Class 1 integrons. The spread of antibiotic resistance genes has been associated with the presence of class 1 integrons, the most common and prevalent in clinical isolates. In 1999 all isolates of clone I harbored 760 bp integrons, which carried genes for aminoglycoside-modifying enzymes. In 2002, 50% of clone I isolates carried not only these integrons, but also combinations of other 1200 and 550 bp structures. This pattern was also detected in 2005 and 2008. Although other mechanisms cannot be discarded, we infer that these elements contributed to the increase in resistance to aminoglycosides in the isolates analysed in the study. In 1999, isolates of clone II harbored a unique 550 bp integron, but another structure of 1200 bp was also present in 2002-2008.

Plasmids. All isolates from both clones presented plasmids of different sizes: 112 kb, 84 kb, 32 kb, 8 kb and 2.5 kb. Some isolates presented all these plasmids at the same time, but others had just some of them. The most frequent sizes were 2.5 kb, 8 kb and 32 kb. Plasmid digestion using *EcoRI*, *PstI* and *HindIII* endonucleases and Southern transfer hybridization experiments with a *bla*_{OXA-40} probe labeled with dUTP-digoxigenin located the *bla*_{OXA-40} carbapenemase gene in the 32 kb plasmid.

Table 1 Clonal relatedness, antibiotic resistance and presence of carbapenemases of clinical isolates of *A. baumannii*.

PFGE	Year (Number of isolates)	Antibiotic resistance						<i>bla</i> _{OXA-40}	<i>bla</i> _{OXA-51}
		CTX	CAZ	IMP	MEM	AMK	GEN		
I	1999 (28)	85 %	68 %	32 %	25 %	18 %	75 %	20 %	100 %
	2002 (52)	91 %	85 %	75 %	85 %	85 %	83 %	91 %	100 %
	2005 (25)	100 %	96 %	100 %	100 %	76 %	100 %	98 %	100 %
	2008 (50)	94 %	63 %	100 %	93 %	45 %	88 %	100 %	100 %
II*	1999 (50)	100 %	90 %	84 %	88 %	52 %	94 %	98 %	100 %
	2002 (17)	100 %	100 %	71 %	82 %	65 %	71 %	98 %	100 %
	2005 (5)	100 %	80 %	80 %	80 %	0 %	80 %	98 %	100 %
Others*	1999 (24)	70 %	58 %	41 %	33 %	8 %	95 %	0 %	100 %
	2002 (13)	58 %	66 %	33 %	20 %	66 %	45 %	0 %	100 %

* No isolates belonged to clone II in 2008 and to other clones in 2005 and 2008. CTX: ceftriaxone, CAZ: ceftazidime, IMP: imipenem, MEM: meropenem, CAZ: ceftazidime, GEN: gentamicin.

4.5 Conclusions:

We detected two *Acinetobacter baumannii* endemic clones, named I and II whose prevalence changed along time. PFGE techniques let us know that these clones corresponded to European clones I and II; in addition sequence of *ompA* and *csuE* genes showed similarity between clone I with SG 3 from the United Kingdom and clone II with SG 1.

A multidrug-resistant phenotype was predominant in the clinical isolates. Molecular techniques to investigate the presence of carbapenemases revealed the presence of the OXA-51 carbapenemase in all isolates and the OXA-40 carbapenemase in a percentage of isolates that increased along the study period.

All isolates showed class 1 integrons ranging in size from 550 to 1200 bp, and plasmids from 2.5 to 125 Kb. Integrons contained aminoglycoside-modifying enzymes and the 32 kb plasmid contained the *bla*_{OXA-40} carbapenemase gene. These mobile genetic structures contributed to the spread of antibiotic resistance.

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