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"Molecular bases of antimicrobial resistance in *Acinetobacter* spp. clinical isolates."

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CERTIFICA:

Que el trabajo de investigación titulado **"MOLECULAR BASES OF ANTIMICROBIAL RESISTANCE IN *ACINETOBACTER* SPP. CLINICAL ISOLATES",** presentado por Sara Martí Martí, ha sido realizado en el Laboratorio de Microbiología del Hospital Clínic de Barcelona, bajo su dirección y cumple todos los requisitos necesarios para su tramitación y posterior defensa frente al Tribunal correspondiente.

Firmada: Dr. Jordi Vila Estapé

Director de la tesis doctoral

Barcelona, Septiembre de 2008

Por fin!!! Parece mentira pero al final todo se acaba. Cuando llegué era "la de Londres" pero con el tiempo me hice un pequeño agujerito en este laboratorio. Ahora ya está, la tesis está escrita y hay que seguir adelante. Pero como Goethe dijo: *"Si yo pudiera enumerar cuánto debo a mis grandes antecesores y contemporáneos, no me quedaría mucho en propiedad"*.

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If we knew what it was we were doing,

it would not be called research, would it?

Albert Einstein

A person who never made a mistake,

never tried anything new.

Albert Einstein

All truths are easy to understand once they are discovered;

the point is to discover them.

Galileo Galilei

The most exciting phrase to hear in science,

the one that heralds new discoveries,

is not “Eureka!” but “That’s funny…”

Isaac Asimov

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I. INTRODUCTION

The miasma theory originated in the Middle Ages and carried on as the main theory of disease transmission for centuries. The miasmic theory of disease alleged that diseases such as cholera or the Black Death were caused by a miasma (Greek language: “pollution”), a poisonous vapour filled with particles from decomposed material. During the Great Plague of 1665, doctors wore masks full of sweet-smelling flowers to keep out the poisonous miasmas; they also sanitized some buildings and drained the accumulation of dirty waters to get rid of the bad smells. However, this way of thinking only worked when something smelled bad; in the winter, all the sanitation measures were forgotten. In the 1800s, this theory developed into the “Bad Air theory” which was used as an explanation for the spread of cholera in London and Paris. Miasmic reasoning prevented many doctors from adopting more hygienic practices such as hand washing between patients because they thought that “lethal agents travelled by air and not lodged beneath a doctor’s fingernail” (136). Although the miasma theory resulted to be incorrect, it proved that there was a relationship between dirtiness and disease; it encouraged cleanliness and opened up the way for the public health reform. Florence Nightingale firmly believed in miasmas and worked in making hospitals clean, fresh and airy (136).

Medical practice has changed greatly since the 19th Century: Semmelweis introduced the use of antiseptics to prevent disease; he postulated that doctors and students were contaminating their hands when performing autopsies on non-refrigerated bodies and were passing the fatal agent to the hospitalized patients. A few years after, Lister developed a system of antiseptic surgery designed to prevent microorganisms from entering wounds. He sprayed with phenol surgical instruments, dressings and incisions; in addition, he instructed surgeons to wash their hands and wear clean gloves before and after operations (169). As the germ theory substituted the miasma theory, sterile surgery became a common

practice. With the posterior discovery of the antimicrobial agents and vaccines, control and eradication of some diseases was a fact. Antibiotics were treated as miracle drugs and there was a huge abuse and misuse. There was a clear perception that antibiotics were Ehrlich's "magic bullet" and they would eliminate all the microorganisms causing disease.

Nowadays, although there has been a huge improvement compared to the times of the miasmic theory, hospitals remain as important focus of infection. Gathering large numbers of infected people under one roof has many advantages but it also favours transmission of infections from one person to another (138). Hospital infections also known as nosocomial infections (Greek *nosos*, disease, and *komeion*, to take care of) are caused by pathogens that are present in the hospital or other type of clinical care facility. Most of these infections emerge while the patient is in hospital but some of them are not detected until the patient has been discharged (168). Pathogens responsible for nosocomial diseases come from either an endogenous or exogenous source. Endogenous sources come from the patient's own microbiota and are responsible for auto-infections with microorganisms from another site within the patient. These pathogens are brought into the hospital by the patient or are acquired by colonisation after admission in the hospital. Exogenous sources come from the hospital environment, other patients, hospital personnel or from an endogenous source and are responsible for cross-contamination (138,168).

In the 21st Century, with the antibiotic resistance spreading, nosocomial infections are an important cause of concern. To the naked eye, hospitals may look clean; however, there are millions of potentially dangerous pathogens on the environment. Cross-infections are becoming a major health concern for admitted patients and also a liability for hospital administrators. These infections generally affect immunocompromised patients in the Intensive Care Unit (ICU) and they have become so common due to four main reasons (232):

- Hospitals congregate huge numbers of ill people whose immune system is often weakened. The shift to outpatient care is leaving in the hospital the most vulnerable patients, who are extremely susceptible to infections.
- Medical staff moves from one patient to another, helping the spread of microorganisms and the cross-contamination. Many hospital personnel still fail to follow basic infection control procedures, such as hand washing between patient contacts.
- Many medical and surgical procedures are very aggressive, by-passing the body's natural protective barriers; these interventions create a cluster of particularly susceptible patients especially in intensive care units.
- Use of antimicrobial agents creates selection pressure for the emergency of resistant strains. Nowadays, medical institutions face a resident flora of "super-bugs", resistant to the most aggressive antimicrobial therapies.

The pathogens responsible for nosocomial infections may be different from those responsible for community-acquired infections. Generally, nosocomial pathogens are specialised for survival in the hospital environment where they have to support a high use of antiseptics and antibiotics. In hospitals, especially in emergency rooms and ICUs, difficulties to predict the susceptibilities of the causative nosocomial pathogens trigger doctors to employ blind therapies which usually involve the utilization of broad spectrum antimicrobial agents to cover the maximum number of possible pathogens (91). Nosocomial infections have always been present in hospitals; however, the nosocomial pathogens have changed over the years. In Semmelweis's time, most nosocomial problems were associated with streptococci. *Staphylococcus aureus* took over during the next decades until the pandemic between 1940 and 1950. In the 70s, the main nosocomial pathogens were Gram-negative bacilli, especially *Pseudomonas aeruginosa* and *Enterobacteriaceae.* In the 90s, methicillin-resistant *S. aureus* (MRSA) and vancomycin-

resistant enterococci (VRE) emerged in hospitals worldwide (232). Nowadays, MRSA and VRE (mainly in USA) remain the major Gram-positive pathogens of concern in hospitals; nevertheless, during the past 20 years there has been an increment in the number of Gram-negative bacilli causing infection principally in ICUs. These units, and their patients, provide an important niche for opportunistic microorganisms, generally harmless for healthy individuals, which are often highly resistant to antibiotics and can spread epidemically among patients. The introduction of newer broad-spectrum antibiotics in hospitals produced an increase in the importance of strictly aerobic Gram-negative pathogens and the selection for multi-drug resistant (MDR) nosocomial pathogens. Among them, *Pseudomonas aeruginosa, Klebsiella pneumonia* and *Acinetobacter baumannii* quickly develop multiple resistance mechanisms to different classes of antimicrobial agents and are important for a considerable proportion of infections in patients in ICUs worldwide (11,54). Within these newly acquired nosocomial pathogens, it is now recognized that *Acinetobacter* spp., especially *A. baumannii,* play an important role in the colonization and posterior infection of patients admitted to hospitals (11).

1. CHARACTERISTICS OF THE GENUS *ACINETOBACTER*

Currently, *A. baumannii* is becoming an important emerging nosocomial pathogen worldwide and is responsible for 2-10 % of all the Gram-negative infections in ICUs (180). These microorganisms are known principally for their role as causative agents of nosocomial pneumonia, and particularly ventilator-associated pneumonia. In addition, they have also been implicated in a variety of nosocomial infections such as bacteraemia, urinary tract infection, and secondary meningitis. Although some cases of community-acquired infection have been described, the importance of *Acinetobacter* spp. lies mainly in their role as nosocomial pathogens where they stand out due to their ability to colonize almost any surface and to acquire antibiotic resistance (11,63). Despite the huge increment in the frequency of infections caused by MDR *Acinetobacter* spp., there is still a lack of awareness of the potential importance of these microorganisms, possibly due to the initial taxonomic confusion (63).

1.1 Taxonomic characteristics

The history of the Gram-negative bacteria which comprise the genus *Acinetobacter* is complex. These ubiquitous bacteria have been independently isolated from different sources by different authors leading to a considerable taxonomic confusion (5).

In 1911, Beijerinck identified the first strains of *Acinetobacter* spp. from soil and were named *Micrococcus calcoaceticus* (5). The genus *Acinetobacter* was created in 1954 by Brisou and Prévot and was composed by Gram-negative saprophytes which did not produce pigments; this genus included both oxidase-negative and oxidase-positive species (11,99). Three years later, Brisou assigned *Acinetobacter anitratum* as the type species (5). Later on, Baumann *et al*. (6) demonstrated by extensive nutritional studies that these strains could be easily separated from the oxidase-positive *Moraxella* group and proposed

to classify them in the genus *Acinetobacter*. In 1971, the "subcommittee on *Moraxella* and allied bacteria" accepted the proposal, and the genus *Acinetobacter* was limited to oxidase-negative strains (118). Within this group, Baumann *et al*. proposed the existence of three species, including *A. calcoaceticus* as type species. At this point, it was still difficult to distinguish the different species based on physiological characteristics; therefore, at the beginning of the 1980s all the strains were generally named *A. calcoaceticus* (5). Indeed, the genus *Acinetobacter* was classified by the "Bergey's Manual of Systematic Bacteriology" in the family *Neisseriaceae* (107), with only *A. calcoaceticus* as species and two subspecies that were *Acinetobacter anitratus* and *Acinetobacter lwoffii* (17). Recent taxonomy developments have allowed the classification of the genus *Acinetobacter* in the family *Moraxellaceae* within the order *Gammaproteobacteria*, which includes the genus *Moraxella, Acinetobacter, Psychrobacter,* and related organisms (160,188), and which constitutes a discrete phylometric branch in superfamily II of the *Proteobacteria* on the basis of 16S rRNA studies and rRNA-DNA hybridization assays (11).

Gram-negative bacteria from the genus *Acinetobacter* have been classified previously under at least 15 different "generic" names, the best known of which are *Bacterium anitratum* (195); *Herellea vaginicola* and *Mima polymorpha* (41); *Achromobacter, Alcaligenes, Micrococcus calcoaceticus*, and "B5W" (106); and *Moraxella glucidolytica* and *Moraxella lwoffii* (19,163). However, in 1986, the taxonomy of the genus *Acinetobacter* was reorganized by combining the results of DNA-DNA hybridizations with the phenotypic characteristics (5). **The genus *Acinetobacter* is now defined as Gram-negative nonfermenting coccobacilli, with a DNA G+C content of 39 to 47 mol%, that are strictly aerobic, no motile, catalase positive, and oxidase negative** (11,160).

In 1986, Bouvet and Grimont proposed twelve different genomic groups or genospecies based on DNA/DNA hybridization studies. Posterior works done by Bouvet and Jeanjean, Tjernberg and Ursing, and Nishimura *et al*. resulted in the description of additional genospecies. At present, studies based on DNA/DNA hybridization have resulted in the description of 31 validated “genomic species”, 17 of which have been given a valid species name (Table 1) (160); numbers have been assigned to the other genospecies. It is important to note that some of the genomic species have been described independently by Bouvet and Jeanjean (18) and Tjernberg and Ursing (213). Due to minor discrepancies in the numbering system, the suffixes BJ or TU are added to the number of the genospecie to indicate which study they come from (216). In addition, there is a close relationship between the genomic species *A. calcoaceticus, A. baumannii*, and the genospecies 3 and 13; therefore, as a result of the difficulties to differentiate the isolates according to their phenotypic characteristics, the term *Acinetobacter calcoaceticus*-*Acinetobacter baumannii* complex is often used (ABC-complex). Nevertheless, some authors still report these isolates as *A. calcoaceticus* subspecies *anitratus*. This situation has led contributors to the Manual of Clinical Microbiology (196) to affirm that the majority of the genomic species cannot be reliably distinguished by phenotypic tests (66,180,196). All this complicated taxonomic history has led to the under-recognition and misclassification of the species in clinical practice (101).

Table 1. Genomic species of the genus *Acinetobacter*

Genomic species name	Type strain	Reference
Acinetobacter calcoaceticus	ATCC 23055	(6,17,213)
Acinetobacter baumannii	ATCC 19606	(17,213)
Acinetobacter baylyi	DSM 14961	(21)
Acinetobacter bouvetii	DSM 14964	(21)
Acinetobacter gerneri	DSM 14967	(21)
Acinetobacter grimontii	DSM 14968	(21)
Acinetobacter haemolyticus	ATCC 17906	(17,213)
Acinetobacter johnsonii	ATCC 17909	(17,213)
Acinetobacter junii	ATCC 17908	(17,213)
Acinetobacter lwoffii	ATCC 15309	(17,20,213)
Acinetobacter parvus	NIPH 384	(148)
Acinetobacter radioresistens	IAM 13186	(150)
Acinetobacter schindleri	NIPH 1034	(147)
Acinetobacter tandoii	DSM 14970	(21)
Acinetobacter tjernbergiae	DSM 14971	(21)
Acinetobacter towneri	DSM 14962	(21)
Acinetobacter ursingii	NIPH 137	(147)
*Acinetobacter venetianus**	ATCC 31012	(43,224)

*** *A. venetianus* is a microorganism found in marine water but it does not have a formal species status.**

1.2 Identification in the laboratory

1.2.1 Identification to the genus level

The members of the genus *Acinetobacter* are short, plump rods (1.0 to 1.5 by 1.5 to 2.5 µm) in the logarithmic phase of growth and they generally adopt a more coccoid shape in the stationary phase with a tendency to group in pairs or also to form chains of a variable length (Fig. 1 & 2). The cell wall is typical of Gram-negative bacteria, however they are difficult to destain and occasionally they can be confused with Gram-positive cocci (1). Single pure cultures of *Acinetobacter* spp. often present variability in the Gram-stain together with variations in cell size and arrangement (11). These microorganisms are no motile even if some “twitching” or “gliding” on semisolid media has occasionally been reported; in addition, they do not form spores and flagellae are absent (216).

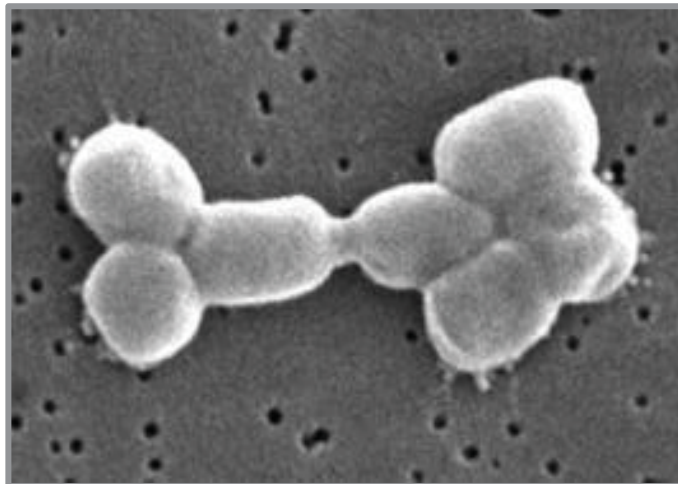

Fig. 1: A cluster of *A. baumannii* visualised by electron microscopy.

(Photography CDC / Janice Carr)

Morphologically, *Acinetobacter* spp. generally form smooth and sometimes mucoid colonies on solid media (Fig. 3), with a colour ranging from white to pale yellow or greyish-white (11,216); nevertheless, some environmental strains have been described to produce a diffusible brown pigment. Some clinical isolates may also show haemolysis on sheep blood agar plates, although this property is never found in the members of the ABC complex (11,160).

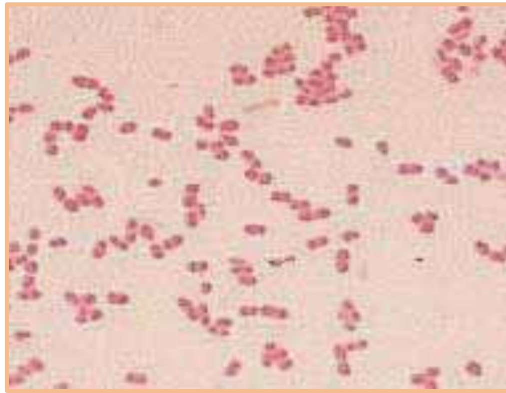

Fig. 2: Gram stain of *A. calcoaceticus*. (Photography CDC / Dr. WA Clark)

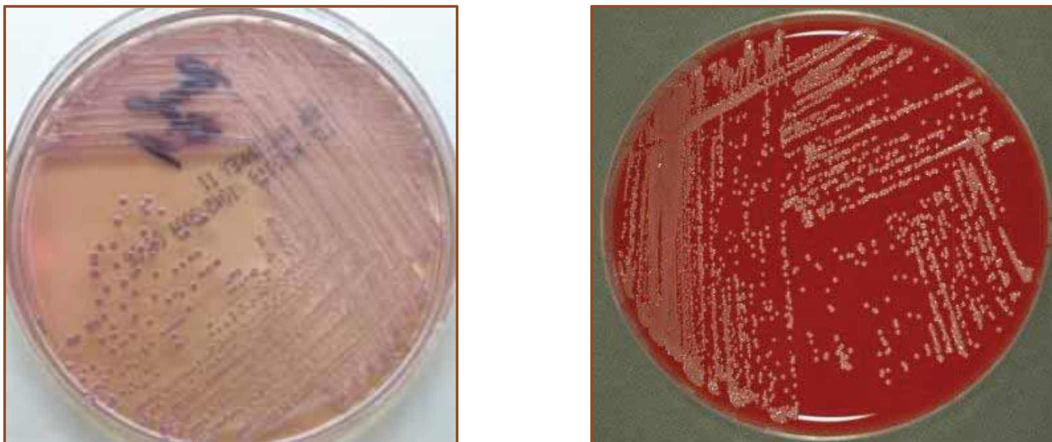

Fig. 3: Bacterial growth of *A. baumannii* in MacConkey and Blood agar plates.

Phenotypically, these microorganisms are strict aerobes, oxidase and indol negatives, catalase positives and nonfermentatives. The oxidase test serves to differentiate the genus *Acinetobacter* from other related nonfermentative bacteria (11,216). Growth occurs at a wide range of temperatures; environmental isolates prefer incubation temperatures between 20-30 ºC in contrast with the clinical isolates that normally grow at 37 ºC or some of them even at 42 ºC (216). They are non-fastidious microorganisms that easily grow in a simple mineral medium with single carbon and energy source; however, few strains can use glucose as carbon source. Most strains are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay (11,216).

Isolation of *Acinetobacter* spp. can be achieved with standard laboratory media such as trypticase soy agar or brain heart infusion agar. Members of the *Acinetobacter* spp. from soil and water can be isolated by growing the samples on Baumann's Enrichment Medium; this methodology is rarely used with clinical specimens. For direct isolation of clinical specimens, the use of selective medium such as MacConkey may be helpful in recognizing colonies but it is preferable to use a selective medium that suppresses the growth of other microorganisms (11,216). A selective medium modified by addition of various antibiotics has been commercialized by Difco as Herellea agar (95). In addition, the Leeds Acinetobacter Medium is effective for the recovery of most *Acinetobacter* genospecies from both clinical and environmental sources (216).

1.2.2 Identification to the genomic species level

As *Acinetobacter* spp. are widespread in nature, typing methods are needed to differentiate strains in epidemiological studies (216). An accurate identification of *Acinetobacter* spp. at the species level is important for the selection of the appropriate therapy because differences in antimicrobial efficacy against strains from different species has been demonstrated (123). Differentiation of the genus *Acinetobacter* from other related bacteria is accomplished by a combination of nutritional tests, including most commercially available diagnostic devices and systems. On the other hand, phenotypic identification to the genomic species level is more difficult because commercial identification systems do not separate between the different genospecies (216), especially within the ABC-complex. Kampfer *et al.* (108) designed a scheme of 22 phenotypic test to differentiate most of the genomic species but this method is extremely laborious and time-consuming. Biotyping methods are based on biochemical tests and can be used for comparative typing of strains (11). Among them, the API 20NE (BioMérieux, France) is currently used in hospital laboratories and although it is reliable, it usually requires complementation with other biochemical analysis such as growth at 44 ºC to identify *A. baumannii* (14).

Serological identification has been attempted with the analysis of capsular types (217) and also with studies of lipopolysaccharide molecules (156). Protein profiles have been used in epidemiological and taxonomic studies and have allowed a successful identification of specific strains during endemic episodes and outbreaks in hospitals (11).

At present, molecular typing is the main methodology to distinguish the individual genomic species. The most commonly used methodologies are the study of the **ribosomic RNA (ribotyping), ribosomic DNA (Amplified 16S Ribosomal DNA Restriction**

Analysis "ARDRA"), tRNA spacer fingerprinting, and the **study of chromosomal DNA** principally by DNA-DNA hybridization and amplified fragment length polymorphism (AFLP). Pulsed-field gel electrophoresis (PFGE), and PCR mediated DNA amplification such as REP-PCR (172) are also typing methodologies but do not allow identification to the species level. Specific gene sequencing is also performed, especially with the 16S-23S rRNA gene intergenic spacer (ITS) region, the *rec*A gene and the *rpo*B gene. However, all these methodologies are laborious and difficult to apply for routine clinical identification (123). Currently, ARDRA and AFLP are the most widely accepted and validated methodologies for identification of *Acinetobacter* to the species level; large libraries of different profiles are available for both, reference and clinical isolates (160).

Recently, a new methodology has been developed by Lin *et al.* (123). The identification is performed with a microsphere-based array that combines an allele-specific primer extension assay and microsphere hybridization. They state that this assay has a high specificity and can discriminate 13 different *Acinetobacter* spp. in less than 9 h. Presumptively, this methodology is highly sensitive, detecting a hundred *A. baumannii* cells per ml of blood and it can differentiate clinical *Acinetobacter* spp. isolates with a 90% identification rate (123).

Other recent developments to identify *A. baumannii* include the detection of the bla_{OXA-51} gene encoding a carbapenemase which is intrinsic from this species, PCR-electrospray ionization mass spectrometry (PCR-ESI-MS), and a new PCR methodology to analyse the differences in the respective *gyr*B genes which allows differentiation between *A. baumannii* and the *Acinetobacter* genospecies 13TU (160).

1.3 Clinically important *Acinetobacter* spp.

Numerous studies support the initial suggestion that *A. baumannii* and its close relatives are the main genomic species associated with outbreaks of nosocomial infection. Isolates belonging to the closely related DNA groups 3 and 13TU have also been implicated in a number of outbreaks in ICUs (11,216). In a large study of 584 *Acinetobacter* isolates realized by Seifert *et al.* (199), over 70% of the strains were classified as *A. baumannii*. This study also identified other genomic species such as *Acinetobacter* genospecies 3, *A. johnsonii* and *A. lwoffii* (199). Other studies have also associated the presence of *Acinetobacter* genospecies 3 (213) and 13TU (210) with hospital infection.

Some reports imply that other genomic species have also been involved in acquired nosocomial infections. *A. haemolyticus* has been described as the causative agent of endocarditis (22). *Acinetobacter* genospecies 3 and *A. junii* have been found responsible of bacteraemia and sepsis in neonatal intensive care and paediatric oncology units (13,40,96,109). *A. johnsonii* has been isolated from patients with catheter-related bloodstream infection (200). *Acinetobacter* genospecie 13TU has been involved in different nosocomial infections (45,77,134). Finally, *A. lwoffi* has been isolated from patients with both nosocomial and community-acquired infections such as meningitis, peritonitis, endocarditis and endophthalmitis (35,193,201,219). Nevertheless, little is still known about the implications and clinical significance of other *Acinetobacter* genomic species. *Acinetobacter* spp. are commonly found in the environment and also as commensals on the human skin flora; this ubiquitous occurrence means that the presence of these isolates in clinical specimens is often considered to be a contaminant (216).

So far, with the exception of *A. calcoaceticus,* the members of the ABC complex are the genomic species more commonly related to infection in hospitals. Indeed, many studies have shown that *Acinetobacter* isolates involved in nosocomial infection frequently belong to the glucose-acidifying variety. However, due to the close relationship between the members of this complex, the majority of isolates are identified as *A. baumannii.* On the other hand, the majority of glucose-negative, non-haemolytic strains found in clinical specimens are mainly identified as *A. lwoffii, A. johnsonii* or *Acinetobacter* genospecies 12; and most of the haemolytic isolates are identified as *A. haemolyticus* or *Acinetobacter* genospecies 6 (216).

1.4 Natural and clinical reservoirs

1.4.1 Reservoirs

Acinetobacter spp. are ubiquitous in nature and have been recovered from soil, water, animals and humans. They have been isolated from freshwater ecosystems, raw sewage and heavily polluted water nearby wastewater treatment plants (50), and have also been found in body lice collected from homeless people (114). However, even though most *Acinetobacter* spp. are ubiquitous, not all of them have their natural habitat in the environment. There is a misconception that *A. baumannii* is also ubiquitous in nature; this microorganism does not seem to be a typical environmental microorganism (160).

Food is known to be a source of Gram-negative rods; although there is few data about the presence of *Acinetobacter* spp. in the food chain, members of this genus have been found in a variety of food items (66). Berlau *et al.* (12) showed that 17 % of the vegetables contained small numbers of *Acinetobacter* spp.; the ABC-complex accounted for 56 % of all strains isolated from fruits and vegetables. They suggest that hospital food could be a potential source for *A. baumannii* acquisition (12). *Acinetobacter* spp. are also

involved in spoilage of foods such as bacon, chicken, eggs and fish, even when stored under refrigerated conditions or following irradiation treatment (216).

The members of the genus *Acinetobacter* are normal inhabitants of human skin and are frequently isolated from moist skin areas, and also from the throat, respiratory and intestinal tract of hospitalized patients; other reservoirs include the medical equipment within the hospital environment as well as the patients and staff (33,216). In the hospital environment, these microorganisms have been isolated from contaminated reusable medical equipment such as humidifiers, ventilator tubing, respirometers, and arterial pressure monitoring devices (9,23,36,96), together with a wide variety of dry environmental objects such as gloves (157), mattresses (205), pillows (231) and other medical or surgical equipments. It is worth to comment that there is a significant difference between the microbial populations found in clinical and other environmental surfaces. Most clinical isolates belong to the ABC complex; by contrast, most of the environmental isolates are generally identified as *A. johnsonii, A. lwoffii* or *Acinetobacter* genospecies 9 (216). *A. calcoaceticus, Acinetobacter* genospecies 3 and genospecies 11 have been found in water, soil and on vegetables; *A. lwoffii, A. radioresistens* and the *Acinetobacter* genospecies 3 have been reported from human skin, and the *Acinetobacter* genospecies 11 has also been isolated from the human intestinal tract. *A. johnsonii* has been isolated from water, soil, human skin and faeces. On the other hand, *A. baumannii* that is the most important nosocomial pathogen within this genus, has been only rarely found on human skin and in human faeces (160).

1.4.2 Survival in the environment

The survival in the environment is a common characteristic of the genus *Acinetobacter*, and as such it has been known since the discovery of these microorganisms

(90). *Acinetobacter* spp. can persist in the environment for many days or weeks, even in dry conditions on particles and dust; this survival is probably due to their ability to resist drying (102) and to the fact that they can grow at different temperatures and pH values (11,216). Indeed, Jawad *et al.* (102) established that the response to drying of *A. baumannii* was similar to that of *Staphylococcus aureus* (102). However, clinical strains were more resistant to desiccation than American Type Culture Collection (ATTC) *A. baumannii* strains possibly due to the repeated subculturing that the ATCC strain has undergone over the years (103). Wendt *et al.* (233) showed that the ability of *A. baumannii* strains to survive under dry conditions depended on the source of the isolates; strains obtained from dry sources survived better than strains isolated from wet sources (233). Posterior studies by Houang *et al.* (97) reported that *A. baumannii* undergoes morphological changes when desiccated; this study suggested that desiccated cells had significantly thicker and more electron-dense cell walls and nucleic acids than those of control specimens (97).

A. baumannii survives desiccation better than other *Acinetobacter* spp. (102,144,160); this, together with the greater susceptibility of the non-baumannii isolates, could explain why *A. baumannii* is the genomic species more frequently implicated in hospital outbreaks (160). *A. lwoffii* has been reported to survive up to 7 days on dry surfaces while ***A. baumannii* can persist up to 25 days** (93). Nevertheless, other Gram-negative bacilli only survive a few hours: *Escherichia coli* survived for 24 hours and *Pseudomonas aeruginosa* less than 24 hours (101).

2. EMERGENCE OF *A. BAUMANNII* AS A NOSOCOMIAL PATHOGEN

A. baumannii is emerging as a cause of numerous global outbreaks with increasing rates of resistance to antimicrobial agents. Multidrug resistant strains have been isolated worldwide and it has been demonstrated that these strains can spread from areas with high rates of antimicrobial resistance to other areas with historically low rates (161).

There are four factors involved in the spread and persistence of an epidemic of nosocomial infection caused by *A. baumannii* (28):

- High adaptability of these microorganisms to adverse environmental conditions: they can spread and persist in the hospital environment for many days.
- Their capacity to rapidly develop multidrug resistance.
- Intensive use of broad spectrum antimicrobial agents in hospitals.
- Increment of patients susceptible of acquiring these infections.

2.1 Epidemiology

The most outstanding characteristic of *A. baumannii* in hospitals is the endemic and epidemic occurrence of multidrug resistant strains. *Acinetobacter* Genospecies 3 and 13TU could have a similar impact in hospitals but probably their involvement in infection has been underestimated due to the difficulties to identify them (44). The pattern of an outbreak varies depending on the hospital condition (whether there is a common source or multiple sources) and the characteristics of the strain, because some strains have a greater tendency for epidemic spread than others (44). In hospitals, an epidemic strain is usually introduce by a colonized patient and transmitted to other patients or to the environment; colonized and infected patients are an important reservoir of *A. baumannii* (29). Although it can occasionally spread through the air in water droplets, the main type of transmission is via the hands of hospital staff (44). An initial colonization may progress into infection

when *A. baumannii* crosses the protective skin barrier (28); it will generally cause mild to severe illness, although in some cases it can be fatal. The severity of the infection will depend on the site of the infection and the own patient's vulnerability to infection (104). *A. baumannii* has also been associated to cases of community-acquired infections, mostly in countries with tropical or subtropical climate. These infections have been reported mainly in patients with some kind of comorbidity and are associated with heavy smoking and excess alcohol consumption (57).

The first descriptions of endemic or epidemic outbreaks date from the 1970s (76). At present, *A. baumannii* is responsible for an increasing number of nosocomial infections, either in the form of epidemic outbreaks or with an endemic occurrence throughout the time without a specific reservoir. Highly similar but not epidemiologically related strains have been isolated at different locations and different times. These clones are usually resistant to antibiotics, genetically stable and very successful in hospitals; their spread could be related to the transfer of patients between hospitals or to the presence in the community at a low level and occasionally spreading in hospitals due to the selective pressure from antimicrobial agents (44). Clones I and II are mainly responsible for outbreaks in hospitals in north-west Europe, however clone I has also been isolated in Spain, South Africa, Poland and Italy whereas clone II has also been identified in Spain, Portugal, South Africa, France, Greece and Turkey. On the other hand, clone III has been mainly found in France, Belgium, The Netherlands, Italy and Spain (221,222).

2.2 Infections caused by *A. baumannii*

At first, *Acinetobacter* spp. were mainly isolated from patients in surgical or medical wards and the infections were mostly related to post-surgical urinary tract infections. Improvements in the resuscitation techniques have changed the types of infections caused

by this microorganism (104). *Acinetobacter* spp. have been isolated from patients with infections such as septicaemia, pneumonia, endocarditis, meningitis, skin and wound infection and urinary tract infection (11).

- ***Nosocomial pneumonia***

Nowadays, the **nosocomial pneumonia** is the most important infection caused by *A. baumannii*, especially since the application of mechanical ventilation procedures (104). Several studies have reported that *A. baumannii* would be responsible for 3-5% of the nosocomial pneumonia and it is emerging as an important complication of mechanical ventilation (11,132). Nevertheless, in patients in the ICU with mechanical ventilation, *A. baumannii* would be responsible of 15-24% of the pneumonias (75,79). Although high mortality rates have been reported, it is difficult to determine whether the patients would have survived in the absence of the pneumonia. Factors such as advance age, chronic lung disease, immunosuppression, surgery, use of antimicrobial agents and presence of invasive devices, increase the risk of acquiring nosocomial pneumonia (11).

Recently, there have also been reports on **community-acquired pneumonia**, mainly in tropical regions of Australia and Asia. This infection has been associated to an elevated mortality rate and it generally occurs during the rainy season, among patients with some form of comorbidity or associated with heavy smoking and alcohol abuse (2,57,160).

- ***Nosocomial bloodstream infections***

Bacteraemia is currently one of the infections with a higher mortality rate in hospitals. Despite this, mixed infections are frequent and it has been suggested that bacterial synergy is important in cases of bacteraemia (104); as a result, it is difficult to determine the real morbidity and mortality attributable to *A. baumannii* (29). This infection

can affect predisposed patients after invasive clinical procedures, and the factors associated with poor prognosis are a severe underlying disease, pneumonia, septic shock, disseminated intravascular coagulation, mechanical ventilation and inappropriate antimicrobial therapy (29).

- ***Meningitis***

Before 1967, few cases of **meningitis** due to *A. baumannii* were reported, and they were mostly community-acquired; however, since 1979 most of the cases are hospital acquired (11). This infection has been associated with adult men undergoing lumbar punctures or neuro-surgical procedures and the main risk factor is the presence of ventricular catheters (11). Mortality may be as high as 70 %, even though the real cause of dead is usually difficult to determine (160).

- ***Urinary Tract Infection***

Urinary tract infections are infrequent and occur mainly in elderly debilitated patients, those in the ICUs and patients with permanent urinary catheters (11). Isolation of *A. baumannii* from urine is not always associated with infection because this microorganism can colonize the urethra of patients with urinary catheters (94).

Other infections: Occasionally, there have been reports **of native-valve infective endocarditis, peritonitis, cholangitis and septic complications** (11). Nowadays, *A. baumannii* has also been isolated from wounds of combat casualties from Iraq or Afghanistan (160).

2.3 Risk factors for infections with *A. baumannii*

Numerous studies have tried to identify the predisposal factors for the development of nosocomial infections by *A. baumannii*. Acquisition and spread of these microorganisms has been related to several variables. Within the patient population, the most susceptible to acquire these infections are those who have undergone major surgery, those with severe underlying disease (malignancy, burns or immunosuppression) and also the elderly (11).

Previous antimicrobial therapy is the risk factor most frequently involved in acquisition of MDR *A. baumannii*, with carbapenems and third-generation cephalosporins as the most commonly implicated, and followed by fluoroquinolones, aminoglycosides and metronidazole. Other risk factors are prolonged ICU stay, severity of illness, sex, mechanical ventilation, tracheostomy, hydrotherapy, transfusions and the presence of catheters (58).

2.4 Clinical impact of *Acinetobacter* spp.

Currently, there is a debate on the real clinical impact of *A. baumannii*; especially, there are controversial points of view on whether this microorganism really increases morbidity and mortality in patients. Some investigators believe that *A. baumannii* infections are responsible for an increment in patient mortality, while other investigators suggest that these infections occur on critically ill patients and argue that the mortality is due to their underlying diseases (44,55,57). An additional problem is the difficulty to determine the real frequency of infections caused by *A. baumannii* because the isolation of this microorganism from a patient could be due to a colonisation rather than an infection (223). Several authors have reported that colonisation of patients with resistant *A. baumannii* isolates could lead to the development of important infections; however, Mahgoub *et al.* (127) suggest that although infections produced by highly resistant bacteria

have a poor prognosis, colonisation with completely resistant *A. baumannii* isolates appears to be a marker associated with certain risk factors and does not necessarily produce a poorer outcome (127). In a review of matched cohort and case-control studies, Falagas *et al.* (55) concluded that patients colonised or infected with *A. baumannii* had a statistically significant higher mortality than control patients. However, this review cannot determine the attributable mortality due to the acquisition of *A. baumannii* (55). Nevertheless, clear conclusions are difficult to be obtained due to the great methodological heterogeneity found in the different studies reported in the literature (160).

On the other hand, within patients with community-acquired pneumonia or bacteraemia, mortality is generally high. Nevertheless, although the published data suggests that these infections are associated with considerable mortality, this data could be affected by publication bias, being accepted for publication only the reports of patients with severe community-acquired infections (57).

Even though there is no agreement on the real mortality produced by *A. baumannii* infections, it is important to note that this microorganism can lead to complications within the hospital environment (86). Infections with these microorganisms also bring logistical and economical problems to the hospital; infected patients have to be isolated and cannot move to other wards or hospitals (86). During outbreaks, environmental decontamination procedures or even ward closure are economically important for the hospital. An increment in the length of hospital stay to treat the infections caused by *A. baumannii* represents an additional hospitalization cost per patient. Lee *et al.* (117) analysed the impact of these infections on clinical and economic basis among patients with MDR *A. baumannii* bacteraemia. They found that patients with bacteraemia due to MDR *A. baumannii* had an additional hospitalization of 13.4 days and US$ 3.758 of additional costs compared with bacteraemia due to non-MDR *A. baumannii* (117). Therefore, the resistance characteristics

of *A. baumannii* have a negative impact on health and economic outcomes but also represent a difficult challenge for diagnosis testing, treatment and controlling the emergence and spread of multidrug resistant bacteria (135).

2.5 Virulence and pathogenicity

Colonization with *A. baumannii* is more frequent than infection, suggesting that the pathogenicity of this species is normally low (44). Although it has always been considered a microorganism of low virulence, the appearance of fulminant community-acquired pneumonia suggest that sometimes it can be highly pathogenic and cause invasive diseases (104). *In vitro* studies have also demonstrated that it can cause lethal infections in immunosupressed animals, with a death rate between 75% and 100% (185). Several virulence and pathogenicity factors have been described so far but more studies on this area are still needed.

- ***Lipopolysaccharide (LPS)***

Presence of **lipopolysaccharide and lipid A** is responsible for lethal toxicity in mice and high fever in rabbits (3,104). The LPS acts in synergy with the capsular exopolysaccharide and it is involved in resistance to complement in human serum. In Gram-negative bacteria, capsular polysaccharide blocks the access of complement to the microbial cell wall and prevents the triggering of alternative pathways. Approximately 30% of *Acinetobacter* strains produce exopolysaccharides which are thought to protect bacteria from host defences. Experimental studies suggest that exopolysaccharide-producing *Acinetobacter* strains are more pathogenic than non-producing strains (104). In a mouse model, *A. baumannii* LPS has been found to be the major immunostimulatory component leading to proinflammatory response during pneumonia (44). The presence of a

polysaccharide capsule made of L-rhamnose, D-glucose, D-glucuronic acid and D-mannose probably makes the surface of the strains more hydrophilic (11).

- ***Quorum-sensing***

Quorum-sensing is a regulatory mechanism in Gram-negative bacteria which is involved in important microbial activities such as extracellular enzyme biosynthesis, biofilm formation, antibiotic biosynthesis and extracellular virulence factors (208). In *Acinetobacter* clinical isolates there have been described four different quorum-sensing signal molecules. This could be an important mechanism for autoinduction of multiple virulence factors (104) and could be responsible for the control of various metabolic processes including biofilm formation (44).

- ***Genetic exchange***

Acinetobacter spp. are described as **"naturally transformable"**, however, it is unknown whether *A. baumannii* is also naturally competent (161). Acquisition of additional genetic material can facilitate pathogenicity and survival of the microorganism. DNA sequencing has identified genomic islands containing virulence genes associated with cell-envelope biogenesis, antibiotic resistance, autoinducer production, pilus biogenesis and lipid metabolism (44,209). **Conjugative plasmids, transposons and integrons** are important in the dissemination of genetic determinants of resistance in the *Acinetobacter* spp. (161).

- ***Biofilm formation***

Biofilm formation is very important regarding public health due to its role in certain infectious diseases, especially in a variety of device-related infections. The biofilm is a congregation of microbial cells surrounded by an extracellular polymeric substance matrix

(49). *A. baumannii* is able to form **pilus-mediated biofilm** on glass and plastics. On medical devices, the biofilm can constitute a niche for the bacteria, favouring the colonization and infection of the patients. Pili on the surface of the bacteria interact with human epithelial cells; these pili together with hydrophobic sugars of the LPS could promote the adherence to host cells as a first step in patient colonization (44).

- ***Outer Membrane Proteins (OMPs)***

Outer membrane protein A (OmpA) has been associated with induction of cytotoxicity (25,44). Choi *et al.* suggested that this protein binds to eukaryotic cells and translocates to the nucleus causing the nuclear localization of these proteins and inducing cell death *in vitro* (25).

- ***Stress-response mechanisms***

A. baumannii has an extraordinary ability to adapt to different conditions; this suggests that this microorganism must possess effective stress-response mechanisms (44).

2.6 Multidrug resistance

A. baumannii is intrinsically resistant to commonly used antimicrobial agents such as aminopenicillins, first and second generation cephalosporins and chloramphenicol (44). Nevertheless, the major concern associated with *A. baumannii* is the remarkable ability to rapidly develop antimicrobial resistance. To date, some strains of *A. baumannii* have become resistant to almost all currently available antimicrobial agents, including carbapenems, which were considered the drug of choice for the treatment of infections caused by this microorganism (180). Practices in the ICU contribute to the development of resistance because the use of antimicrobial agents per patient and surface area are significantly higher (29). In addition, the difficulty to eradicate these bacteria has allowed

them to colonise niches left vacant after the eradication of other more susceptible microorganisms (223). However, the antimicrobial resistance of *A. baumannii* varies among countries, centres and even among the different wards within the same hospital (29), and it has been suggested that the problem of resistance could be associated with a limited number of successful lineages (44). Other factors related to the variation on resistance rates among hospitals would be the differences in antimicrobial usage, infection-control practices and climate (70).

Until the early 1970s, *Acinetobacter* infections could be treated with gentamicin, minocycline, nalidixic acid, ampicillin or carbenicillin (11). Nevertheless since the 1970s, *Acinetobacter* spp. clinical isolates have been increasing the level of resistance to antimicrobial agents. First, they acquired resistance to the aminopenicillins, ureidopenicillins, first and second generation cephalosporins, cephamycins, aminoglycosides, chloramphenicol and tetracyclines (11,86). Imipenem remained as the most active drug; unfortunately, carbapenem-resistant *A. baumannii* clinical isolates have been isolated in hospitals around the world and in some areas the frequency of these resistant strains can exceed 25% (11,44). Despite its toxicity, colistin remains as a last resort antimicrobial agent to treat infections caused by multiresistant *A. baumannii* isolates. Unfortunately, the increment in the use of polymyxins has resulted in the emergence of colistin resistant pathogens in the order of 5 % to 28 % of the clinical isolates (115). In addition, several authors have reported an increasing number of colistin heteroresistance and tolerance in the clinical environment (88,89,112,122), including a report by Li *et al.* (122) demonstrating for first time heteroresistance in *A. baumannii* clinical isolates which were susceptible to colistin on the basis of MIC (122).

In *A. baumannii*, as in many members of the Gram-negative nonfermenter group, the acquisition of resistance to one class of antibiotics is often accompanied by resistance to

other antibiotic classes (135). A mechanism of multidrug cross-resistance is the acquisition of genetic resistance genes which confer resistance to a variety of antimicrobial classes. Most cases of cross-resistance involve a single class of antimicrobial agents, however, when the mechanism of resistance is an efflux pump, resistance to several classes of antimicrobial agents can be observed (135). Therefore, multidrug resistance is nowadays relatively common and complicates the treatment and the eradication of this microorganism from the hospital environment. Nevertheless, this multidrug resistance pattern affects mainly *A. baumannii* clinical isolates which are usually more resistant than non-*baumannii* species (101).

2.7 Treatment.

Evidence accumulated during the last 10 years suggest that an initial inappropriate antimicrobial treatment is an important factor contributing to the excess mortality in critically ill patients (173). A precise differentiation between colonization and infection is essential to avoid inappropriate antibiotic use (128). Peleg *et al.* (159) highlight the risk of antibiotic misuse and the necessity of restrictive rules for the prescription of carbapenems in order to avoid the emergence of antibiotic resistance in *A. baumannii* (159,180). Currently, only a few of the major antimicrobial agents are effective for the treatment of severe nosocomial infections caused by *A. baumannii*: β-lactam antibiotics should be used only after extensive *in vitro* susceptibility tests; ticarcillin can be useful combined with sulbactam, ceftazidime or imipenem; aminoglycosides can be used successfully combined with an effective β-lactam antibiotic (11). Sulbactam combined with β-lactam antibiotics has proved to be efficient against infections caused by *A. baumannii* isolates with a moderate resistance to imipenem; however, for pan-resistant strains, colistin remains the drug of choice (66). Among the new antimicrobial agents, tigecycline has a good activity

against multiple clinical isolates of *A. baumannii* (110); *in vitro* studies have shown that tigecycline has bacteriostatic activity against *A. baumannii*, including imipenem resistant strains (154). On the other hand, doripenem is active against strains which lack carbapenemases (145). Based on uncontrolled clinical trials, case reports and retrospective reviews, colistin, ampicillin/sulbactam and doxycycline or minocycline should be considered as an option for the treatment of MDR *A. baumannii* nosocomial infections (101). *In vitro* studies suggest several possible combination therapies, although these studies cannot confirm whether these combinations will be successful as antibiotic therapy (161); even so, combination therapy against MDR *A. baumannii* may be more effective than monotherapy and it may prevent the selection of further resistance in these isolates.

At present, there are also clinical trials involving antibacterial peptides which have shown a potent activity in vitro against MDR *A. baumannii* (218). There are studies on two cationic membrane-active antimicrobial peptides which inhibit the growth of *A. baumannii*: $rBPI_2$ is a recombinant form of the N-terminal domain of the human bactericidal permeability-increasing protein and cecropin P1 which is an antibacterial peptide (161). In addition, Rodríguez-Hernández *et al.* (186,194) have recently presented a report on the activity of cecropin A-Melittin hybrid peptides which suggests that these peptides could be an alternative chemotherapy for colistin-resistant *Acinetobacter* spp. (186,194). However, although the *in vitro* and animal models are important to determine the efficacy and activity of the antimicrobial agents, the results obtained are not always applicable in clinical practice (161).

There are some areas of controversy regarding the selection of the ideal antimicrobial agent to be used and also to determine whether it is more appropriate the use of combination therapy or monotherapy. Murray *et al.* (143) suggested that in a host without significant comorbidities, treatment with a single antibiotic should be adequate; however,

in vitro antimicrobial tests should be performed to have a guide on the selection of the best antimicrobial agent for each nosocomial infection (143).

- ***Treatment of pneumonia caused by carbapenem susceptible A. baumannii***

Carbapenems are recommended for the treatment of these infections because this group of antimicrobial agents has the greatest activity against *A. baumannii*. Within this group, imipenem is the antibiotic most commonly chosen while meropenem is used when the use of imipenem is contraindicated (30).

Sulbactam has a bactericidal activity against *A. baumannii* and its *in vivo* efficacy has been demonstrated in an animal model of pneumonia. The clinical effectiveness is similar to imipenem and it is generally indicated as an alternative to carbapenems in severe infections (30).

Aminoglycosides are usually recommended in combination with **β-lactam antibiotics**, although it is unknown whether this combination has a superior efficacy than monotherapy with β-lactam antibiotics (30).

- ***Treatment of pneumonia caused by carbapenem resistant A. baumannii***

Treatment of this infection has few possibilities when it is cause by carbapenem resistant strains. *In vitro*, **colistin** is one of the antimicrobial agents with a better activity against *A. baumannii* isolates; however, the clinical results after treatment of pneumonia with colistin are variable. In addition, the levels of toxicity recommend the use of this antimicrobial agent only in cases of infections with pan-resistant *A. baumannii* (30).

Sulbactam and the association between **doxycycline and amykacin** have a good activity against strains susceptible to these antimicrobial agents. On the other hand, **Rifampicin** maintains a good activity even in carbapenem resistant *A. baumannii* clinical

isolates; nevertheless, monotherapy with this antibiotic is not recommended because it can induce resistance. Studies based on combined therapy in front of pneumonia caused by MDR *A. baumannii* suggest that combinations of **rifampicin/imipenem** and **sulbactam/imipenem or meropenem**, could be an alternative therapeutic option (30,153).

- ***Treatment of bacteraemia***

The treatment of choice in bacteraemia by *A. baumannii* has not been established. In cases of bacteraemia caused by a non-MDR *A. baumannii*, the best option is the use of monotherapy with a **β-lactam** antibiotic or in association with an **aminoglycoside** (28,29). Several studies also recommend the use of **sulbactam** whenever the isolate is susceptible to this antimicrobial agent and that the combination **ampicillin/sulbactam** has been associated with a decreased mortality (29,101). On the other hand, **colistin** is the only therapeutic option in bacteraemia caused by pan-resistant *A. baumannii* isolates, therefore, the use of this last-resort antimicrobial should be regulated to avoid selection of resistance (28,218).

2.8 Prevention, monitoring and control measures.

In most outbreaks caused by *A. baumannii***,** patients are the most likely reservoir from which spread starts. When these infections become apparent in the hospital, the number of colonized patients is probably already high; therefore, precautions to prevent an outbreak are already too late and all inanimate surfaces can be putative reservoirs for *A. baumannii* (104). In most nosocomial outbreaks, there is a common source of contamination and the elimination of this source leads to a rapid end of the outbreak (221). The best way to prevent outbreaks of nosocomial infections is by applying severe measures of prevention, together with a constant monitoring of the environment and the application of control measures.

- ***Prevention***

Several studies indicate that patient colonization with MDR Gram-negative bacteria is a frequent precursor of a posterior true infection. Hand contamination is an important factor in the transmission of *A. baumannii* infections; this transmission can be controlled by proper hand washing with antiseptic or alcohol-based soaps and appropriate glove use. Some studies have also shown that antibacterial prophylaxis with topical and systemic agents can reduce the number of respiratory tract infections and prevent mortality in critically ill patients (218).

In order to prevent outbreaks of nosocomial infections, it is important to rapidly identify the patients who have a higher risk of acquiring these infections, followed by an appropriate intervention, isolation and treatment of these patients (128). The isolation measures taken to prevent outbreaks of MRSA should be appropriate for the containment of *Acinetobacter* infections. However, in the Netherlands, despite all these measures, the arrival of three patients infected with *A. baumannii* and MRSA produced the spread of *A. baumannii* in 2 out of 3 hospitals whereas spread of MRSA did not occur (15).

- ***Monitoring***

The general pattern encountered in outbreaks caused by *A. baumannii* suggests that multi-site environmental contamination is related to a cross-transmission, while the presence of negative environmental cultures generally suggests the presence of a common source as a reservoir for the outbreak. The first step in the analysis of an outbreak is to take samples of the environment and to identify the isolates; when the environmental cultures are negative and a single strain is found, it is necessary to look for a common source for the outbreak (221).

Surveillance programs are necessary to identify changes in the spectrum of microbial pathogens causing serious infections and to monitor trends in antimicrobial resistance patterns in nosocomial and community-acquired infections. The information obtained with these programs is used to design new approaches for the treatment and control of infections caused by MDR pathogens. Currently, there are several surveillance programs (Table 2) which have been established by the pharmaceutical industry or by the governmental agencies to monitor patterns of microbial resistance (56,105).

A well-planned surveillance study should analyse the changes in bacterial susceptibility and help to control antimicrobial resistance by using the most appropriate antimicrobial agents. All the data accumulation is not valuable if it cannot be used to help to control the spread of resistance. Surveillance is important in order to detect shifts in susceptibility of microorganisms to various antimicrobial agents and to inform the medical community of such changes (7).

Table 2: List of several surveillance programs which have been created worldwide.

Surveillance Program	Reference
SENTRY Antimicrobial Surveillance Program	(70)
European Antimicrobial Resistance Surveillance System (EARSS)	http://www.rivm.nl/earss
European-based Meropenem Yearly Susceptibility Test Information Collection (MYSTIC)	http://www.mystic-data.org
European Network for Antimicrobial Resistance and Epidemiology (ENARE)	http://www.enare.org/publications.html
Surveillance and Control of Pathogens of Epidemiologic Importance (SCOPE)	(234)
Intensive Care Antimicrobial Resistance Epidemiology (ICARE) project	http://www.sph.emory.edu/ICARE/publications.php
European Surveillance of Antimicrobial Resistance (ESBIC)	http://www.esbic.de/esbic/ind_esar.htm

- ***Control measures.***

Infection control measures are needed to slow the emergence and spread of resistant Gram-negative microorganisms such as *A. baumannii.* These control measures include the proper use of instruments, minimization of fluid accumulation, barrier isolation precautions, surveillance and isolation of microorganisms (135). For the management of *A. baumannii* infections, it is important to rapidly determine the source of infection, apply the necessary control procedures (isolation of infected patients and environmental disinfection) and carry out the appropriate antimicrobial use guidelines to prevent more antibiotic pressure on these MDR microorganisms (28,30,143). Further investigation on new antimicrobial treatments is also needed to improve the prognostic of those patients infected with pan-resistant *A. baumannii* strains (28). On the other hand, epidemiological studies should be performed to investigate the global distribution of the actual clones (46).

The main points to follow in order to prevent outbreaks due to *A. baumannii* are a **continuous monitoring** within the hospital, **strict infection control measures**, applying **treatment only when it is indicated** and **rapid discharge** of patients from hospital (127).

3. ANTIMICROBIAL AGENTS

The first scientific observations about the activity of natural antibiotics date from the XIX century. In the 1870s, researchers such as Lister, Billroth or Garré were already aware of the capacity of certain microorganisms to inhibit bacterial growth. Nevertheless, the beginning of the antibiotic era started with the publication in 1929 of Fleming's first description of the penicillin; after this initial step, a great number of antimicrobial agents have been isolated or synthesized to target different infective agents (191). Most antibiotics are natural products elaborated by other bacteria or fungi as weapons to kill off other microbes in the surrounding. However, nowadays there are also semi-synthetic antibiotics which are modifications of natural antibiotics to obtain a better activity, and completely synthetic antibiotics. There are three proven targets for the main antimicrobial groups: **bacterial cell wall biosynthesis, bacterial protein synthesis** and **bacterial DNA replication** (Fig. 4) (230). Currently, the main groups of antimicrobial agents with activity against Gram-negative pathogens are: **β-lactam antibiotics, aminoglycosides, tetracyclines** and **quinolones.**

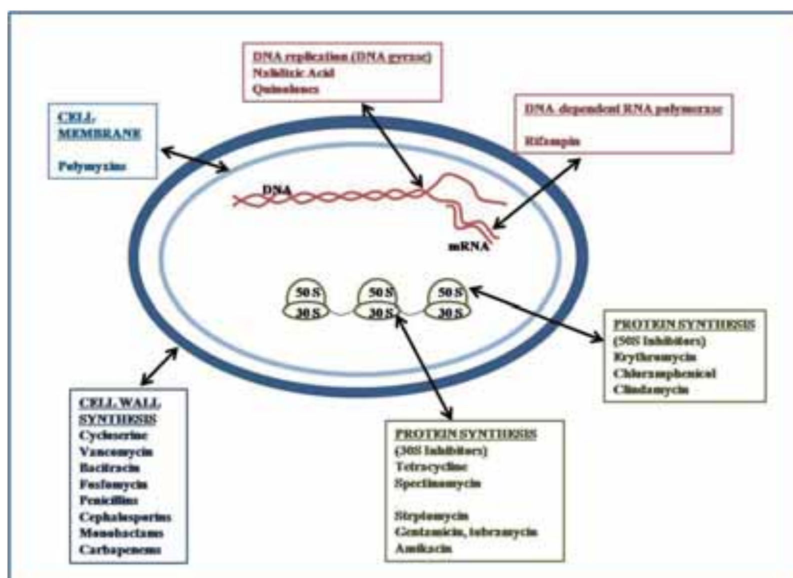

Fig. 4: Basic targets for the antimicrobial agents.

3.1 β-lactam antibiotics

In 1928, Fleming fortuitously discovered the first β-lactam antibiotic (penicillin G) from *Penicillium notatum*, a fungus that accidentally contaminated his bacterial cultures. He concluded that the mould was releasing a substance that was inhibiting bacterial growth and lysing the bacteria. In the following years, further research on this molecule allowed the identification of new compounds with a wider spectrum of activity against pathogens. Nowadays, this family of antimicrobial agents is the most abundant and also the most commonly prescribed for treatment of infections in hospitals (130,140).

- ***Chemical structure and classification***

The name of this family of antimicrobial agents comes from the chemical structure which is characterized by the presence of a **four-membered β-lactam ring** (Fig. 5); these antibiotics need an intact β-lactam ring to exert its bactericidal activity. Further addition of a different secondary ring and side chains gave rise to the different groups of antibiotics within this family and was the determinant factor for the difference in activity; various chemical side chains have been synthetically linked to the ring structures producing a large number of antibiotics with different properties in the host. Many older penicillins display little activity against Gram-negative bacteria, since they do not penetrate the outer membrane; by contrast, the newer β-lactam antibiotics are active against these microorganisms. To date, the β-lactam antibiotics include the **penicillins, cephalosporins, carbapenems, monobactams** and **β-lactamase inhibitors** (Table 3) (130,140).

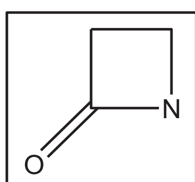

Fig. 5: Basic β-lactam ring structure.

The **penicillins** are a group of either natural or semi-synthetic β-lactam antibiotics that are characterized by the presence of a β-lactam ring and a **thiazolidine ring**; this structure is defined as **6-amino-penicillanic acid** and its presence is essential for the activity of these antimicrobial agents (Fig. 6). Hydrolysis of the C=O union by the activity of β-lactamases gives rise to a bacteriologically inactive molecule. The differences of activity among the members of this group are due to the lateral chain found in the position 6 of the 6-amino-penicillanic acid (130,140).

The **cephalosporins** are structurally similar to the penicillins; however, the members of this group present a **dihydrothiazine ring** instead of the thiazolidine ring which is present in the penicillins. As for the penicillins, the introduction of additional side chains will give rise to different cephalosporins with a different level of antimicrobial activity (Fig. 6) (130,140).

The **carbapenems** are also structurally very similar to the penicillins, but the sulphur atom in the position 1 of the structure has been replaced with a metil group (Fig. 6). The additional side-chains and its special disposition confer an important **affinity towards the Penicillin Binding Proteins (PBPs)** and make this group of antibiotics resistant to a great part of β-lactamases (130,140).

The **monobactams** are monocyclic compounds derived from the **3-aminomonobactamic acid**. Their major characteristic is the presence of the β-lactam ring alone and not fused to another ring (Fig. 6) (130,140).

Currently, the clinical use of **β-lactamases inhibitors** is exclusively based on clavulanic acid, tazobactam and sulbactam (Fig. 7). The **clavulanic acid** has a structure similar to the penicillins but the sulphur atom has been substituted for an oxygen atom which increases the affinity of these molecules for the β-lactamases. On the other hand, the

sulbactam and the **tazobactam** have an oxidation of the sulphur present in the β-lactam ring (130,140).

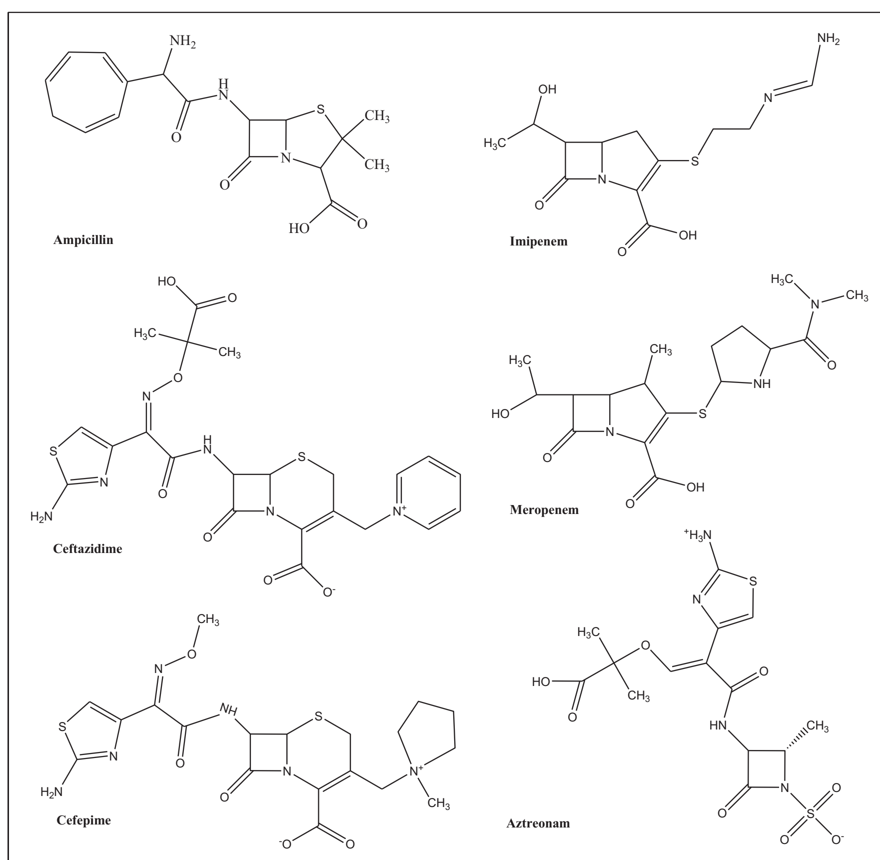

Fig. 6: Basic structure of the β-lactam antibiotics.

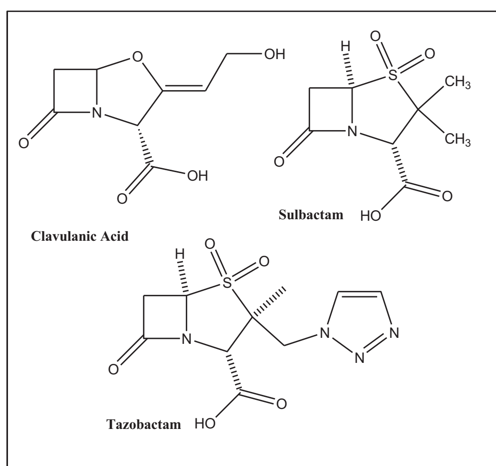

Fig. 7: Structure of the main β-lactamase inhibitors.

Table 3: Classification of the main β-lactam antibiotics (140).

Class	Group	β-lactamic
Penicillins	Natural Penicillins	Penicillin G, Penicillin V
	Aminopenicillins	Ampicillin, Amoxicillin
	Ureidopenicillins	Mezlocillin, Piperacillin
	Carboxipenicillins	Carbenicillin, Ticarcillin
	Isoxazolic penicillins	Oxacillin, Cloxacillin, Dicloxacillin
Cephalosporins	1st generation	Cefazolin, Cephalotin, Cefradin, Cephalexin, Cefradoxil
	2nd generation	Cefuroxime, Cefamandole, Cefaclor, Cefuroxime axetil
	3rd generation	Cefotaxime, Ceftriaxone, Ceftazidime, Cefoperazone, Cefibute, Cefixime, Cefpodoxime, Cefdinir
	4th generation	Cefepime
	Cephamicines	Cefoxitin, Cefotetan
	Oxacefems	Moxalactam
	Carbacefems	Loracarbef
Carbapenems		Imipenem, Meropenem, Ertapenem, Doripenem
Monobactams		Aztreonam
β-lactamase inhibitors	Clavulanic acid	
	Sulbactam	
	Tazobactam	

- ***Mode of action***

The **peptidoglycan** is a network of strands of peptide and glycan that confer strength to the bacterial cell wall; it is mainly composed by alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAMA) (230). β-lactam antibiotics are **bactericidal substances** that inhibit the synthesis of this peptidoglycan layer. Cell wall destruction occurs as consequence of the inhibition of the last step in the synthesis of the peptidoglycan; this final step is facilitated by transpeptidases known as **Penicillin-**

Binding-Proteins (PBPs). The β-lactam antibiotics bind to and inhibit the PBPs involved in the transpeptidation (cross-linking) of the peptidoglycan. A region of this antibiotic is structurally similar to the last two amino acids of the pentapeptide D-alanine-D-alanine; this facilitates a covalent union to the active site of the transpeptidase, which irreversibly binds to the PBP active site, preventing the normal cross-linking of the peptide chains in the peptidoglycan layer and therefore, disrupting cell wall synthesis. Without the restrictive presence of the cell wall, the high osmotic pressure inside the cell bursts the inner and outer bacterial membranes (130,140,230).

Generally, the β-lactamase inhibitors have a low antimicrobial activity. Nevertheless, they contain the β-lactam ring and their sole purpose is to prevent the inactivation of the β-lactam antibiotics. These inhibitors have a strong affinity for the β-lactamases (higher than the β-lactam antibiotics), to which they will attach and form an irreversible union, protecting the β-lactam antibiotics from the hydrolysing action of the β-lactamases (130). Sulbactam, in contrast to the other β-lactamase inhibitors, has clinically relevant antimicrobial activity against certain microorganisms, such as the *Acinetobacter* spp. This molecule has an antimicrobial activity on its own by binding to the PBP2 of these microorganisms, rather than working exclusively as a β-lactamase inhibitor. Sulbactam can be used as a combination therapy with other antimicrobial agents, or as monotherapy to treat sulbactan-susceptible *Acinetobacter* infections (119,160).

- ***Mechanisms of resistance***

The effectiveness of the β-lactam antibiotics relies on their ability to reach the PBPs with the β-lactam ring intact and to bind to them. There are several mechanisms of resistance to β-lactam antibiotics and they are generally due to **point mutations on the chromosome** or to the **acquisition of mobile elements** such as plasmids or transposons

(Fig. 8). The resistance to β-lactam antibiotics can be due to the expression of a single mechanism of resistance or to the additive effect of several mechanisms (130,140).

- ✓ Reduced membrane permeability: the Gram-negative, non-fermentative bacilli such as *A. baumannii* are intrinsically resistant to many of the β-lactam antibiotics. These antimicrobial agents are generally hydrophilic molecules which penetrate de bacteria through the Outer Membrane Proteins (OMPs) present in the bacterial membrane. Alteration of these OMP can result in the resistance to different types of β-lactam antibiotics.
- ✓ Efflux pumps: These energy dependent efflux pumps boost the antibiotic outside the bacterial cell. This mechanism is used to confer resistance to different types of antimicrobial agents.
- ✓ Target modification: The β-lactam antibiotics have to attach to the PBPs in order to be bactericidal. The modification of the target will produce a reduced affinity of the β-lactam antibiotic towards the target PBP.
- ✓ β-lactamase production: This is the major mechanism of resistance to β-lactam antibiotics. These enzymes are structurally similar to the PBPs and will form a non-covalent union with the antibiotic; the hydrolysing activity of these enzymes opens up the β-lactam ring, inactivating the β-lactam antibiotic. The genes encoding for these enzymes may be inherently present on the bacterial chromosome or may be acquired via plasmid transfer; gene expression may be induced by exposure to β-lactam antibiotics. The production of a β-lactamase does not necessarily exclude all treatment options with β-lactam antibiotics; in some cases, they may be co-administered with a β-lactamase inhibitor.

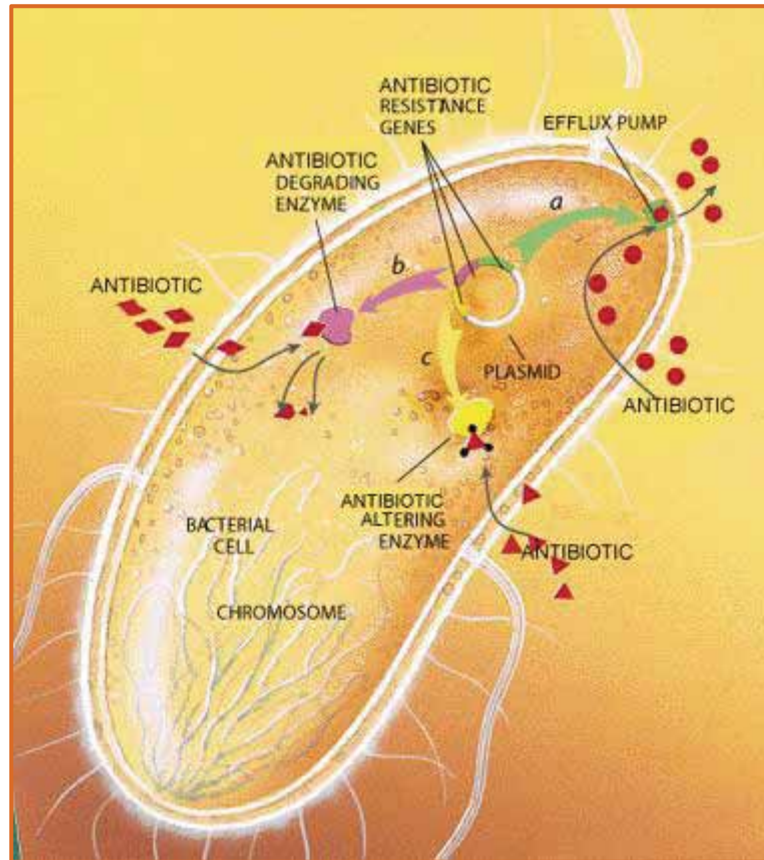

Fig. 8: Graphical representation of the main mechanisms of resistance to the antimicrobial agents (New Scientist. March 2003: 177(2385): 4).

3.2 Aminoglycosides

The **aminoglycosides** are a wide group of broad-spectrum antimicrobial agents formed mainly by naturally synthesized substances. Streptomycin was the first aminoglycoside introduced in clinical training; this antibiotic was isolated from a strain of *Streptomyces griseus*, in 1944. Later on, other members of this family were isolated from different *Streptomyces, Bacillus* and *Micronospora* spp. Toxicity and rapid spread of resistance encouraged researchers to develop new semi-synthetic molecules by chemical modification of the existing ones (10,80).

- ***Chemical structure and classification***

The aminoglycosides are multifunctional hydrophilic sugars that possess several amino and hydroxyl functionalities; they are considered polycationic substances and show a binding affinity for nucleic acids, especially for the prokaryotic rRNA (113). Their structure consists of two or more amino sugars connected by glycosidic linkages to an aminocyclitol nucleus which is generally located in a central position (Fig. 9). Based on their chemical structure, they should be called **aminoglycoside-aminocyclitols** but this name has been abbreviated for simplicity. The aminoglycosides are classified into different groups depending on the nature of their central aminocyclitol nucleus (Table 4) (10,80). In most clinically used aminoglycosides, the amino sugar is **2-desoxystreptamine** (Fig. 11); however, in streptomycin and derivatives, the aminocyclitol nucleus is **streptidine** (Fig. 10), and in the fortimicin series is **fortamine** (Fig. 12) (139). Currently, the aminoglycosides are still active against most Gram-negative aerobic bacilli; however, in severe infections they are generally administered together with a β-lactam antibiotic because this combination has proven to have a synergic effect (80).

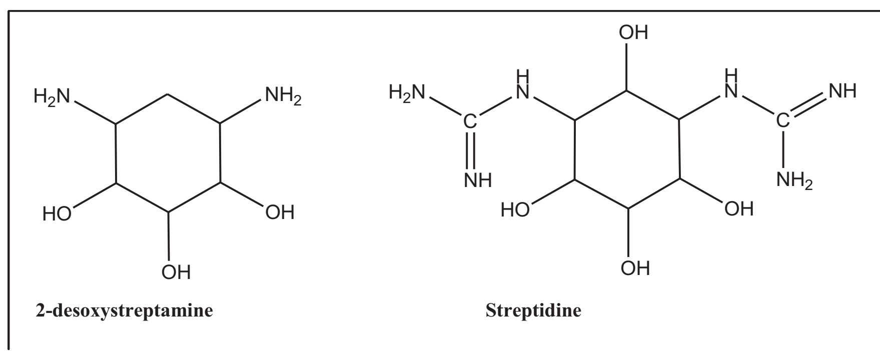

Fig. 9: Graphical representation of 2-desoxystreptamine and streptidine; the main aminocyclitol nucleus in the aminoglycosides.

Table 4: Aminoglycoside classification according to the nature of their central aminocyclitol nucleus (80).

Sugar component	Substitution	Antibiotic
Streptidine		Streptomycin
2-desoxystreptamine	4-monosubstituted	Apramycin
	4,5-disubstituted	Ribostamycin, Paromomycin, Neomycin, Butirosin, Lividomycin
	4,6-disubstituted	Kanamycin, Amikacin, Tobramycin, Dibekacin, Arbekacin, Gentamicin, Isepamicin, Sisomicin, Netilmicin
Aminocyclitol		Spectinomycin, Hygromycin, Fortimicin, Dactimicin, Trospectomicin

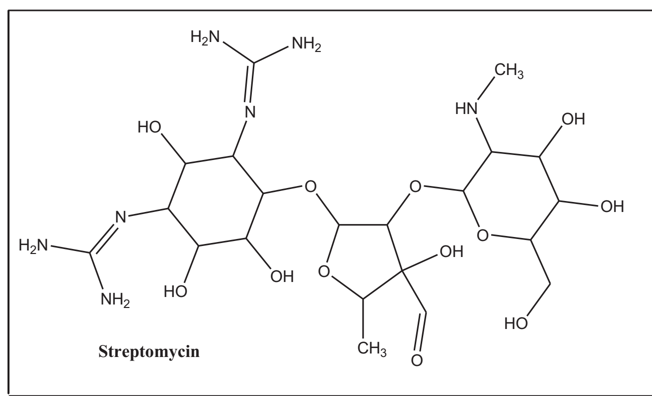

Fig. 10: Graphical representation of streptomycin.

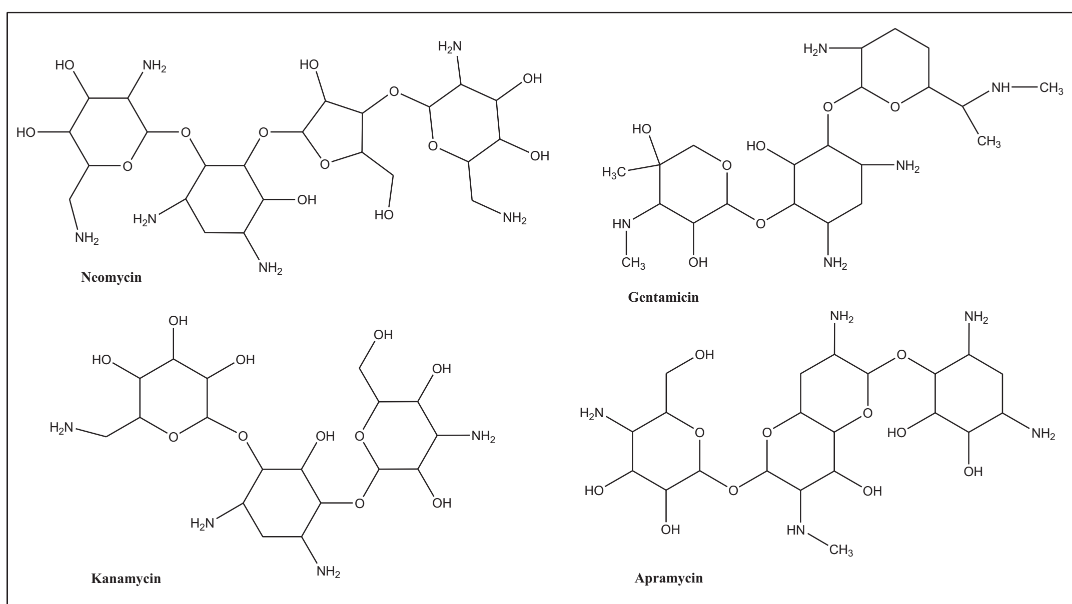

Fig. 11: Graphical representation of aminoglycosides containing 2-desoxystreptamine as a central sugar component: Apramycin, Neomycin, Gentamicin and Kanamycin A.

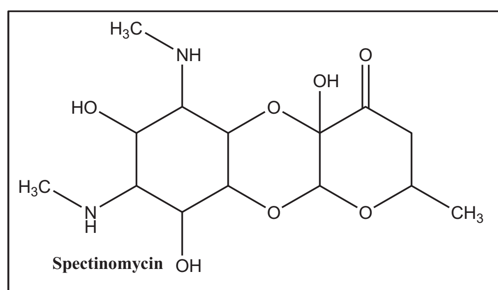

Fig. 12: Graphical representation of spectinomycin.

- ***Mode of action***

Aminoglycosides are antimicrobial agents with **bactericidal activity** as a result of the inhibition of protein synthesis and the alteration in the integrity of the bacterial cell membrane. The target of these antibiotics is the bacterial ribosome to which they covalently attach forming an irreversible union. Penetration of the aminoglycosides through the bacterial membrane is accomplished by an **energy-dependent phase I (EDP-I)** active transport mechanism. Once inside the cell, the aminoglycoside binds to the 30S subunit of the ribosome, triggering a second **energy-dependent transport system (EDP-II)** which will accelerate the intracellular accumulation of the drug. Although this union does not prevent the formation of the initiation complex for the protein synthesis, it perturbs the elongation process by impairing the proofreading process. Aberrant cell membrane proteins are produced leading to an altered permeability and an additional entrance of aminoglycosides inside the cell (10,80,139). Different classes of aminoglycosides bind to different sites on the rRNA depending on the structural complementarity between both structures or they can also bind to the same site in more than one conformation. The affinity of the aminoglycosides to the prokaryotic rRNA is at least 10-fold higher than to the eukaryotic rRNA; nevertheless, this is not a big difference in affinity and it can explain the toxic effects of these antimicrobial agents (72,113).

- ***Mechanisms of resistance***

Aminoglycoside resistance is caused by the presence of one or more of the following mechanisms: loss of permeability of the bacterial cell to the drug, ribosomal alterations that prevent the drug from binding to its site of action, or inactivation of the drug by aminoglycoside-modifying enzymes produced by the bacteria (Fig. 8) (80,113,139).

- ✓ Reduced membrane permeability: This resistance mechanism produces a decrease in drug uptake and accumulation inside the bacterial cell due to membrane impermeabilization, reduction in the transport through the inner membrane or the presence of efflux pumps. The level of resistance produced is moderate but it may result in cross-reactivity to all aminoglycosides (80,113,139).
- ✓ Efflux pumps: In *E. coli*, AcrD has been involved in the efflux of aminoglycosides; deletion of this gene decreased the MIC of amikacin, gentamicin, neomycin, kanamycin, and tobramycin. In addition, active efflux by MdfA has also been observed for neomycin, kanamycin and hygromycin A. Nevertheless, the clinical significance of this mechanism of resistance is still unclear compared to the active efflux of other antimicrobial classes (139,187). In *P. aeruginosa*, the extrusion of aminoglycosides is specific to MexXY-OprM, an efflux pump that recognizes a wide range of substrates (133,141).
- ✓ Target modification: Mutations at the site of aminoglycoside attachment is responsible for the reduction in the affinity of the aminoglycoside for the ribosome and may interfere with ribosomal binding. Alteration of the ribosomal binding sites is mainly responsible for the **resistance to streptomycin** because this antibiotic binds to a single site on the 30S subunit of the ribosome. Resistance to the other aminoglycosides is uncommon since they bind to multiple sites. The ***arm*A (aminoglycoside resistance methyltransferase) gene** is involved in the acquisition of resistance to deoxystreptamines and fortimicin and it was initially characterised in *Klebsiella pneumoniae*. This self-defence mechanism involves the post-transcriptional methylation of the ribosomal RNA using S-adenosyl-methionine as a cofactor and it has been developed by aminoglycoside-producing microorganisms to avoid suicide.

This mechanism has always been associated to the presence of the *bla*$_{CTX-M-3}$ on an IncL/M plasmid (71,72,80,113,139).

✓ Aminoglycoside-modifying enzymes (AMEs): Enzymatic modification is the most common type of aminoglycoside resistance. The antibiotic becomes inactive and it cannot properly bind to the ribosome. The genes encoding for AMEs are usually found on plasmids or transposons which facilitate the rapid acquisition of the drug-resistance phenotype. To date, a large number of genes have been characterized and are responsible for the synthesis of three types of enzymes (Fig. 13) (80,113,139):

- **N-Acetyltransferases (AAC)** – catalyzes an acetylation reaction of an amino group, using the acetyl-coenzyme A as a donor.
- **O-Adenyltransferases (ANT)** – catalyzes an ATP-dependent adenylation of hydroxyl group.
- **O-Phosphotransferases (APH)** – catalyzes an ATP-dependent phosphorylation of a hydroxyl group.

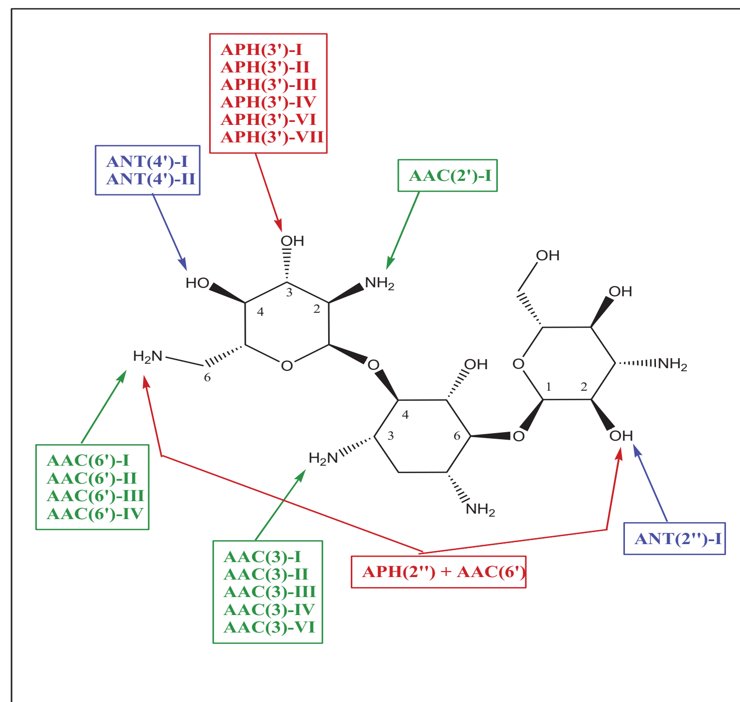

Fig. 13: Activity of the main aminoglycoside-modifying enzymes on a molecule of Kanamycin B.

3.3 Tetracyclines

Tetracyclines are broad-spectrum antibiotics with activity against a great variety of Gram-positive and Gram-negative pathogens. In addition to their therapeutic use, these antimicrobial agents are used in plant agriculture and are also added at subtherapeutic levels to animal feeds because they have proved to be useful as growth promoters in animals. The first members of the tetracycline group were discovered in the late 1940s and they were a natural product of *Streptomyces aureofaciens* and *S. rimosus*. Later on, some semi-synthetic analogs were designed to improve the solubility of these substances and enhance the oral absorption. At present, there are three generations of tetracyclines which have been grouped depending on their discovery year (Table 5): first generation (1948 to 1963), second generation (1965 to 1972) and third generation (glycylcyclines) (26,175,182).

- ***Chemical structure and classification***

Tetracycline molecules are made of a linear fused tetracyclic nucleus (four rings designated A, B, C and D) to which different functional groups are attached. The simplest tetracycline with antibacterial activity **is 6-deoxy-6-demethyltetracycline** and it is regarded as the minimum pharmacophore (Fig. 14). The basic structural characteristics to preserve the antimicrobial activity is the maintenance of the linear fused tetracycle and the conservation of the keto-enol system near to the phenolic D ring (26). Several structure-activity studies have shown that to maintain the antimicrobial activity, all the rings in the linear fused tetracycline nucleus must be six membered and purely carbocyclic; however, there is one exception, the 6-thiatetracyclines, which have a sulphur atom at the position 6 of the ring and still maintain their antimicrobial activity. These compounds, together with the anhydrotetracyclines, the 4-epi-anhydrotetracyclines and chelocardin, have been

classified as atypical tetracyclines because their mode of action is slightly different to the majority of tetracyclines. Nevertheless, their high toxicity makes them of no interest as therapeutic substances and this is probably due to their ability to interact non-specifically with eukaryotic cells (26,175).

The rapid acquisition of resistance to first and second class tetracyclines promoted an intensive search of new tetracycline antibiotics. As a result, in the early 90s, a third class of tetracycline analogues, the **9-glycinyltetracyclines, (glycylcyclines)** was discovered (Fig. 15). The addition of a bulky N,N-dialkylamine side chain to the position 9 of minocycline produced a compound which retained the antimicrobial activity and was also active against bacteria containing tetracycline resistance genes (*tet* genes) responsible for both, the efflux of the previous tetracyclines and ribosomal protection (26,175).

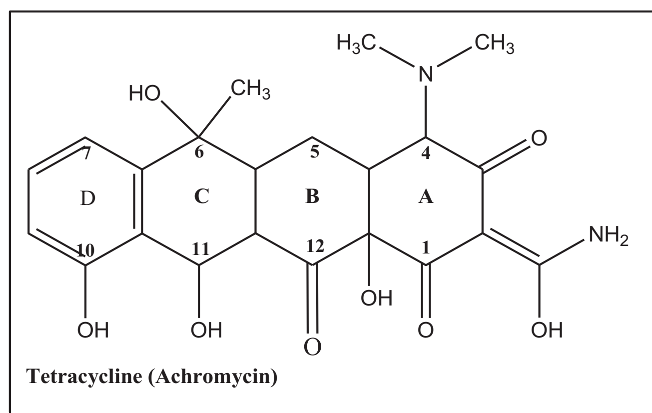

Fig. 14: Basic structure of the tetracycline antimicrobial agents.

Table 5: Principal members of the tetracycline class (175).

Generic name	Trade name	Group (Year of discovery)
Chlortetracycline	Aureomycin	1st generation (1948)
Oxytetracycline	Terramycin	1st generation (1948)
Tetracycline	Achromycin	1st generation (1953)
Demethylchlortetracycline	Declomycin	1st generation (1957)
Rolitetracycline	Reverin	1st generation (1958)
Limecycline	Tetralysal	1st generation (1961)
Clomocycline	Megaclor	1st generation (1963)
Methacycline	Rondomycin	1st generation (1965)
Doxycycline	Vibramycin	2nd generation (1967)
Minocycline	Minocin	2nd generation (1972)
Tigecycline	Tygacil	3rd generation (1993)

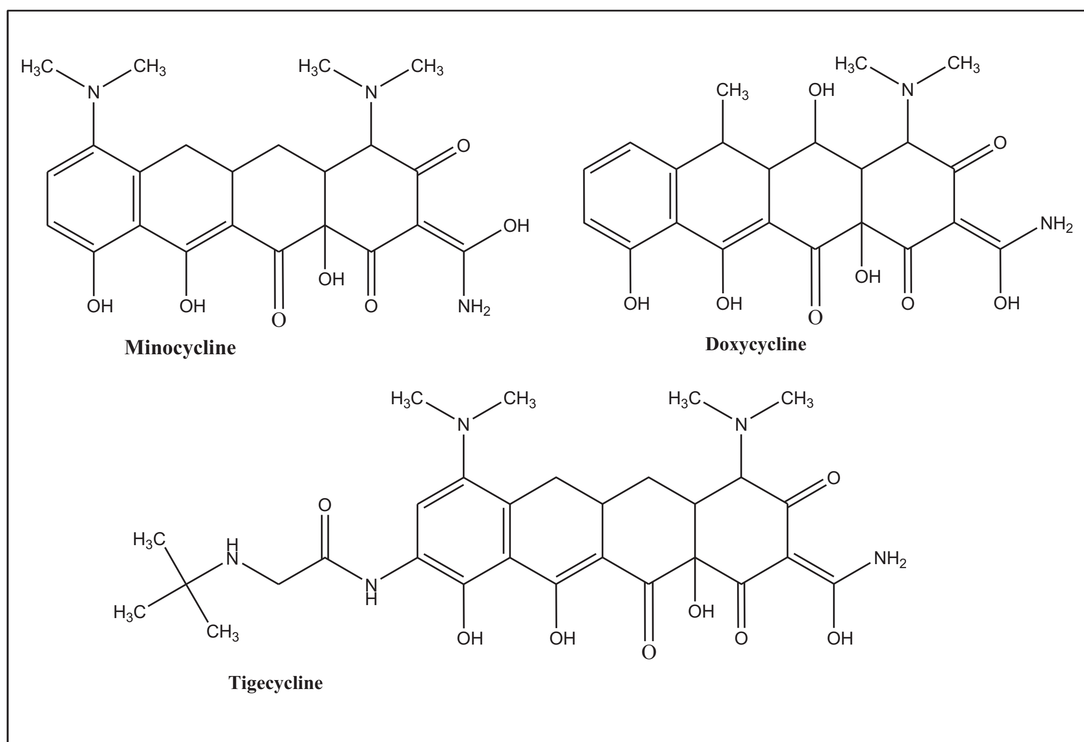

Fig. 15: Graphic representation of second and third generation tetracyclines: Doxycycline, Minocycline and Tigecycline.

- ***Mode of action***

Tetracyclines inhibit bacterial protein synthesis by binding to the ribosomal complex and preventing the association of aminoacyl-tRNA with the bacterial ribosome. In Gram-negative bacteria, tetracyclines traverse the outer membrane through porin channels and after accumulation in the periplasmic space, they cross the cytoplasmic membrane driven by an energy-dependent proton motive force. Inside the bacterial cell, there is a reversible union between the tetracycline molecule and the prokaryotic 30S ribosomal subunit and as a consequence of that, the bacterial protein synthesis stops. The **bacteriostatic** effect of tetracyclines is due to this reversible union between the antibiotic and the ribosome; diluting out the concentration of antibiotic inside the bacterial cell can reverse the effect, and the protein synthesis starts again (26,175,182).

Tetracyclines also inhibit protein synthesis in mitochondria by binding to the 70S ribosomes in these organelles. On the other hand, these antimicrobial agents interact weakly with the 80S ribosome of eukaryotic cells, creating a relatively weak inhibition of protein synthesis; hypothetically, this is the reason for the selective antimicrobial properties of the tetracyclines which have limited side effects in humans (26,175,182).

- ***Development of resistance***

Tetracyclines have been widely used as **growth promoters** in animal feeds, for veterinary therapy and also as prophylaxis. Antibiotics used as growth promoters are associated to a high risk for selection of resistance because they are used continuously at subtherapeutic levels. Over the years, enough data has been collected to suggest that all this inappropriate use of these antimicrobial agents has as a consequence the selection of resistant animal pathogens (such as *Salmonella* spp., *Campylobacter* spp. and *Yersinia* spp.) and commensals (such as *E. coli* and enterococci); these pathogens exist in both

human and animals. Although it is not possible to demonstrate that the level of resistance in human pathogens is aggravated by the use of antibiotics in animal feed, there are increasing concerns that this could constitute a human health hazard (26).

In the 1960s, the Swann Committee concluded that antibiotics used for treatment in human infections or those that can produce cross-resistance to other antibiotics, **should not be used as animal promoters;** in the 1970s, the subtherapeutic use of tetracyclines was banned (26).

- ***Mechanisms of resistance***

Microorganisms become resistant to tetracyclines mainly by acquisition of the **tetracycline resistance genes** (*tet* genes); these genes generally encode for efflux pumps or ribosomal protection proteins. Nevertheless, bacteria can occasionally become resistant to tetracyclines by other mechanisms such as enzymatic inactivation, decreased accumulation within the cell or target modification (Fig. 8).

✓ Efflux pumps: The *tet* efflux genes (*tet*A to *tet*E) encode for membrane associated proteins which export tetracycline out of the cell by exchanging a proton for a tetracycline-cation complex. This mechanism reduces the concentration of antibiotic inside the cell and protects the ribosomes from the action of tetracycline. These genes generally confer resistance to tetracycline and doxycycline but, the *tet*(B) gene also confers resistance to minocycline. At present, glycylcyclines are not affected by these efflux pumps; however, strains resistant to this group of antibiotics have been obtained *in vitro*, suggesting that bacterial resistance may develop with the clinical use (26,175,182). It is thought that tigecycline overcomes these mechanisms of resistance because of steric hindrance due to a large substituent at position 9 (155). In addition to these tetracycline-specific efflux pumps, this class of antimicrobial agents can also be

expelled out of the cell by multidrug efflux systems such as: CmeABC in *Campylobacter jejuni* (78); MexAB, MexCD and MexXY in *P. aeruginosa* (133,141), or AdeABC and AdeIJK in *A. baumannii* (38,160).

- ✓ Ribosomal protection proteins: The *tet* genes encoding for ribosomal protection proteins confer a wider spectrum of resistance than the above described efflux pumps. These cytoplasmic proteins protect the ribosomes from the action of tetracycline, doxycycline and minocycline; at present, glycylcyclines remain unaffected. The ribosomal protection proteins have a GTPase activity and it is thought that the energy from the GTP hydrolysis releases the tetracycline from the ribosome (26,155,175,182).
- ✓ Enzymatic inactivation: The *tet*(X) gene encodes for a protein that chemically modifies tetracycline in the presence of oxygen and NADPH. It is the only example of this mechanism of resistance to tetracyclines and it has only been found in *Bacteroides* spp. (26,175).
- ✓ Decreased accumulation: The presence of mutations that modify the permeability of the outer membrane can have an effect on the level of resistance to tetracyclines and other antibiotics. In addition, the presence of other efflux pumps not specific for tetracyclines can also reduce the concentration of tetracyclines inside the bacterial cell (175).
- ✓ Target modification: Mutations in two different positions of the rRNA 16S gene have been involved in resistance to tetracyclines in *Propionibacterium cutania* and *Helicobacter pylori* (175) .

3.4 Quinolones

Quinolones are synthetic antimicrobial agents with a broad-spectrum of activity. These antibiotics have experienced a significant evolution since the discovery of the nalidixic acid, the first member of this group, in 1962. Initially, nalidixic acid had a limited spectrum of action and was mainly used to treat Gram-negative urinary tract infections. Since then, structural modifications of the initial molecule have resulted in the development of the fluoroquinolones, a large and expanding group of synthetic compounds which have considerably increased the initial spectrum of activity of the first quinolones. Currently, there are **four-generations of quinolones** with a wide range of clinical applications (4,52,192).

- ***Chemical structure and classification***

The first quinolone, nalidixic acid, was obtained as an impurity during the manufacture of quinine. The basic chemical structure of the quinolones is based on the **1,4-dihydro-4-oxo-pyridine molecule**, which has a carboxylic acid substituent at position 3 (Fig. 16); this substituent together with the carbonyl group at position 4 seems to be essential for the activity of the quinolones (226). Their aromatic core is smaller than the typical intercalators and they have bulky substituents which makes unlikely their classification as part of the intercalator group (206). Currently, there are four generations of quinolones in use; their classification depends on the different substituents and the position of the substitution (Table 6).

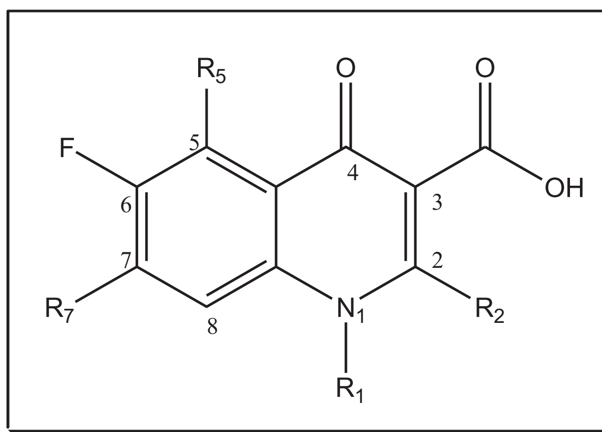

Fig. 16: Basic structure of the quinolones.

First generation quinolones (Fig. 17) contain the original naphthyridine core. However, some compounds in this group, such as pipemidic acid, have a piperazine substitution at the position 7 which gives them certain activity against *P. aeruginosa* (4). The members of this first generation are predominantly used for the treatment of urinary tract infections because they reach high concentrations in the urinary tract. They have moderate Gram-negative activity and are not commonly used since other more evolved quinolones have been discovered (152,226).

Second generation quinolones (Fig. 17) have a cyclic diamine at position 7 and a fluorine atom at position 6 in the quinolone nucleus. Ciprofloxacin is probably the most popular member of this group and also one of the most commonly utilized antibiotics worldwide. They have an excellent activity against Gram-negative bacteria, atypical pathogen coverage and moderate activity against *Staphylococcus aureus* and *Streptococcus pneumoniae* (152,226).

Third generation quinolones (Fig. 17) differ from the previous group in the substituents located at positions 1, 7 and 8 of the quinolone nucleus; these compounds can

have two or three fluor atoms within the molecule. They retain the expanded Gram-negative and atypical intracellular activity of the second generation quinolones but, they also have an improved activity against Gram-positive cocci and some activity against anaerobes (152,226).

Fourth generation quinolones (Fig. 17) maintain the Gram-negative activity and have an improved Gram-positive and anaerobic activity (152).

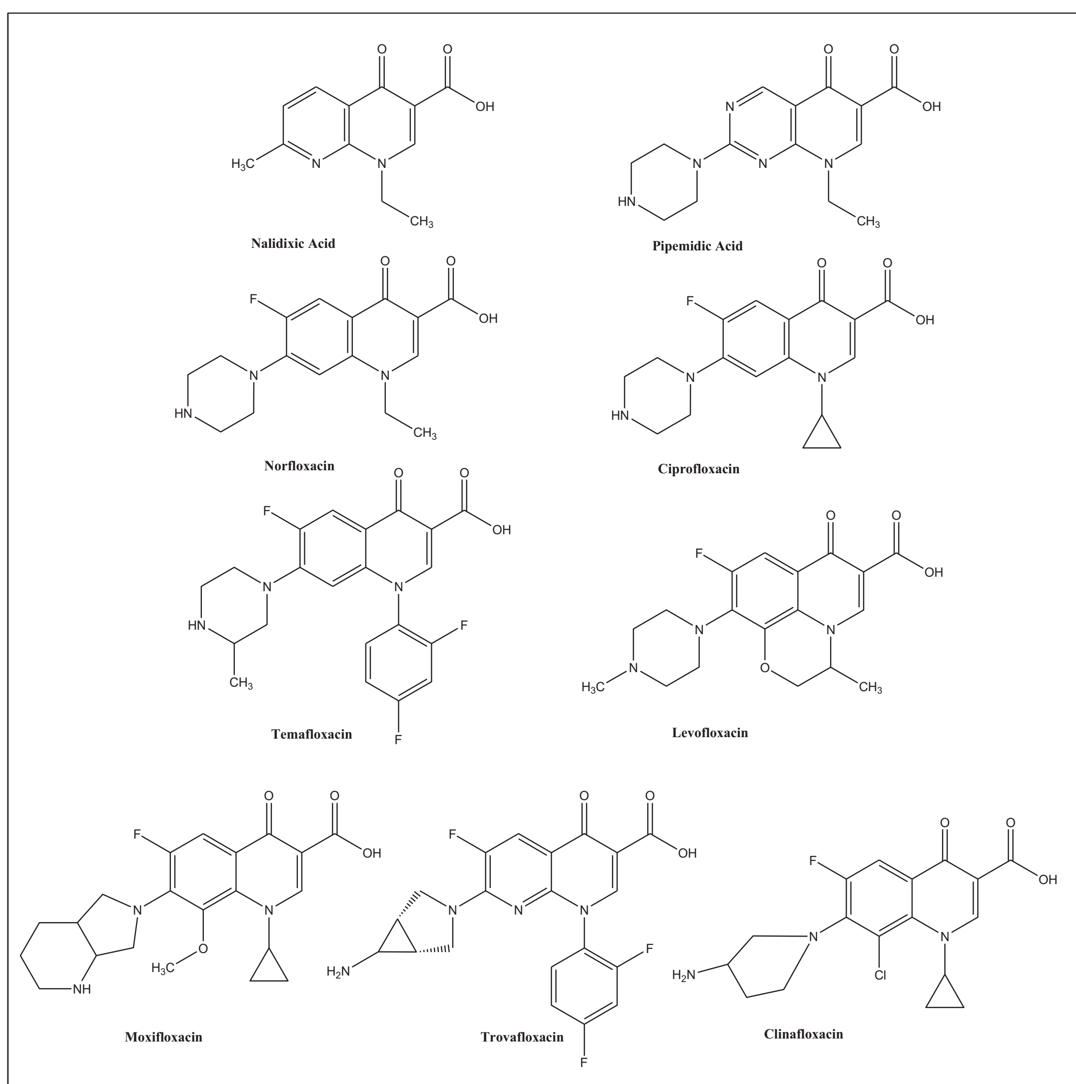

Fig. 17: Structure of the main antimicrobial agents from the four quinolone generations.

Table 6: Quinolone classification (31).

Classification	Activity	Antibiotics
1st Generation	*Enterobacteriaceae* in the urinary tract	Nalidixic Acid, Pipemidic Acid, Oxolinic Acid, Cinoxacin, Piromidic Acid, Flumequine
2nd Generation	Aerobic Gram negative bacilli	Norfloxacin, Pefloxacin, Enoxacin, Nadifloxacin, Tosufloxacin, Ofloxacin, Ciprofloxacin
3rd Generation	Gram negative bacilli & aerobic Gram positive cocci	Levofloxacin, Sparfloxacin, Grepafloxacin, Temafloxacin
4th Generation	Gram negative bacilli, Gram positive cocci & anaerobic microorganisms	Clinafloxacin, Gatifloxacin, Moxifloxacin, Gemifloxacin, Trovafloxacin; Sitafloxacin

- ***Mode of action***

Quinolones are bactericidal compounds that inhibit protein synthesis by inhibiting the activity of two homologous type II topoisomerases: **the DNA gyrase and the topoisomerase IV**. These enzymes are involved in maintaining the integrity of the supercoiled DNA helix during the processes of replication and transcription; without them, the DNA would remain unwound and too large to fit into the cell (52). The DNA gyrase is a tetramer composed of 2 A-subunits and 2 B-subunits which are encoded by the *gyr*A and *gyrB* genes. This enzyme catalyzes the ATP-dependent negative supercoiling of DNA and it is known to play an important role in the transcription and replication of DNA. The topoisomerase IV, which also has a tetrameric structure (A_2B_2), is encoded by the *par*C and *par*E genes and it is involved in the decatenation of daughter replicons following DNA

replication (162,192,226). The DNA gyrase and the Topoisomerase IV cleave double-stranded DNA, pass another strand of DNA through the opening, and re-ligate the cut ends, using ATP as energy source, to produce supercoils. Quinolones do not prevent the cleavage of the DNA but they bind to the DNA gyrase in the presence of DNA; after duplex-strand breakage, they fix the enzyme-DNA complex together, and as a result, free DNA is released causing chromosomal disruption and cell death (Fig. 18) (162,206,226).

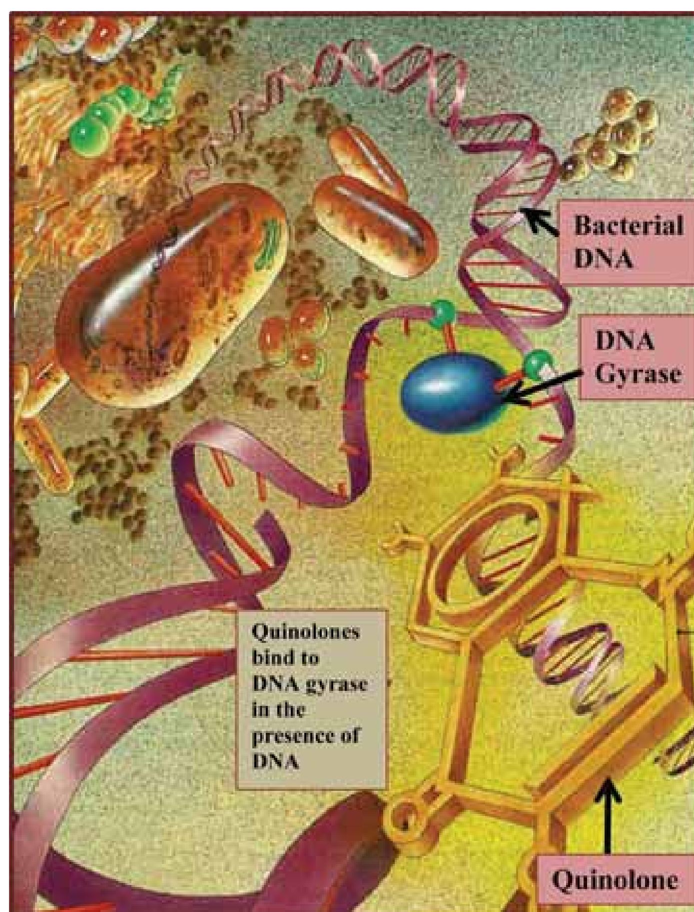

Fig. 18: Representation of the mode of action of fluoroquinolones (74).

- ***Mechanisms of resistance***

The quinolones, but especially the fluoroquinolones have been used as prophylaxis and also to treat a great variety of infections; in addition, they have also been extensively used in veterinary practice, either for animal treatment or as growth promoters. The high level of use, and sometimes misuse, can be responsible for the rapid development of bacterial resistance (190). The mechanisms of quinolone resistance are mainly due to chromosomal mutations that involve modification of the topoisomerases genes, and decreased accumulation inside the cell, either due to a decreased permeability of the membrane or an overexpression of efflux pump systems (Fig. 8). However, the presence of mobile elements carrying resistance gene has recently been reported as a mechanism of reduced resistance to quinolones; these mechanisms have potential for horizontal transfer of quinolone resistance genes (190,226).

- ✓ <u>Topoisomerase mutations</u>: The quinolone targets are different in Gram-negative and Gram-positive microorganisms. In Gram-negative bacteria, the main target is the DNA gyrase and the mutations involved in quinolone resistance occur mainly in a region of the *gyr*A gene known as the **quinolone-resistance determining region (QRDR)**. The presence of a single mutation results in high-level resistance to nalidixic acid; however, with the fluoroquinolones it is necessary additional mutations in the *gyr*A or *par*C genes (190,192,226).
- ✓ <u>Decreased membrane permeability</u>: All the quinolones may cross the bacterial outer membrane through porins while the hydrophobic quinolones can also enter the cell using other non-porin pathways. Although LPS-defective mutants present an increased susceptibility to hydrophobic quinolones, the resistance associated to permeability has usually been associated to porin decreased expression (especially OmpF) (190,192,226).

- ✓ Efflux pumps: Different efflux systems pump out quinolones and other antimicrobial agents from the bacterial cell. Analysis of the *E. coli* genome has revealed the presence of at least 37 putative drug transporters (190).
- ✓ Plasmid-mediated resistance: The first report of plasmid-mediated resistance was found in a *Klebsiella pneumonia* isolate. Currently this mechanism is found in several Gram-negative pathogens. The *qnr* gene is generally located within an integron and transmitted by plasmid transfer between isolates. It encodes a protein that protects the DNA gyrase from the activity of the quinolones conferring low level quinolone resistance (226). A new variant of the aminoglycoside acetyltransferase Aac(6')-Ib also produces a reduced susceptibility in front of ciprofloxacin; the presence of this modified Aac(6')-Ib-cr (cr = ciprofloxacin resistance) enzyme, together with the *qnr* gene, results in a four-fold increment in the level of resistance to ciprofloxacin (183). Recently, a new plasmid-mediated mechanism of resistance has been described by Yamane *et al.* (238); the *qep*A gene encodes an efflux pump which confers resistance to hidrophylic quinolones such as norfloxacin and ciprofloxacin (238).

3.5 Polymyxins

The polymyxin family was discovered in 1947 from different species of *Bacillus polymyxa* and includes five chemically different compounds (polymyxins A to E). Among them, the only clinically valuable compounds are polymyxin B and especially polymyxin E (colistin), two structurally similar compounds which only differ in one aminoacid. In 1960, colistin became available for clinical use, but a decade later, it was replaced by other antibiotics which were considered less toxic. Currently, the emergence of multidrug-resistant Gram-negative pathogens and the lack of new antimicrobial agents have led to the recovery of this old antibiotic; even though its toxicity, colistin remains as a last resource treatment for some diseases caused by these multidrug-resistant pathogens (59,121,242).

- ***Chemical structure and classification***

Colistin is a multicomponent lipopeptide antibiotic that was isolated in 1949. This antimicrobial agent can be commercially found in two different forms: colistin sulphate (polycation) for oral and topical use and colistimethate sodium (polyanion) for parenteral use (121). Structurally, colistin consists of a **polycationic cyclic heptapeptide and a tripeptide side chain linked to a fatty acid chain** through an α-amide linkage (Fig. 19). Two different colistin molecules are obtained depending on the fatty acid chain: colistin A contains a 6-methyl-octan-oic acid and colistin B contains a 6-methyl-eptanoic acid (Fig. 20). Different pharmaceutical preparations of colistin may contain different amounts of these two molecules (59,121,242).

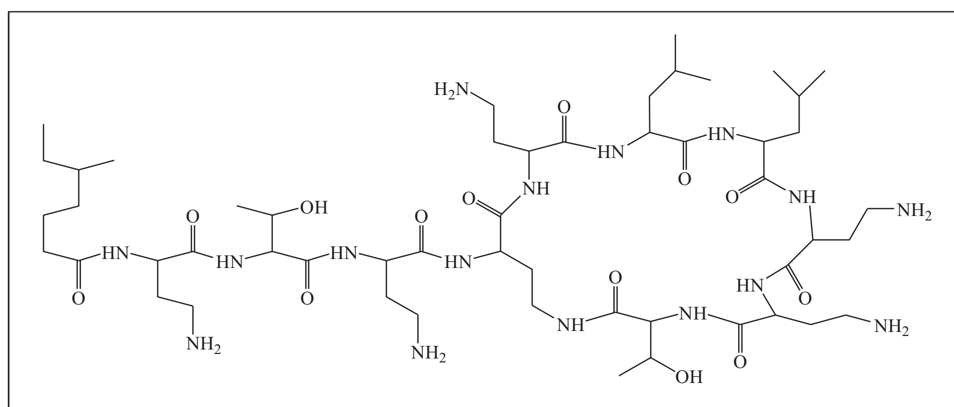

Fig. 19: Structural representation of the colistin molecule

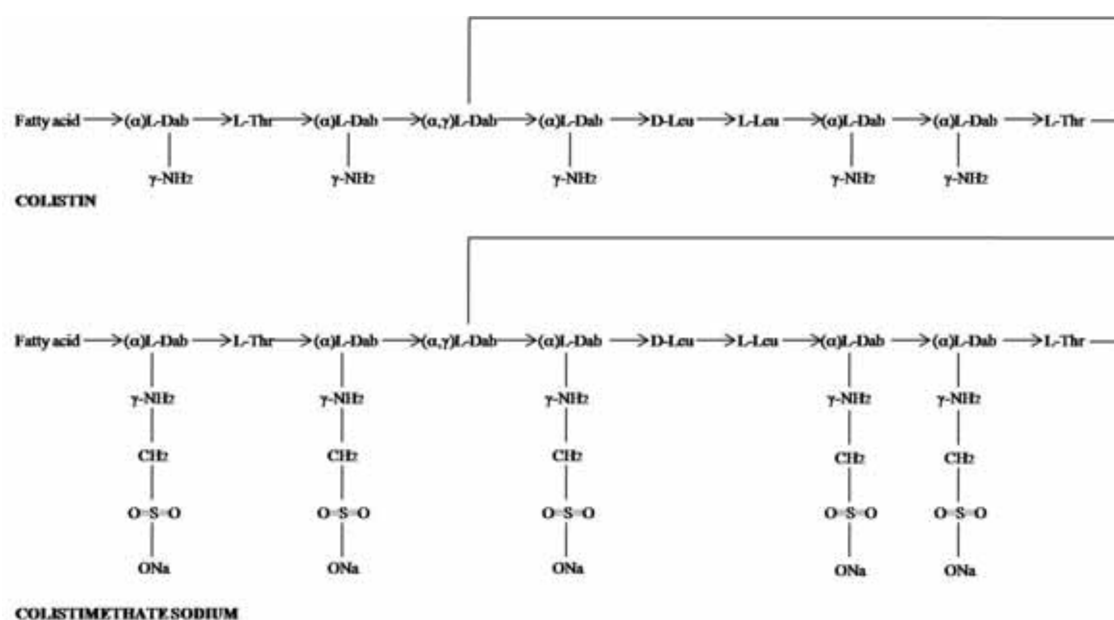

Fig. 20: Structure of colistin and colistimethate sodium; the fatty acid is 6-methyloctanoic acid in colistin A or 6-methylheptanoic acid in colistin B. "Dab"-Diaminobutyric acid; "Leu"-Leucine; "Thr"-Threonine (59).

- ***Mode of action***

Polymyxins are bactericidal agents with a detergent-like mechanism of action over the bacterial cell membrane. These antibiotics interact with the LPS and are taken inside the cell by a "self-promoted uptake" pathway. The initial binding of colistin to the bacterial membrane is due to an electrostatic interaction between the polycationic peptide ring and

the anionic LPS. As a direct consequence of this union, the calcium and magnesium bridges that stabilize the LPS are displaced, resulting in the disruption of the cell membrane. An additional interaction between the fatty acid side chain and the LPS contributes to the insertion of the colistin into the membrane and causes an increase in cell permeability. Fissures in the affected cell membrane allow the leakage of cell contents and the subsequent cell death (59,242). In addition to the direct antibacterial activity, colistin has also a potent anti-endotoxin activity, although the significance of this function for *in vivo* antimicrobial action is not clear (59).

- ***Toxicity***

Nephrotoxicity and neurotoxicity are the most common side-effects with parenteral administration of colistin. Nevertheless, the high levels of toxicity obtained in the initial clinical studies were certainly due to a lack of understanding of the pharmacokinetics, pharmacodynamics and toxicodynamics, together with the use of inappropriate doses. Data from recent studies suggest that the toxicity caused by the polymyxins is less frequent and severe than previously reported. Recent reports on toxicity in patients with cystic fibrosis, suggest that the nephrotoxicity of this antimicrobial agent is similar to the aminoglycosides and the neurotoxic effects are usually mild and resolve rapidly after discontinuation of the treatment with colistin (60,121,212).

The considerable difference between old and recent studies regarding colistin toxicity can be due to a compilation of factors: first, the formulations used at present may be better purified; second, the fluid supplementation and supportive treatment has also improved and finally, the doses of colistin administered during the first years were higher probably due to a lack of knowledge on the pharmacodynamics and pharmacokinetics (61).

- ***Mechanisms of resistance***

There are several mechanisms of resistance to colistin which involve mostly changes in the outer membrane. The most common mechanism of resistance is based on **LPS modification** that reduces the initial interaction between colistin and the cell membrane. However, resistance can also be due to reduced levels of specific OMPs and reduction in Mg^{+2} and Ca^{+2} content (Table 7) (59,242). Different microorganisms acquire resistance by different mechanisms but it is a cause of concern that microorganisms, such as *A. baumannii*, could become resistant to colistin which is considered a last resource treatment for infections with multidrug-resistant pathogens.

Table 7: Major mechanisms of resistance polymyxins in Gram-negative bacteria (242).

Microorganism	Mechanisms of resistance to polymyxins
Pseudomonas aeruginosa	Lipid A modifications with L-Ara4N (controlled by PmrA/PmrB)
Salmonella enterica serovar Typhimurium	Lipid A modification with L-Ara4N and PEtn (controlled by PmrA/PmrB)
Escherichia coli	Lipid A modification with L-Ara4N and PEtn (controlled by PmrA/PmrB)
Klebsiella pneumoniae	Increased production of capsule polysaccharide
Burkholderia cenocepacia	A complete LPS inner core oligosaccharide is required
Helicobacter pylori	Lipid A modification
Yersinia pestis	Lipid A modification with L-Ara4N (controlled by PmrA/PmrB)
Vibrio cholerae	Presence of OmpU (regulated by ToxR)

4. MECHANISMS OF RESISTANCE TO THE ANTIMICROBIAL AGENTS IN *ACINETOBACTER BAUMANNII*

All microorganisms can be killed with the appropriate concentration of antibiotic but, patients cannot tolerate high antibiotic doses. Therefore, bacteria are described as resistant when they cannot be killed with therapeutic levels of antibiotics. Clinical resistance depends on the type of bacteria, location of the infection, immune status of the patient, and the concentration of antibiotic within the body (87).

Resistance to an antimicrobial agent can be intrinsic or acquired. **Intrinsic or natural resistance** is a naturally occurring attribute due to the biology of the microorganism; it is mainly the consequence of cellular impermeability and activity of efflux pumps. On the other hand, **acquired resistance** occurs when previously susceptible bacteria develop resistance to an antibiotic by mutations or through the acquirement of exogeneous DNA. Mutations are spontaneous events which confer a great advantage over the susceptible bacterial population; all the susceptible bacteria will be killed by the antibiotic leaving a resistant subpopulation of mutated bacteria. Favourable mutations can be transferred to other microorganisms via insertion sequences and transposons present in plasmids (87).

The resistance of *A. baumannii* to antimicrobial agents is mediated by all of the major resistance mechanisms that are known to occur in bacteria, including cellular impermeability or active efflux of the antibiotic, inactivation or modification of the antimicrobial agent, and modification of the antimicrobial target sites.

4.1 Permeability changes and active efflux of the antimicrobial agent

Non-fermentative bacteria are a challenge for healthcare management because they represent the problem of multidrug resistance to the extreme. These pathogens present a high intrinsic resistance and are able to rapidly acquire mechanisms of resistance to other antimicrobial agents. Intrinsic resistance to common antibiotics is principally due to the outer membrane which is more impermeable than the outer membrane of other Gram-negative pathogens. The outer membrane of non-fermenters is between 10 and 100-fold more impermeable than the outer membrane of *E. coli*. The presence of efflux systems also contribute to the intrinsic and acquired resistance of these microorganisms (135).

In *A. baumannii*, broad-spectrum antimicrobial resistance is due to a variety of intrinsic mechanisms of resistance together to an extraordinary rapid acquirement of new ones. The diminished permeability of the outer membrane, which may be even more restrictive than that of *P. aeruginosa*, is an important feature for the intrinsic resistance of these bacteria (170).

4.1.1 PAPER I - Review:

"Porins, efflux pumps and multidrug resistance in Acinetobacter baumannii".

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Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*

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Acinetobacter baumannii **is an opportunistic pathogen, causing infections mainly in patients in intensive care units where the extensive use of antimicrobial agents can select for the emergence of multiresistant strains. In fact, since strains resistant to all antimicrobial agents have been reported,** ***A. baumannii*** **is considered the paradigm of multiresistant bacteria. Both acquired and intrinsic resistance can contribute to multiresistance. The ability to acquire multidrug resistance can be due to either the acquisition of genetic elements carrying multiple resistant determinants or mutations affecting the expression of porins and/or efflux pump(s), which can affect unrelated antimicrobial agents. Meanwhile, intrinsic resistance can be generated by the interplay of decreased permeability and constitutive expression of active efflux systems and it too can affect unrelated antimicrobial agents. This review is focused on the current knowledge of porins and efflux pump(s) in this microorganism.**

Keywords: permeability, intrinsic resistance, *A. baumannii*

Introduction

Thirty-two different genomic species are currently accepted in the *Acinetobacter* genus and *Acinetobacter baumannii* is, undoubtedly, the most frequently isolated species of greatest clinical interest.[1] Since isolates resistant to all antimicrobial agents have been described,[2] this species can be considered the paradigm of multiresistant bacteria. Several factors can favour the acquisition of multiresistance: one is the ability to survive in environmental and human reservoirs. Numerous publications have reported the presence of *Acinetobacter* spp. in different hospital environments, either as the source of an outbreak or in metastatic locations.[3,4] *Acinetobacter* spp. may survive on dry surfaces longer than reported for *Staphylococcus aureus* and *Pseudomonas aeruginosa*[5] and there is no difference between the survival times of sporadic and outbreak strains of *A. baumannii*.[6] Survival is probably due to the minimal nutritional requirements needed by *Acinetobacter* spp. to grow and its ability to grow at different temperatures and pH values.[7] *A. baumannii* may also contribute to the bacterial flora of the skin, particularly in regions such as the axilla and groin.[8] *Acinetobacter* spp. have also occasionally been found in the oral cavity and respiratory tract of healthy individuals.[9] However, the carrier state in these zones is more common in hospitalized patients, particularly during an epidemic outbreak. Colonization of the intestinal tract by *Acinetobacter* spp. is controversial. While some authors suggest that it is an unusual event,[10] others report that the gastrointestinal tract is the most important reservoir of resistant strains.[11] The difference is probably due to the epidemiological situation, i.e. whether there is an epidemic outbreak or not. The second factor widely influencing the acquisition of multiresistance is the acquisition of genetic elements. Among these elements, plasmids, transposons and integrons have been reported. In the early 1980s, Goldstein and colleagues[12] demonstrated the presence of a plasmid containing three resistance genes, one gene encoding a β-lactamase TEM-1 and two genes encoding aminoglycoside-modifying enzymes [APH(3′)(5′)I and ADD(3″)(9)]. Transposons may also play an important role in ensuring the establishment of new resistance genes. Ribera *et al*.[13] partially characterized a transposon carrying the *tetR* and *tet*(A) genes, encoding a regulatory protein and a tetracycline resistance determinant. In the last 5 years, a plethora of papers has been published reporting the implication of the integrons in *A. baumannii* as genetic elements that carry different antibiotic resistance genes.[14–20] On comparing the genome of a multiresistant *A. baumannii* strain versus a fully susceptible strain, Fournier *et al*.[21] recently found that the resistant strain carried a 86 kb resistance island in which 45 resistance genes were clustered. This island also contained two operons associated with arsenic and mercury resistance, respectively and four *qacE1* genes encoding small multidrug resistance (SMR) efflux pumps, which confer low-level resistance to ammonium antiseptics.

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The third factor favouring the acquisition of multiresistance is the intrinsic resistance of these microorganisms, which can be explained by the low permeability of certain antibiotics through the outer membrane, the constitutive expression of some efflux pumps or the interplay between the two processes. This review is focused on the current knowledge of outer membrane permeability as well as outer membrane proteins (OMPs) and efflux pumps associated with antibacterial agent resistance characterized to date.

OMPs

Porins are proteins able to form channels allowing the transport of molecules across lipid bilayer membranes, that show little permeability for hydrophilic solutes. They provide membranes with multiple functions. Porins can act as potential targets for adhesion to other cells and binding of bactericidal compounds to the surface of Gram-negative bacteria. Variations in their structure as a mechanism to escape from antibacterial pressure or regulation of porin expression in response to the presence of antibiotics are survival strategies that have been developed by many bacteria. Porins may play a significant role in mechanisms of resistance.

One of the limitations of our knowledge of *A. baumannii* is the lack of information concerning its OMPs and the permeability properties of this outer membrane. Until now, only a few OMPs have been reported and their functions remain unclear.[22] The small number and size of porins could explain the decrease in *A. baumannii* outer membrane permeability (less than 5%) when compared with other Gram-negative organisms.[23] The outer membrane in *A. baumannii* is less permeable to antimicrobial agents than that in *Escherichia coli*. In accordance with Sato and Nakae[24] the coefficient of permeability to cephalosporins is between 2- and 7-fold larger in *P. aeruginosa* than in *Acinetobacter* spp. They therefore suggested that the intrinsic cause of the resistance to antimicrobial agents could be attributed to the small number of porins as well as their small size. However, another possibility to maintain this intrinsic resistance to antimicrobials could be the low level of constitutive expression of one or several active efflux systems in *A. baumannii* or to the interplay between both low permeability and constitutive expression of efflux pump(s).[7]

In several reports the decreased expression of some OMPs has been shown to be associated with antimicrobial resistance in *A. baumannii*.[25–27] The major OMP of *A. baumannii* described to date is the heat-modifiable protein HMP-AB.[28] These porins show a different mobility following SDS–PAGE without heating and after 10 min at 95°C.[29] The HMP-AB gene encodes a protein of 346 amino acids with a molecular mass of 35 636 Da and is assembled in the membrane in a similar manner to monomeric porins.[28] Sequence comparison of HMP-AB with other OMPs revealed a clear homology with the monomeric OMP A (OmpA) of Enterobacteriaceae and the OMP F (OprF) of *P. aeruginosa*. Secondary structure analysis indicated that HMP-AB has a 172-amino-acid N-terminal domain that spans the outer membrane by eight amphiphilic beta strands and a C-terminal domain that apparently serves as an anchoring protein to the peptidoglycan layer. Analysis of the amino acid sequence reveals the typical structure of Gram-negative bacterial porins: a highly negative hydropathy index, absence of hydrophobic residue stretches, a slightly negative total charge, low instability index, high glycine content and an absence of cysteine residues. This porin belongs to the OmpA family. Porins of this family are known as slow porins that allow the penetration of β-lactams and saccharides up to approximately 800 Da.[28,29] Slow porins belonging to this family allow a much slower diffusion of small solutes but allow the diffusion of much larger solutes that cannot penetrate through the OmpF channel of *E. coli*.[30] Therefore, in organisms that lack the classical trimeric porin, the protein of this family functions as the major porin and contributes to the high levels of intrinsic resistance.[30]

The OmpA from different species of *Acinetobacter* has recently been described and characterized.[31] The sequenced fragment was found to be homologous among *A. baumannii*, *Acinetobacter radioresistens* and *Acinetobacter junii*. However, the authors did not mention the similarity between this OmpA and the above-mentioned HMP-AB. In the *A. radioresistens* (KA53) strain, the OmpA was found to be a secreted emulsifier. It is known that bio-emulsifiers play an important role in bacterial pathogenesis, quorum sensing and biofilm formation, regulating adhesion to surfaces.[31]

Three other OMPs have been reported to be missing in the imipenem-resistant strains of *A. baumannii*: one is a 33–36 kDa protein,[32] another is a 29 kDa protein, designated CarO[27,33] and, finally, a 43 kDa protein, which shows significant peptide homology with OprD from *P. aeruginosa*.[34] On studying CarO by mass spectrometry Siroy *et al*.[33] detected another 25 kDa protein that they called Omp25, together with CarO. Both 25/29 kDa proteins adopted a typical β-barrel conformation, however, only one of these proteins (CarO) displayed pore-forming properties. No binding site for imipenem could be detected in CarO, suggesting an unspecific monomeric channel function rather than a specific function.[33] It is important to mention that the protein OprD of *P. aeruginosa* has been demonstrated to be involved in the uptake of basic amino acids, small peptides and of imipenem and meropenem.[30] Therefore, CarO may function as a carbapenem-unspecific channel and the OprD-like protein may function as a carbapenem-specific channel.

Another OMP in *A. baumannii* is OmpW, which shows high homology with OmpW found in *E. coli* and *P. aeruginosa*. Its function in *A. baumannii* remains unclear, however, we have recently found that the expression of this OMP was decreased in an '*in vitro*' colistin-resistant *A. baumannii* mutant (Sara Martí, unpublished data). However, based on the observation that OmpW expression was dramatically decreased in a ceftriaxone-resistant strain of *Salmonella* Typhimurium, it was recently proposed that OmpW might also be involved in the uptake of this antibiotic.[35]

More in-depth studies are necessary to elucidate the role of these OMPs in multidrug resistance and to fully characterize the complex structure of the outer membrane of *A. baumannii* that confers its special permeability features.

Efflux-pump-mediated resistance

In Gram-negative bacteria, the outer membrane limits the rate of antimicrobials entering the cell and the multidrug efflux pumps actively export multiple, structurally-distinct classes of antimicrobials out of the bacteria.[36] Efflux transporters are expressed in all living cells, protecting them from the toxic effects of

organic chemicals. Bacterial multidrug resistance has often been associated with overexpression of these transporters. The antimicrobials expelled out of the cell have to cross the low permeability outer membrane in order to enter again; therefore the efflux pumps work synergistically with the low permeability of the outer membrane.[36] An increased efflux of antibiotic from the bacterium produces a reduction in drug accumulation and an increment in the MIC. The most common antimicrobials expelled by the efflux pumps are macrolides, tetracyclines and quinolones.[37] In all the metabolic processes there is generally a high degree of specificity in the transport of proteins and enzymes, although multidrug efflux pumps recognize a broad range of structural and chemically different substrates.[38]

The multidrug efflux systems have been grouped into six families:[36] the ATP binding cassette (ABC) family, the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) family, the multidrug and toxic compound extrusion (MATE) family, the SMR family and the drug/metabolite transporter (DMT) superfamily.

ABC-type efflux pumps are ATP-dependent multidrug transporters and use ATP as a source of energy to expel the antimicrobials out of the cell. The members of this family are rarely involved in acquisition of resistance to antimicrobials in Gram-negative bacteria. The other types of efflux pumps are drug-proton antiporters. Antimicrobial expulsion is accomplished utilizing the proton motive force as the driving force for efflux.[36,39] The major efflux pumps involved in multidrug resistance belong to this group of proton-motive-force-dependent exporters, with the most important group being the RND family, as well as the MFS and SMR families.[36]

In *A. baumannii*, efflux-pump-mediated resistance to antimicrobials is generally associated with the MFS and RND family (Table 1). Using a comparative genomic approach Fournier *et al.*[21] recently attempted to identify all the resistance genes present in the *A. baumannii* multidrug-resistant strain AYE, which was epidemic in France. Most of the resistance genes found in this strain had been acquired from other bacteria such as *Pseudomonas*, *Salmonella* or *E. coli* and were clustered in an 86 kb region or island. This resistance island (AbaR1) contained 45 genes predicted to be associated with resistance to antimicrobial drugs, heavy metals and antiseptics. Outside of this island, 46 ORFs were putatively associated with resistance to antimicrobials of which 32 ORFs were associated with the RND family, seven with the MFS, two with the MATE family and one with the SMR family. Moreover, one gene was associated with the ABC superfamily and another with the DMT superfamily.[21]

Major facilitator superfamily (MFS)

The MFS efflux pumps are not normally multidrug transporters, but more usually function as specific exporters for certain classes of antimicrobial agents.

Tet efflux pumps

The two main mechanisms of resistance to tetracycline in Gram-negative bacteria are the expression of an efflux pump or a ribosomal protection system. The Gram-negative *tet* efflux genes can be present on transposons that are inserted into plasmids from a variety of incompatibility groups, most of which are conjugative. Ribera *et al.*[13] identified the *tet*(A) gene in a Tn*1721*-like transposon. Their results suggested that there is a horizontal transfer among different genera of Gram-negative bacteria that share the same ecological niche. This gene encodes a membrane-associated efflux protein that confers resistance to tetracyclines. These efflux pumps belong to the MFS and exchange a proton for a tetracycline–cation complex.[40] In Gram-negative bacteria, there is one gene encoding for an efflux protein and there is another gene encoding for a repressor protein. The system is regulated by the presence of tetracycline. In the absence of tetracycline, the repressor protein blocks the transcription of the structural genes. The process starts when a tetracycline–Mg^{2+} complex

Table 1. Efflux pumps described in *Acinetobacter baumannii* and their activity in front of several antimicrobial agents

Efflux pump	Family	Antibiotics	NCBI accession number(s)
Tet(A)	MFS	tetracycline	AAO38186
Tet(B)	MFS	tetracycline, minocycline	
CmlA	MFS	chloramphenicol	CAJ77032
AdeABC	RND	aminoglycosides, β-lactams, chloramphenicol, erythromycin, tetracyclines and ethidium bromide; reduced susceptibility to fluoroquinolones	AAL14439, AAL14440, AAL14441
AbeM	MATE	norfloxacin, ofloxacin, ciprofloxacin, gentamicin, 4′,6-diamino-2-phenylindole (DAPI), triclosan, acriflavine, Hoechst 33342, daunorubicin, doxorubicin, rhodamine 6G and ethidium bromide	BAD89844

MFS, major facilitator superfamily; RND, resistance-nodulation-division; MATE, multidrug and toxic compound extrusion.

binds to the repressor protein, changing the conformation of this repressor and allowing the transcription of the efflux structural and repressor genes.[40]

In *A. baumannii* the main efflux pumps in this category are: Tet(A) and Tet(B). The efflux determinant Tet(A) confers resistance to tetracycline and Tet(B) confers resistance to tetracycline and minocycline.[41] These efflux pumps do not affect the new tetracyclines such as glycylcyclines. Recently, Martí and colleagues[41] analysed the prevalence of the *tet*(A) and *tet*(B) genes in a collection of 79 tetracycline-resistant *A. baumannii* strains that were not epidemiologically related. They found that 66% of the strains carried the *tet*(B) gene and 13.6% the *tet*(A) gene. None of the strains analysed had both genes. Guardabassi *et al.*[42] suggested that these two efflux pumps were infrequently found among *Acinetobacter* spp. from an aquatic environment.

CmlA and MdfA efflux pumps

The chloramphenicol resistance gene (*cmlA*) encodes for an efflux pump that confers resistance to chloramphenicol and it has recently been described by Fournier *et al.*[21] as forming part of a 86 kb resistance island in *A. baumannii* strain AYE. MdfA is a transporter described in several Enterobacteriaceae, we have recently identified an MdfA orthologue (42.7%) in an *A. baumannii* clinical isolate (data not shown). Bacteria expressing MdfA exhibit multidrug resistance, affecting among others ciprofloxacin and chloramphenicol.

Resistance-nodulation-division (RND) family

AdeABC efflux pump

This family of efflux pumps expels the antimicrobial by utilizing the proton motive force as the driving force for efflux.[36] Overexpression of this normally cryptic, antimicrobial efflux pump confers resistance to aminoglycosides, β-lactams, chloramphenicol, erythromycin, tetracyclines and ethidium bromide.[39,43] In addition, AdeB has been associated with acquisition of reduced susceptibility to fluoroquinolones.[44] Most of the multidrug transporters belonging to this family interact with a membrane fusion protein (MFP) and an OMP. This interaction allows the antimicrobial agent to pass across the inner and the outer membranes of the bacteria without accumulating in the periplasm. Therefore, AdeABC is a three-component efflux pump where AdeA is the MFP, AdeB is the multidrug transporter and AdeC is the OMP.[39] The three genes that encode for these three-component efflux pumps are contiguous in the genome and directly oriented which suggests that they form an operon.[43] The regulator gene appears next to the gene encoding the MFP, followed by the gene encoding the transporter protein and, finally, the gene encoding the OMP; the MFP and the transporter protein are generally co-transcribed.[45] This efflux pump is regulated by a two-component regulatory system (AdeRS): AdeS is a sensor kinase and AdeR is a response regulator.[43] The genes that encode these two proteins are located in front of the *adeABC* genes and are transcribed in the opposite direction. The sensor protein monitors the environmental conditions and activates or inactivates the response regulator protein which controls the expression of the efflux pump.[43] The presence of the OMP AdeC is not essential for resistance because this efflux pump may be associated with other OMPs such as AdeK. This OMP belongs to a new efflux pump identified in *A. baumannii* but which is still being characterized.[43]

Multidrug and toxic compound extrusion (MATE) family

Efflux pump AbeM

This is a multidrug efflux pump that belongs to the MATE family of transporters and has recently been identified by Su *et al.*[46] The AbeM protein shows homology with NorM, but also shows similarities with PmpM in *P. aeruginosa*, VcmA in *Vibrio parahaemolyticus*, YdhE in *E. coli* and HmrH in *Haemophilus influenzae*. The presence of this efflux pump confers more than a 4-fold increase in the MICs of norfloxacin, ofloxacin, ciprofloxacin, gentamicin, 4′,6-diamino-2-phenylindole (DAPI), triclosan, acriflavine, Hoechst 33342, daunorubicin, doxorubicin, rhodamine 6G and ethidium bromide. Moreover, it also produces a reproducible 2-fold increase in the MICs of kanamycin, erythromycin, chloramphenicol, tetraphenylphosphonium chloride (TPPCl) and trimethoprim.[46] This family of efflux pumps is associated with two energy sources: the proton motive force and the sodium ion gradient.[45] AbeM utilizes the proton motive force to expel the antimicrobial out of the cell.[46]

The prevalence of the overexpression of the efflux pumps in *A. baumannii* clinical isolates has only been studied using some efflux pump inhibitors, such as reserpine and MC 207,110.[47,48] These inhibitors are notoriously non-specific and may affect multiple efflux systems disproportionately, however, this does not preclude their utility for detecting the compounded efflux effects of multiple systems. Ribera *et al.*[48] found that in 45% of the *A. baumannii* epidemiologically unrelated clinical isolates, the MIC of nalidixic acid decreased at least 8-fold in the presence of MC 207,110. In contrast, when the MIC was determined in the presence of reserpine, the MIC of ciprofloxacin decreased at least 4-fold in 33% of the *A. baumannii* clinical isolates without affecting nalidixic acid.[47] These disparities in the proportion and degree of changes in the MICs of these two antimicrobial agents implies the functioning of multiple efflux pumps.

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Transparency declarations

None to declare.

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4.2 Inactivation or modification of the antimicrobial agent

Microorganisms produce **antibiotic modifying enzymes** to protect themselves from the effect of the antimicrobial agents; β-lactam antibiotics and aminoglycosides are commonly inactivated by these enzymes. *A. baumannii* produces 4 different types of β-lactamases and also 3 classes of aminoglycoside-modifying enzymes (Table 8).

4.2.1 β-lactamases

The group of β-lactamases identified so far in *A. baumannii* includes more than 50 different enzymes, or their allelic forms (44), and according to their nucleotide sequences, they can be classified in four groups, named class A to class D β-lactamases. Class A, C, and D have a serine at their active site, while the class B enzymes have four zinc atoms at the active site (64). Some of these enzymes are intrinsically found in *A. baumannii* while others have been acquired through natural transformation or by plasmid conjugation (Table 8). Currently, transposons and integrons are very important in the dissemination of these genetic mechanisms of resistance (161).

- ***Ambler class A β-lactamases***

This group of enzymes include narrow and extended-spectrum β-lactamases (ESBL) (Table 8).

✓ Narrow-spectrum enzymes: They are mainly active against benzylpenicillins. TEM-1 and TEM-2 are active against aminopenicillins, SCO-1 against penicillins and CARB-5 against carboxypenicillins (44).

✓ ESBL: The extended-spectrum β-lactamases inactivate benzylpenicillins but also some cephalosporins and monobactams (64). PER-1 has mainly been found in Turkey, Korea, France, Belgium and Bolivia and confers resistance to penicillins and

extended-spectrum cephalosporins. VEB-1 has produced outbreaks in France and Belgium; SHV-12 has been reported from China and in the Netherlands, where they also reported a TEM-116. Recently, a TEM-92 extended-spectrum β-lactamase has also been detected in an Italian hospital and structural analysis showed that it was associated with a Tn3-like transposon (53). Finally, CTX-M-2 hydrolyzes cefotaxime and ceftriaxone and has been isolated form an epidemic strain in a neurosurgical ward in Japan and in Bolivia (161).

- ***Ambler class B β-lactamases***

Class B metallo-β-lactamases (MBLs) are characterized by the presence of a metal ion in the active site (usually zinc) and can inactivate penicillins, cephalosporins and carbapenems. In *A. baumannii*, three groups of acquired MBLs have been identified (Table 8): IMP-like, VIM-like and SIM-1 (64,165).

- ✓ IMP MBLs: This group of enzymes is mostly detected as part of a class 1 integron. At present, six representatives of this group have been identified: IMP-1, IMP-2, IMP-4 (also identified in an *A. junii* clinical isolate), IMP-5, IMP-6 and IMP-11. Carbapenem resistance due to these enzymes is becoming a problem in Korea and the Pacific rim nations (161,165).
- ✓ VIM & SIM MBLs: These two groups of enzymes are rarely found in *A. baumannii*; to date, only the VIM-2 and SIM-1 have been reported from Korea (161,165).

- ***Ambler class C β-lactamases***

Class C β-lactamases are chromosomally encoded cephalosporinases which are common to all strains of *A. baumannii* (Table 8). These enzymes hydrolyze penicillins and cephalosporins, but not cefepime or carbapenems. Phylogenetic analysis suggests that the

genes encoding for these enzymes (*bla* genes) have a common ancestor, and it has been proposed to classify them in a distinct family of β-lactamases: the *Acinetobacter*-derived cephalosporinases (ADCs). To date, 28 bla_{ADC} genes have been found in *A. baumannii* (161); although these genes are normally expressed at low level, their function can be upregulated with the presence of an efficient promoter upstream of the gene. **Insertion sequences (IS)** are small mobile genetic elements with a simple genetic organization which are capable of inserting themselves at multiples sites in a target molecule. They can produce mutations as a result of their translocation, but also activate the expression of the adjacent genes. In *A. baumannii*, the presence of the IS*Aba1* sequence upstream the chromosomal bla_{ADC} genes results in the overexpression of the ADC-type β-lactamases; it is thought that this insertion element serves as a switch to turn on these genes (44,129).

- ***Ambler class D β-lactamases***

The most prevalent carbapenemases in *Acinetobacter* spp. are the carbapenem-hydrolysing class D ß-lactamases (CHDLs) which are divided into four phylogenetic subgroups (Table 8): Subgroup 1 (OXA-23-like) contains the OXA-23, -27 and -49 ß-lactamases; subgroup 2 (OXA-24-like) is composed of OXA-24, -25, -26 and -40 and shares 60% amino acid identity with respect to the subgroup 1. Subgroup 3 includes the OXA-51 variants, which are intrinsic to *A. baumannii*, and shares 56% and 63% amino acid identity with subgroups 1 and 2 respectively. The presence of the insertion sequence IS*Aba1* upstream of this gene is thought to enhance the expression of the intrinsic OXA-51 carbapenemases. Finally, subgroup 4 holds the recently characterized OXA-58 enzymes that shares 59% amino acid identity with OXA-51 and less than 50% with subgroups 1 and 2 (44,165).

Table 8: β-lactamases identified in *A. baumannii* (44).

β-lactamase	Class	Target drug	Location
TEM-1, -2	A	Aminopenicillins	
SCO-1	A	Penicillins	Plasmid
CARB-5	A	Carboxypenicillins	
PER-1, VEB-1, TEM-92, TEM-116, SHV-12	A	Benzylpenicillins, cephalosporins, monobactams	Plasmid or chromosomal
CTX-M-2	A	Cefotaxime, ceftriaxone	Plasmid
IMP	B	Carbapenems	Class 1 integron
SIM-1, VIM-2	B	Carbapenems	Class 1 integron
ADC	C	Cephalosporins	Chromosomal (intrinsic to *A. baumannii*)
OXA-23-like	D	Carbapenems	Plasmid
OXA-24-like	D	Carbapenems	Chromosomal, plasmid (202)
OXA-51-like	D	Carbapenems	Chromosomal (Intrinsic to *A. baumannii*)
OXA-58-like	D	Carbapenems	Plasmid or chromosomal

4.2.2 Aminoglycosides-modifying enzymes

Resistance to aminoglycosides occurs by specific chemical modifications of the hydroxyl or amino groups of the antibiotic. These modifications are catalyzed by O-phosphoryltransferases (phosphotransferases), N-acetyltransferases (acetylases) and O-adenyltransferases (adenylases). Several studies have been performed to determine the types of aminoglycoside-modifying enzymes (AMEs) which are present in *A. baumannii*. Most of the genes coding for these enzymes are only present in some clinical isolates and they are associated to mobile genetic elements such as plasmids, transposons or within integron-type structures, suggesting that they are acquired by horizontal transfer (44). Seward *et al.* analyzed a collection of *Acinetobacter* spp. from 11 countries and concluded that the different genes are not restricted to specific geographical areas; similar integron

structures have been found in isolates from different locations worldwide (161,203). Nevertheless, *A. baumannii* strains isolated from nine different hospitals in Spain showed that the spread of amikacin resistance was directly related to an epidemic strain which had a phosphotransferase APH(3')-VIa (229); this is indeed, the phosphotransferase most commonly isolated in *Acinetobacter* spp.

To date, at least nine different aminoglycoside-modifying enzymes have been found in *Acinetobacter* spp. (Table 9); more than one enzyme can be present in each isolate and in different combinations (44). AMEs are not exclusive from *A. baumannii*; studies have shown that *A. haemolyticus* and other related species are also intrinsically resistant to these antibiotics due to the synthesis of a specific chromosomally encoded N-acetyltransferase AAC(6') (223). Recently, a new type of acetyltransferase, AAC(6')-Iad, has been discovered and it is thought that this enzyme could play an important role in the acquisition of amikacin resistance among *Acinetobacter* clinical isolates in Japan (48).

Table 9: Aminoglycoside-modifying enzymes identified in *Acinetobacter* spp. (44).

Name	Class	Target drug	Plasmid, integron or chromosomal
AAC(3)-Ia	Acetyltransferase	Gentamicin	Class 1 integron
AAC(3)-IIa	Acetyltransferase	Gentamicin, Tobramycin	
AAC(6')-Ib	Acetyltransferase	Tobramycin, Amikacin	Class 1 integron
AAC(6')-Ih, AAC(6')-Iad	Acetyltransferase	Tobramycin, Amikacin	Plasmid
APH(3')-Ia	Narrow spectrum phosphotransferase	Kanamycin	
APH(3')-VI	Phosphotransferase	Amikacin, Kanamycin	Plasmid
ANT(2'')-Ia	Adenyltransferase	Gentamicin, Tobramycin	Class 1 integron
ANT(3'')-Ia	Adenyltransferase	Streptomycin, Spectinomycin	Class 1 integron

4.3 Modification or protection of the antimicrobial target sites

A. baumannii can also become resistant to different antimicrobial agents by modifying the target sites of the antibiotic. This mechanism of resistance affects β-lactam antibiotics, aminoglycosides, tetracyclines and also quinolones.

4.3.1 Modification of PBPs

The main mechanism of resistance to β-lactam antibiotics is the production of β-lactamases but, occasionally resistance to imipenem has also been associated to the modification of certain PBPs. An *in vitro* mutant selected by Gehrlein *et al.* showed a hyper-production of a 24 kDa PBP and the production of other six PBP at a low level. In another study, the absence of a 73.2 kDa PBP2a conferred a low level resistance to imipenem and/or meropenem, while the additional absence of a 70.1 kDa PBP2b was associated to a higher level of resistance to both antimicrobial agents. It is important to note that these isolates had also a loss of OMPs and they were producing β-lactamases; this illustrates the interplay between different mechanisms of resistance to antimicrobial agents (62,161,165,227).

4.3.2 Methylation of 16S rRNA

Aminoglycoside resistance is mainly due to the production of AMEs; however, recent studies have identified several *A. baumannii* strains producing the 16S rRNA methylase ArmA. In 2005, Galimand *et al.* (72) studied the worldwide dissemination of this resistance methylase gene. They concluded that this gene was associated to a transposon TN*1548* and the spread was due to conjugation and transposition. They also suggested that this was the cause for the dissemination of aminoglycoside resistance by 16S rRNA methylation in *Enterobacteriaceae* and in *A. baumannii* (72). Further reports

have identified an *arm*A plasmid-encoded gene in closely related *A. baumannii* clinical isolates in North America, China and also in Korea (47,111,239).

4.3.3 Ribosomal protection proteins

The only gene identified in *A. baumannii* encoding a ribosomal protection protein is *tet*M which was first described by Ribera *et al.* (177); it encodes a protein that protects the ribosome from tetracycline, doxycycline and minocycline. This gene has 100% homology to the same gene of *Staphylococcus aureus* suggesting a horizontal transfer of genetic material between Gram-positive and Gram-negative bacteria (177).

4.3.4 Mutations in gyrA and parC genes

In *A. baumannii*, resistance to quinolones is often due to mutations in the quinolone resistance-determining regions of the *gyr*A and *par*C genes. These mutations have as a consequence a lower affinity of the quinolone for the enzyme-DNA complex (161). The most common mutations in *A. baumannii* are Ser-83 and Gly-81 in the GyrA protein, while ParC usually presents mutations in the Ser-80 and Glu-84. Mutations in the position Ser-83 of the GyrA are sufficient to produce CMI values over 4 mg/L to nalidixic acid or fluoroquinolones; however, the presence of double mutations, in *gyr*A and *par*C, produce a high level resistance to quinolones (116,226,236).

5. GENETICS OF RESISTANCE IN *ACINETOBACTER BAUMANNII*

As it has been mentioned above, the genus *Acinetobacter* is **well suited for genetic exchange** and has been described as being “naturally transformable”. This remarkable capacity for the acquisition of foreign genetic material, especially antibiotic resistance genes, favours the survival of this microorganism in the hospital environment. A second important characteristic is their **ability to resist desiccation** which increases the difficulty to eradicate these pathogenic agents from the environment. The combination of all these conditions has transformed *A. baumannii* into a pathogen difficult to eliminate, while 30 years ago was probably not even considered as a pathogen.

5.1 Modes of gene transfer

The three major types of gene transfer have been described in *A. baumannii*: **transformation, conjugation and transduction** (Fig. 21).

- ✓ Transformation: It involves the **transfer of a naked DNA fragment from a donor to a recipient bacterium**. In 1969, transformation was described for first time in an *Acinetobacter* spp. strain. Several posterior studies on *Acinetobacter* strain BD413 (highly competent strain of *A. calcoaceticus*) have determined that although competence occurs throughout the bacterial cell cycle, the early exponential growth phase has the highest transformation rate. Similar experiments on *Acinetobacter calcoaceticus* strain NCIB 8250 showed two peaks of competence, at early exponential growth phase and at the beginning of the stationary phase. Further studies to determine the basis for DNA uptake have found an association between the DNA uptake and certain components involved in the assembly of the type IV pilus (216).

- ✓ Conjugation: **Transfer of DNA between two bacteria which are temporarily in physical contact**. The first description of conjugation was reported in 1976 in an *Acinetobacter calcoaceticus* strain. The vector used for conjugation was a broad-host-range plasmid RP4 and was able to mobilize chromosomal genes between different *A. calcoaceticus* mutant derivatives. Chromosomal transfer by conjugation has also been reported with the naturally occurring *Acinetobacter* plasmid pAV1 (216).
- ✓ Transduction: This mechanism involves the **transfer of bacterial DNA by bacteriophages**. Several bacteriophages have been isolated which are active against specific strains of *Acinetobacter*. Although most of them are lytic phages, Herman *et al.* described a temperate phage P78 which lysogenizes its host strain. Nevertheless, this phage is specific for its host strain and cannot be used for genetic studies in *Acinetobacter* spp. (216).

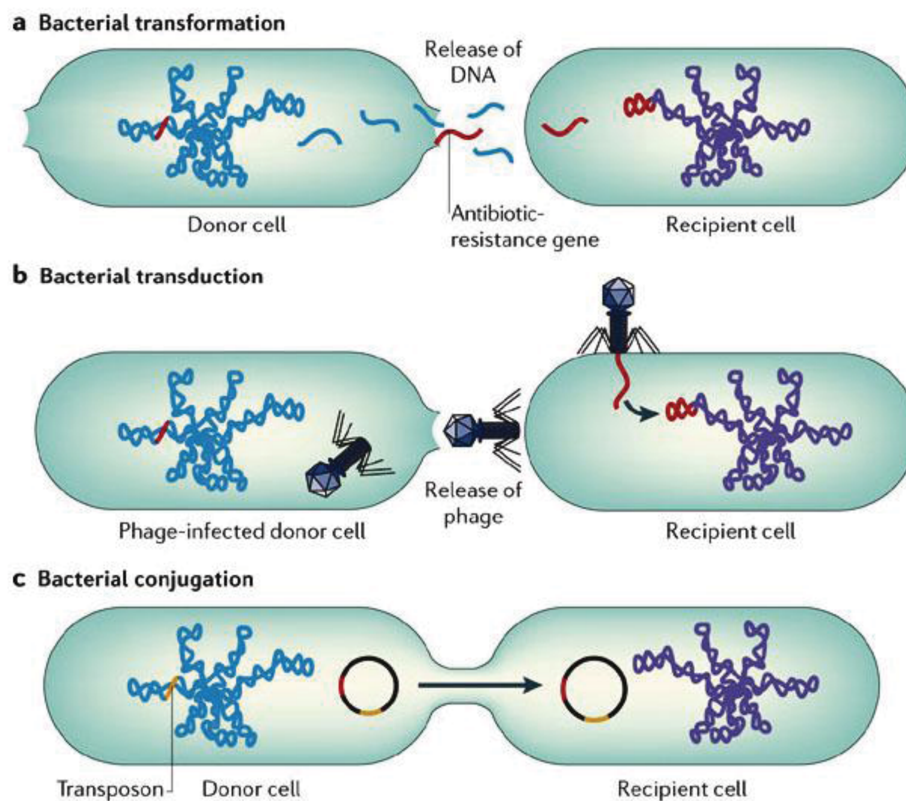

Fig. 21: Mechanisms of gene exchange in bacteria (© 2006 Nature Publishing Group) (69).

5.2 Mobile genetic elements

The members of the genus *Acinetobacter* have a tendency to rapidly develop resistance to the antimicrobial agents; they are intrinsically resistant to many antibiotics and have a great ability to acquire new resistance mechanisms. **Plasmids, transposons** and **integrons** are generally major contributing factors in the acquisition and transfer of these mechanisms of resistance (223).

- ***Plasmids***

Plasmids are small, circular DNA molecules that are present in many bacteria and can exist independently from the host chromosome (Fig. 22). They have relatively few genes but contain their own replication origin which allows them an autonomous replication. Although the genetic information present in the plasmids is not essential, the presence of antibiotic resistance genes confers a great advantage for the survival of the microorganism. A specific type of plasmids, conjugative plasmids, can transfer copies of themselves to other bacteria during conjugation (167).

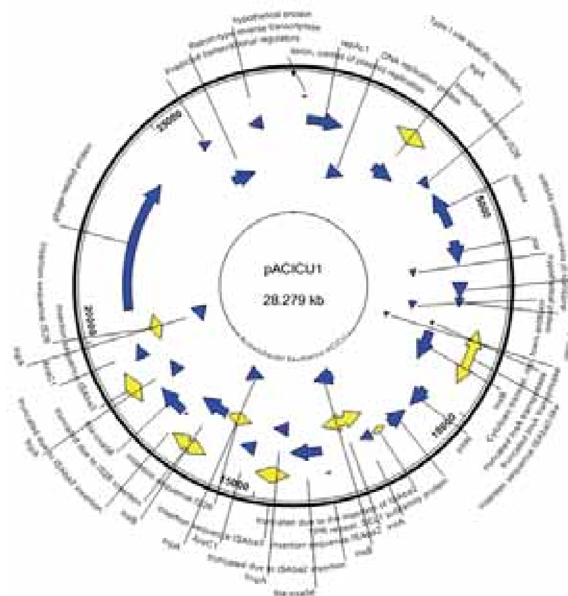

Fig. 22: Schematic representation of an *A. baumannii* plasmid (98).

The results for the presence of plasmids in *Acinetobacter* spp. are variable; while some studies suggest the presence of plasmids in over 80 % of the isolates, others have reported them in less than 30 % of *Acinetobacter* isolates (216,223). The main reason for this variability is probably the difficulty in breaking the cell wall of these microorganisms. Nevertheless, most of the plasmids found in these species seem to be relatively small (<23 kb) and are mainly associated with resistance to antibiotics. Due to the small size of these plasmids, they probably lack conjugative functions; indeed, few studies have been able to demonstrate plasmid-mediated transfer of resistance genes (216). Large plasmids have also been described in *Acinetobacter* spp. and they are usually associated to widespread transfer of resistance genes. Recently, Naiemi *et al.* (146) have reported a 65 kb plasmid containing genes encoding two ESBL enzymes (SHV-12 and TEM-116) and aminoglycoside resistance. A few months before, a similar plasmid had been responsible for an outbreak of aminoglycoside-resistant and ESBL-producing *Enterobacter cloacae*. After extensive analysis of the plasmid, they concluded that similar plasmids with identical organizations were present in the outbreak strains and also in five other Gram-negative isolates of four different species (146). This report supports the theory on interspecies plasmid transfer.

- *Transposons*

Transposons are DNA segments that originate in one chromosomal location and can move around the genome (Fig. 23). These transposable elements contain genes responsible for transposition but also some additional genes which are usually related to antimicrobial resistance or toxin production. Transposons are also located within plasmids, which act as the source and the target for other transposons; this contributes to the spread of antibiotic resistance genes. In fact, multiple drug resistance plasmids are usually formed by transposon accumulation in a single plasmid. In addition, the movement of transposons is also responsible for turning on and off chromosomal genes (167).

Fig. 23: Structure of a bacterial composite transposon.

Several reports have identified chromosomally located transposons in *Acinetobacter* spp. which carry multiple antibiotic resistance genes. Ribera *et al.* (176) partially characterized a transposon carrying the *tetR* and *tet*A genes which encodes for a regulatory protein and a tetracycline resistance determinant (176). Recently, whole genome comparison determined the presence of several transposons in resistance islands, suggesting that this structures are important for the island dynamics and for the rapid acquisition of other foreign resistance genes (67). Transposons may play an important role in ensuring that novel genes become established in a new gene pool. This is important because even if the plasmids are unstable, the resistance genes within the transposon do not get lost (216,223).

- ***Integrons***

Integrons are conserved genetic elements encoding a site-specific recombination system that allows the insertion, deletion and rearrangement of discrete genetic cassettes within the integron structure (Fig. 24) (216). A large number of *Acinetobacter* clinical isolates have integrons incorporated into their chromosome; it is especially frequent the presence of these mobile elements in epidemic strains, possibly because most of the cassettes identified are associated with antibiotic resistance. Indeed, it has been suggested that integrons are an important contribution to the dissemination of antibiotic resistance genes (73,216,223). The integrons identified in *A. baumannii*, show a high prevalence of genes encoding for AMEs and β-lactamases. There are three main classes of integron structures: **Class 1 integrons** include the gene encoding the Int1 integrase (*intI1*) and are principally associated with the *sul1* gene. **Class 2 integrons** are characterized by the presence of a defective *intI* gene (*intI2**). Finally, **class 3 integrons** present the *intI3* gene which encodes an IntI3 integrase which has a 60.9 % homology with the IntI1 integrase of class 1 integrons. Within *Acinetobacter* spp., class 1 integrons are the most commonly described (73,223).

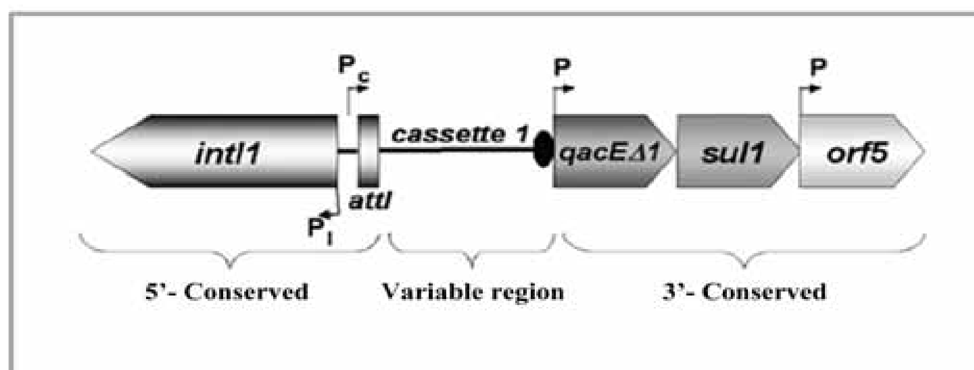

Fig. 24: General structure of class 1 integrons: two conserved segments flanking a variable region (© Léon, G & Roy, PH).

- *Insertion Sequences*

Insertion Sequences or IS elements are the simplest transposable elements which are made of a phenotypically cryptic short DNA sequence containing only the genes encoding the necessary enzymes for its transposition (transposase). These genes are surrounded at both ends by identical or very similar sequences of nucleotides which are between 10 to 40 bp long and have a reversed orientation; each IS element has its own characteristic inverted repeats (Fig. 25). The main function of the IS elements is to activate the expression of neighbouring genes by providing efficient promoters; nevertheless, they have also been associated with gene disruption, deletion, rearrangement, recombination and transfer (129,167).

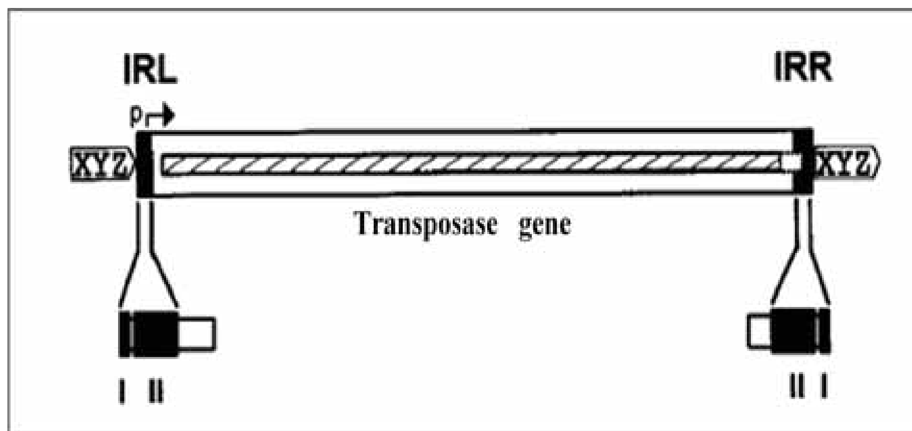

Fig. 25: Structure of an IS elements with two Inverted Repeats: (IRL) left inverted repeat; (IRR) right inverted repeat. XYZ represents short direct repeats generated as a consequence of insertion. The promoter "p" is partially localized in the IRL. Domain I represents the recognition site for Tpase-mediated cleavage & Domain II contains the nucleotides necessary for sequence-specific recognition and binding by the Tpase (129).

In *A. baumannii*, the IS elements act as strong promoters of β-lactamase production and they also contribute to acquire other resistant phenotypes. The presence of the IS$_{Aba1}$ element (IS*4* family) is generally associated with the overexpression of AmpC and OXA-51 β-lactamases; however, due to the mobility of this insertion element, it may have a significant role in controlling the expression of a variety of genes in *Acinetobacter* spp. (161,197). In *A. calcoaceticus*, the IS*1236* which belongs to the IS*3* family, has been associated to the prevention of *p*-hydroxybenzoate metabolism; in *A. haemolyticus*, the IS*17* from the IS*903* family, was responsible for insertional inactivation of the aminoglycoside resistance gene *aac(6')-Ig*. An insertion sequence has also been reported from *Acinetobacter* genospecies 13; the IS*18*, a member of the IS*30* family, was associated to the activation of the intrinsic silent gene *aac(6')-Ij* (189). A recent publication by Vallenet *et al*. (220) reports that the proportion and distribution of IS elements depends on the different *Acinetobacter* strains. They compared the genomic sequences of the MDR human isolate *A. baumannii* strain AYE, with a susceptible *A. baumannii* SDF strain isolated from body lice and a soil-living *Acinetobacter* strain (*A. baylyi* ADP1). The SDF strain had the largest number of IS elements and were mainly IS*Aba6* and IS*Aba7*, while the *A. baumannii* strain AYE had twenty-one copies of IS*Aba1*; although IS*Aba1* is thought to be an IS element intrinsic from *A. baumannii*, this element was not present in the susceptible SDF strain (220).

5.3 Resistance islands

Genomic islands are clusters of genetic elements found within the chromosome which have been acquired from other microorganisms by horizontal gene transfer (Fig. 26). These islands are between 10 and 100 kb in length, and can be divided into different subtypes: “ecological islands”, “saprophytic islands”, “symbiosis islands” and

"pathogenicity islands" (PAIs) which contribute to the pathogenic efficiency of the bacteria. Sequencing of several bacterial genomes has revealed that PAIs are more widespread than it was initially thought (85). Indeed, sequencing of several *Acinetobacter* genomes has revealed the presence of different genomic islands in these microorganisms (67,209,220). Fournier *et al*. (67) described a 86 kb resistance island in the epidemical MDR *A. baumannii* strain AYE which contained 45 of the 52 resistance genes found in this strain; in the same position, in the susceptible *A. baumannii* strain SDF, there was a 20 kb genomic island which did not contain any of the resistance genes. Within the resistance island in the AYE strain, there were three class 1 integrons, four different transposons (a truncated Tn*5393*, a truncated Tn*1721*, and IS*1*-like transposable element and a Tn*21*-like transposon) and five insertion sequences from the IS*15* and IS*26* classes (67).

Recently, Smith *et al*. (209) have sequenced the complete genome of *A. baumannii* ATCC 17978 and have identified 28 putative alien islands, 16 of which contain genes directly implicated in virulence. The largest pathogenicity island (133,740 bp) contained eight genes homologous to the Legionella/Coxiella Type IV virulence/secretion apparatus. In addition, the other alien islands had genes encoding antibiotic resistance genes, heavy metal resistance, iron uptake and metabolism, fimbrial genes, autoinducer processing and cell envelope biogenesis (209).

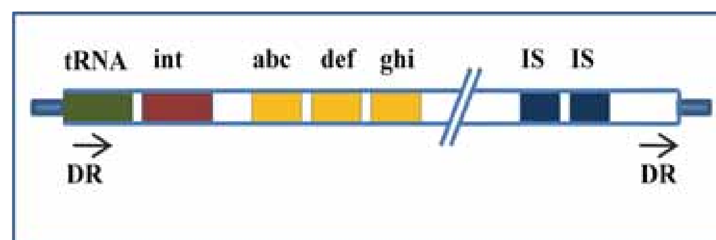

Fig. 26: Schematic model of a genomic island. The transferred DNA block is linked to a tRNA gene and flanked by direct repeats (DR). Integrase gene (int); genes encoding specific function (abc, def, ghi); Insertion Sequence (IS) (85).

5.4 Biofilm formation

As well as being highly resistant to the antibiotics, the members of the *Acinetobacter* spp., and especially *A. baumannii*, are highly resistant to desiccation; these microorganisms can persist during long periods of time on inanimate surfaces. In addition, *A. baumannii* is one of the most common causes of device-related nosocomial infections, possibly because it is able to resist physical and chemical disinfection. Biofilm formation is a possible explanation for the resistance to desiccation and disinfection; furthermore, it could also be responsible for an important number of the device-related nosocomial infections (49,214).

A biofilm is composed by a congregation of bacterial cells which are irreversibly associated to a solid surface and enclosed within a polysaccharide matrix (Fig. 27). Biofilm-associated bacteria differ from their planktonic equivalents in some of the genes that are transcribed; up- and down-regulation of several genes have been described in the attaching cells. These structures have been found in a variety of surfaces such as living tissues, medical devices and natural aquatic systems. Indeed, the solid-liquid interface which is formed between a surface and an aqueous medium is a perfect environment for bacterial attachment and growth. On medical devices, biofilms are mainly composed by bacterial cells and an extracellular polymeric substance (EPS) matrix which although is primarily made of polysaccharides, it varies in chemical and also physical properties; these structural attributes are considered universal but, every microbial biofilm community is unique (49).

In *A. baumannii* ATCC 19606, *in vitro* biofilm formation occurs mainly at the liquid-air interface and produces a biofilm-ring structure just above the surface of the medium; furthermore, this structure grows upwards from the liquid-air interface onto the

walls of the tube (Fig. 28). Within this biofilm, bacterial cells are attached to each other by pili-like structures and there are channels to provide nutrients to the individual bacteria and remove the waste products. A polycistronic operon involved in pili assembly (*csu* genes) has been described in this strain as a requirement for pili formation and therefore initial bacterial attachment to the surface (214). In addition, a biofilm-associated protein (Bap) has been described in *A. baumannii* which may have a function in supporting the development of the mature biofilm structure. This protein is a surface-expressed protein that is structurally similar to bacterial adhesins and its disruption produces a reduction in the volume and thickness of this biofilm (125).

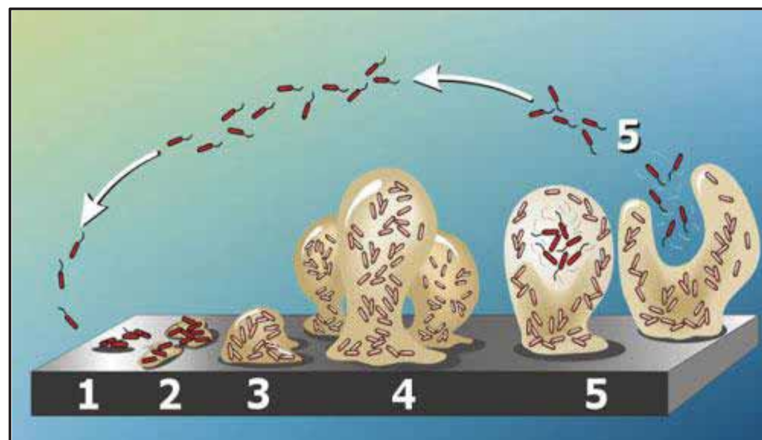

Fig. 27: Mechanism of biofilm formation. 1 & 2: reversible and irreversible attachment to the surface; 3 & 4: maturation phases; 5: biofilm fragments are released (© Davies, DG).

Fig. 28: Biofilm ring-structure formed by *A. baumannii*.

II. WORK JUSTIFICATION

Acinetobacter baumannii, as the most important representative member of the genus *Acinetobacter*, is considered a paradigm among the emerging MDR nosocomial pathogens. This microorganism is responsible for an increasing number of infections due to its long survival in the hospital environment and its capacity to rapidly develop resistance to the antimicrobial agents. *A. baumannii* can survive longer than other microorganisms on dry surfaces possibly due to the ability to grow at different temperatures and pH values, and the need of minimal nutritional requirements. The main cause of concern is the ability of *A. baumannii* to produce epidemic outbreaks and to be able to persist in hospitals with an endemic occurrence; these aspects make the control and eradication of this microorganism very difficult.

At present, most of the *A. baumannii* clinical isolates are multidrug resistant; in fact, strains resistant to all the available antimicrobial agents have been reported. Colistin is regarded as the last resource treatment for some diseases caused by these MDR pathogens; however, colistin resistant *A. baumannii* isolates have already been reported. This microorganism is intrinsically resistant to many antimicrobial agents due to the interplay of decreased membrane permeability and the constitutive expression of active efflux systems. In addition, *A. baumannii* is able to rapidly acquire new mechanisms of resistance; both, intrinsic and acquired resistance contribute to the actual level of multiresistance found in many clinical isolates.

Acquired multidrug resistance can be due to the acquisition of mobile genetic elements carrying multiple resistant determinants but also to mutations affecting the expression of porins or efflux pumps. In fact, the overexpression of these transporters has been often associated with multidrug resistance.

The genus *Acinetobacter* is well suited for genetic exchange, favouring this bacterium for the survival in the hospital environment. The ability to rapidly acquire antimicrobial resistance together with the high resistance to desiccation has transformed *A. baumannii* into a pathogen difficult to eradicate. Biofilm formation is a possible explanation for this resistance to desiccation and disinfection, and it could also be responsible for an important number of device-related nosocomial infections.

Although *A. baumannii* is the main pathogen within this genus, other *Acinetobacter* spp. have also been related to nosocomial infection. The main problem is the difficulty to correctly identify the different genomic species and as a result, most clinical isolates are identified as *A. baumannii*. However, with the exception of *A. calcoaceticus*, the members of the ABC complex are commonly related to infections in hospitals and occasionally, other genomic species have also been described as the cause of nosocomial infections. It is therefore important to determine whether *A. baumannii* is the only pathogenic member of this genus or the other *Acinetobacter* spp. can also become a problem.

In spite of being an emerging problem within ICUs worldwide, there is still a lack of information on their mechanisms of virulence and resistance to antimicrobial agents, desiccation and disinfection.

Why and how do they survive in the hospital environment? If biofilm is important to prevent desiccation and it is also involved in device-related infections, this mechanism should be studied more in depth. In addition, in order to develop further therapeutic strategies, we need to investigate in depth the molecular bases of antimicrobial resistance. This information may be useful to find possible modifications on the current antimicrobial agents which could help to circumvent the mechanisms of resistance.

III. OBJECTIVES

Probably, the most important factors in the success of *Acinetobacter baumannii* as a nosocomial pathogen are the ability to acquire multidrug resistance and the capacity to survive for long periods of time in the hospital environment. The study of these two factors was the main reason for taking up this work.

Main objectives

- ✓ To study the mechanisms of resistance to different antimicrobial agents in *Acinetobacter* spp. clinical isolates, focusing in the identification of novel efflux pumps and porins.
- ✓ To study the effect of biofilm formation in the clinical success of *A. baumannii* isolates.

Specific targets

In order to accomplish these main targets, the study was carried out following some more specific objectives which can be divided in three different parts: a) the study of *A. baumannii* clinical isolates; b) the study of non-baumannii clinical isolates and c) the study of biofilm formation.

Study of *A. baumannii* clinical isolates

- ➢ To study the prevalence of tetracycline-specific efflux pumps and β-lactamase enzymes in a Spanish collection of clinical isolates. Additionally, to study the effect of the Insertion Sequence ISAba1 in front of these β-lactamase enzymes.
- ➢ To study the activity of novel antimicrobial agents in front of *A. baumannii* clinical isolates.

- To identify and characterize new efflux pumps by PCR with degenerate primers synthesised from homologous areas in the genes coding for these efflux pumps in other microorganisms.
- To identify and characterize by 2D-electrophoresis new efflux pumps and porins involved in favouring a reduced permeability to antimicrobial agents.
- To identify and characterize new mechanisms of resistance to quinolones and colistin by SDS-PAGE and 2D-electrophoresis by comparing the protein maps of *in vitro* isogenic mutants of *A. baumannii.*

Study of non-baumannii clinical isolates

- To study and identify the mechanisms of resistance to antimicrobial agents in non-baumannii *Acinetobacter* spp., especially in clinical isolates from the ABC-complex.

Study of biofilm formation

- To analyse the biofilm formation of a collection of *A. baumannii* clinical isolates. The main objective of this part is to study the relationship between biofilm production and other clinical or microbiological characteristics.

IV. RESULTS

A. RESISTANCE TO CEPHALOSPORINS

PAPER 2: Prevalence of IS(Aba1) in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates.

PAPER 3: *In vitro* activity of Ceftobiprole against *Acinetobacter baumannii* clinical isolates.

➢ PAPER 2:

Prevalence of IS(Aba1) in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates.

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Prevalence of IS$_{Aba1}$ in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates

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insertion sequence; resistance; ceftazidime; *Acinetobacter baumannii*.

Abstract

Seventy-five *Acinetobacter baumannii* strains belonging to different pulsetypes, plus one ceftazidime-susceptible strain, from a pulsetype in which all strains were resistant, were included in this study. The minimum inhibitory concentration of ceftazidime was determined by the microdilution method. The bla_{ADC}-like gene, the IS$_{Aba1}$ element and the IS$_{Aba1}$ located in the bla_{ADC}-like promoter were detected by PCR. The objective of the study was to determine the prevalence of IS$_{Aba1}$ in a collection of epidemiologically unrelated *A. baumannii* clinical isolates. The bla_{ADC}-like gene was detected in 74 (97.3%) out of the 76 strains analysed. In these 74 strains, 51 (69%) were positive for the IS element and it was not detected in 23 (31%) strains. Among the *A. baumannii* strains containing the IS element, 40 (78.4%) had the IS element located in the promoter region of the bla_{ADC}-like gene. In a high percentage of *A. baumannii* clinical isolates carrying the IS$_{Aba1}$, this is inserted into the promoter region of the bla_{ADC}-like gene. In addition, two clinical isolates belonging to the same pulsetype, one with and one without the IS$_{Aba1}$, can be found in the clinical setting, suggesting the potential acquisition or loss of this genetic element in the hospital environment.

Introduction

Acinetobacter baumannii is currently considered to be one of the most important Gram-negative bacteria causing infections in immunocompromised patients, mainly in intensive care units (Bergogne-Berezin & Towner, 1996). These microorganisms are considered to be the paradigm of multiresistant bacteria (Vila, 1998). The β-lactam antibiotics, mainly ceftazidime and carbapenems, previously showed *in vitro* activity. However, currently, the percentage of clinical isolates demonstrating resistance to ceftazidime are very high and resistance to carbapenems is steadily increasing (Henwood *et al*., 2002; Nordmann & Poirel, 2002). The mechanisms of resistance to the β-lactam antibiotics most often found in Gram-negative bacteria, such as the production of β-lactamases, changes in permeability, increase in the efflux and modification of the affinity of the PBPs, have been described in this microorganism (Vila, 1998; Nordmann & Poirel, 2006). The most prevalent mechanism of resistance to β-lactam antibiotics in *A. baumannii* is the synthesis of β-lactamases. Among these enzymes, both chromosomal and plasmid-mediated β-lactamases have been described. To date, eight class C cephalosporinases have been reported in *Acinetobacter* spp. (Perilli *et al*., 1996; Bou & Martinez-Beltran, 2000; Hujer *et al*., 2005; Beceiro *et al*., 2007). The AmpC cephalosporinase, now called ADC, has been found to be overproduced in at least 50% of the *A. baumannii* clinical isolates (Danes *et al*., 2002; Corvec *et al*., 2003). Previous studies have identified the insertion sequence IS$_{Aba1}$ in the chromosome of some strains of these microorganisms (Corvec *et al*., 2003; Segal *et al*., 2004, 2005). This insertion sequence can be inserted into the promoter region of the bla_{ADC}-like gene favouring the overexpression of this gene due to a promoter sequence located in this genetic element (Corvec *et al*., 2003; Segal *et al*., 2004).

The main objective of this study was to analyse the prevalence of IS$_{Aba1}$ in a collection of epidemiologically unrelated *A. baumannii* clinical isolates.

Materials and methods

Bacterial strains

Two hundred and twenty-one *A. baumannii* strains were isolated from 25 different hospitals in Spain in a multicentre study. The *A. baumannii* species was identified by amplified ribosomal DNA restriction analysis (Fernandez-Cuenca

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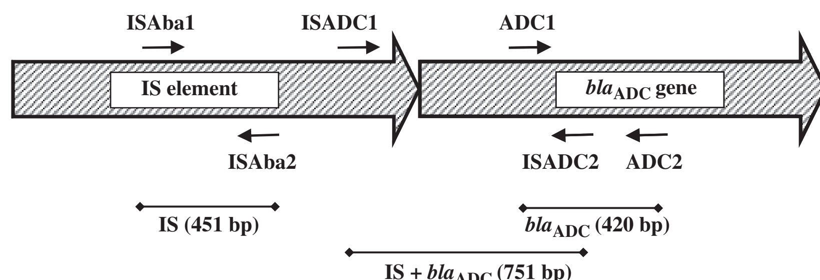

Fig. 1. Location of the primers used in this work.

et al., 2004). Genotyping of these strains was performed by analysis of chromosomal DNA by digestion with a low frequency of cleavage restriction enzymes and pulsed-field gel electrophoresis (PFGE) (Gautom, 1997), and by REP-PCR as described previously (Vila *et al.*, 1996).

Susceptibility testing

Minimum inhibitory concentrations of ceftazidime were determined by a standard microdilution system according to the CLSI guidelines (Clinical and Laboratory Standards Institute, 2000) or by an *E*-test (AB Biodisk, Solna, Sweden). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

Detection of the bla_{ADC}-like gene, IS element and IS located in the bla_{ADC}-like promoter by PCR

The bla_{ADC}-like gene, the IS element and the location of IS upstream of the bla_{ADC}-like gene were detected by PCR using the following primers (Fig. 1): for the bla_{ADC}-like gene amplification, ADC1 5′-CCGCGACAGCAGGTGGATA-3′ and ADC2 5′-TCGGCTGATTTTCTTGGTT-3′ (annealing temperature, 51 °C), for the IS element ISAba1 5′-CATTGG CATTAAACTGAGGAGAAA-3′ and ISAba2 5′-TTGGAAA TGGGGAAAACGAA-3′ (annealing temperature, 52 °C) and to detect the location of IS in the promoter region of the bla_{ADC}-like gene, ISADC1 5′-GTTGCACTTGGTCGAATGA AAA-3′ and ISADC2 5′-ACGTCGCGAGTTGAAGTAAG TT-3′ (annealing temperature, 51 °C). The PCR was carried out for 30 cycles using the following program: 92 °C for 1 min, annealing temperature for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products obtained were resolved in a 1.5% agarose gel electrophoresis and stained with ethidium bromide (0.5 μg mL^{-1}).

Results

Two hundred and twenty-one *A. baumannii* strains were isolated from 25 different hospitals in Spain and the susceptibility to different antimicrobial agents was determined in all (Fernandez-Cuenca *et al.*, 2004). A genotypic analysis by PFGE and REP-PCR defined 75 different types. In the present study, 76 *A. baumannii* strains were chosen for analysis. Seventy-five strains were representative of each pulse type. In addition, an extra strain (strain AC29) was also studied, which was susceptible to ceftazidime but belonged to one pulsetype in which all the strains were resistant to ceftazidime.

Table 1. Prevalence of the IS_{Aba1} and IS_{Aba1} located in the promoter region of the bla_{ADC}-like genes in a collection of epidemiologically unrelated *Acinetobacter baumannii* strains

	No. of strains	Ceftazidime		
		Susceptible	Intermediate	Resistant
IS_{Aba1}+	51	6 (11.7%)	2 (4%)	43 (84.3%)
IS_{Aba1} – ADC+	40	0	0	40 (100%)
IS_{Aba1} – ADC –	11	6 (54.5%)	2 (18.2%)	3 (27.3%)
IS_{Aba1} –	23	16 (69.5%)	3 (13%)	4 (17.5%)

The PCR of the bla_{ADC}-like gene, IS element and IS located in the bla_{ADC}-like promoter generated three amplicons of 420, 451 and 751 bp, respectively. In 74 (97.3%) out of the 76 strains analysed, the bla_{ADC}-like gene was detected and 51 (69%) of these strains were positive for the IS element. The strain AC29, which was susceptible to ceftazidime, did not have the IS element. Among the *A. baumannii* strains containing the IS element, 40 (78.4%) had the IS element located in the promoter region of the bla_{ADC}-like gene (Table 1). All these strains were resistant to ceftazidime, whereas only 27.3% of the *A. baumannii* strains without the IS element in the promoter region were resistant to this β-lactam antibiotic (Table 1).

Discussion

Although some extended-spectrum β-lactamases affecting ceftazidime have been described in *A. baumannii* (Navia *et al.*, 2002; Poirel *et al.*, 2003, 2005), the main mechanism of resistance to this antimicrobial agent in this microorganism is the overexpression of cephalosporinase AmpC, now designated as ADC (Hujer *et al.*, 2005). Recent studies have

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shown that transcription of the bla_{ADC}-like gene is dependent on the promoter sequence within an IS element (IS_{Aba1}) inserted into the upstream region of this gene (Corvec *et al.*, 2003; Segal *et al.*, 2004). In this present study, the IS element was found in 69% of the epidemiologically unrelated strains analysed, being slightly higher than the 47% found by Segal *et al.* (2005). This difference can be explained either by the low number (17 strains) of *A. baumannii* strains analysed in their study, or the predominance of an IS-negative strain among their strains, because no epidemiological relationship was evaluated among these. From the total number of strains analysed, the IS element was located upstream of the bla_{ADC}-like gene in 54%. These data are in agreement with that of Corvec *et al.* (2003), who found the IS element located in the upstream of the bla_{ADC}-like gene in 52.4%.

It is worth mentioning that the IS element was found in only one of the two *A. baumannii* strains (AC029, ceftazidime susceptible and AC031, ceftazidime resistant) from the same hospital, belonging to the same pulse type. These data suggests that the IS element can be acquired or lost by an *A. baumannii* strain in the hospital environment.

All 40 *A. baumannii* strains with the IS located in the promoter region of the bla_{ADC}-like gene were resistant to ceftazidime in comparison with only three strains that had the IS element but not located upstream of the bla_{ADC}-like gene. The resistance to this β-lactam antibiotic in these three strains may be due to a mechanism of β-lactam resistance other than the overexpression of the bla_{ADC}-like gene. In *Acinetobacter* spp., other IS elements associated with activation of aminoglycoside-modifying enzymes have been described (Rudant *et al.*, 1998). Rudant *et al.* (1998) reported the IS17 and IS18 adjacent to the *aac(6′)-Ig* and *aac(6′)-Ij* in two strains of *Acinetobacter haemolyticus* and *Acinetobacter* sp. 13, respectively. They suggested that the location of these IS elements upstream of the genes may promote expression of the genes.

In summary, in a high percentage of *A. baumannii* clinical isolates from Spain carrying the IS_{Aba1}, this element is inserted into the promoter region of the bla_{ADC}-like gene. In addition, two clinical isolates belonging to the same pulse type, one with and one without the IS_{Aba1}, can be found in the clinical setting, which suggests the potential acquisition or loss of this genetic element in the hospital environment.

Acknowledgements

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➢ PAPER 3:

In vitro activity of Ceftobiprole against *Acinetobacter baumannii* clinical isolates.

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(Under review)

1

2 **In vitro activity of Ceftobiprole against *Acinetobacter baumannii***

3 **Clinical Isolates**

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18 Short running title: Activity of Ceftobiprole in *Acinetobacter baumannii*

19 Keywords: *Acinetobacter* spp., Ceftobiprole

20 **Abstract**

21 Ceftobiprole is a new cephalosporin with activity against Gram-positive and
22 Gram-negative pathogens. This study evaluates the in vitro activity of ceftobiprole in
23 a collection of fifty-eight *Acinetobacter baumannii* clinical isolates, showing that the
24 activity of ceftobiprole was superior to ceftazidime and cefepime when the bla_{ADC}-
25 like gene was not expressed.

26

27 *Acinetobacter baumannii* is a nosocomial pathogen involved in a wide range of
28 infections such as bacteraemia, secondary meningitis, pneumonia and urinary tract
29 infections. This microorganism is currently one of the most important Gram-negative
30 pathogens causing infections in immunocompromised patients (1). *A. baumannii* has
31 been considered the paradigm of multiresistant bacteria because there are strains
32 resistant to all antimicrobial agents (10). β-lactam antibiotics, mainly carbapenems,
33 were the first choice against these microorganisms. However, in the last decade,
34 resistance to these antimicrobial agents has appeared in hospitals worldwide due to
35 the production of β-lactamases, changes in permeability, increase in the efflux and
36 modifications of the affinity of the PBPs (7,10).

37 Ceftobiprole is a broad spectrum cephalosporin which has successfully
38 completed phase III clinical trials and is currently waiting for approval in both the
39 US and Europe (11). Its bactericidal activity against certain β-lactam-resistant Gram-
40 positive microorganisms is regarded as the main advantage of this antimicrobial
41 agent in front of the older cephalosporins. Several studies have demonstrated that
42 ceftobiprole has a strong affinity for the penicillin-binding proteins PBP2 and PBP2x
43 from staphylococci and pneumococci. However, this antimicrobial agent can also

44 bind to other PBPs of resistant Gram-positive and Gram-negative bacteria (2,6). For
45 instance, in *Escherichia coli,* ceftobiprole has a good affinity for PBP3 and PBP2,
46 while in *Pseudomonas aeruginosa*, the greatest affinity is for PBP1a and PBP3 (4).
47 *In vitro* studies suggested that ceftobiprole has a low potential for selection of
48 resistance; this conclusion was obtained after serial passages in the presence of
49 subinhibitory concentrations of this antibiotic (2,5,11). Previous studies have
50 proposed that the activity of ceftobiprole against Gram-negative bacteria such as *P.*
51 *aeruginosa* is comparable to the activity of other known antimicrobial agents such as
52 ceftazidime and cefepime (2,6,8). The purpose of this study was to evaluate the *in*
53 *vitro* activity of ceftobiprole in a collection of *Acinetobacter baumannii* clinical
54 isolates.

55 Fifty-eight non-related *A. baumannii* clinical isolates were chosen for this
56 study. Ceftobiprole was obtained from Johnson & Johnson Pharmaceutical Research
57 and Development (Raritan, NJ, USA); ceftazidime and cefepime were obtained from
58 Sigma-Aldrich (Madrid, Spain). All drugs were prepared and dissolved as
59 recommended by the manufacturer. MICs were determined by the microdilution
60 method as described by the Clinical and Laboratory Standards Institute (CLSI) (3).

61 The bla_{ADC}-like gene, the Insertion Sequence IS_{Aba1} and the location of IS in
62 the promoter region of the bla_{ADC}-like gene were detected by PCR with the following
63 primers: ADC1: $^{5'}$ CCG CGA CAG CAG GTG GAT A $^{3'}$ and ADC2: $^{5'}$ TCG GCT
64 GAT TTT CTT GGT T $^{3'}$ for the bla_{ADC}-like gene; ISAba1: $^{5'}$ CAT TGG CAT TAA
65 ACT GAG GAG AAA $^{3'}$ and ISAba2: $^{5'}$ TTG GAA ATG GGG AAA ACG AA $^{3'}$ for
66 the IS element. Finally, for the location of the IS_{Aba1} upstream the bla_{ADC}-like gene,
67 the primers used were ISADC1: $^{5'}$ GTT GCA CTT GGT CGA ATG AAA A $^{3'}$ and
68 ISADC2: $^{5'}$ ACG TCG CGA GTT GAA GTA AGT T $^{3'}$.

69 Fifty-eight *A. baumannii* clinical isolates were separated into two groups
70 depending on the presence or absence of the IS_{Aba1} in front of the *bla*$_{ADC}$-like gene.
71 The MICs for 50% and 90% of the organisms (MIC_{50} and MIC_{90} values respectively)
72 for ceftobiprole, ceftazidime and cefepime are shown in Table 1. The results show
73 that in *A. baumannii*, the overall resistance of the isolates to ceftobiprole (70.7 %) is
74 slightly lower than the resistance to ceftazidime (77.6 %) or cefepime (75.9 %).
75 Although the MIC_{50} is similar for the three antibiotics, the MIC_{90} shows that the level
76 of resistance to ceftazidime and cefepime is higher than the resistance to
77 ceftobiprole. This is confirmed by the range of MICs obtained for the three
78 antimicrobial agents.

79 Thirty-one of the clinical isolates were expressing the *bla*$_{ADC}$-like gene (Table
80 1). In this group, there was practically no difference in the activity of the three
81 antibiotics. However, when the *bla*$_{ADC}$-like gene was not expressed, the resistance to
82 ceftobiprole (37 %) was lower than the resistance to ceftazidime (51.9 %) and
83 cefepime (51.9 %) (Table 1). In this case, the range of MICs obtained for the three
84 antimicrobial agents is wider for ceftobiprole than for ceftazidime and cefepime. In
85 addition, the MIC_{50} of ceftobiprole is the only sensitive value of the whole study.

86 Approximately half of the isolates used in this study (31) were expressing the
87 *bla*$_{ADC}$-like gene because the IS_{Aba1} was located in the promoter region of this gene.
88 The class C, AmpC β-lactamase, is an enzyme that hydrolyses the lactam ring in
89 cephalosporins, conferring resistance to β-lactam antibiotics. The presence of the
90 Insertion Sequence IS_{Aba1} in the promoter region of the *bla*$_{ADC}$-like gene favours the
91 overexpression of this gene (9). In a previous experiment with Gram-negative
92 microorganisms, Queenan *et al.* (8) suggested that in *P. aeruginosa* strains producing
93 high levels of AmpC, the MICs for all the cephalosporins, including ceftobiprole,

94 were over 16 mg/L (8). Our results with *A. baumannii* corroborate these findings in
95 *P. aeruginosa.* However, when the bla_{ADC}-like gene was not expressed, the activity
96 of ceftobiprole against *A. baumannii* was better than the activity of ceftazidime and
97 cefepime. Over 60 % of the isolates had a MIC of 8 mg/L or less for ceftobiprole
98 which is a good percentage in a multiresistant microorganism such as *A. baumannii.*

99 In summary, when the bla_{ADC}-like gene was expressed, the activity of
100 ceftobiprole was similar to that of ceftazidime and cefepime. However, based on the
101 MIC_{50} and MIC_{90} values, ceftobiprole was the most active cephalosporin tested
102 against *A. baumannii* clinical isolates when the bla_{ADC}-like gene was repressed.

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108 **Transparency declarations**

109 None to declare

110

111

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147

148 **Legend to figure**

149

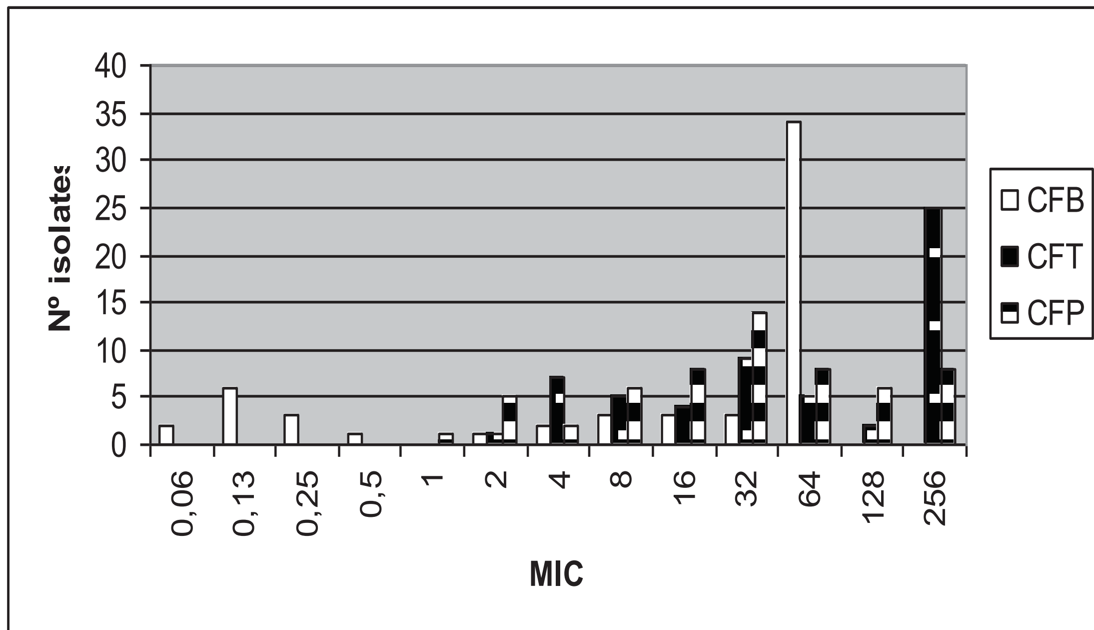
150 Graphic representation of the activity of ceftobiprole, cefepime and ceftazidime

151 in *Acinetobacter baumannii* clinical isolates

152 Table 1. Activity of ceftobiprole, cefepime and ceftazidime in *Acinetobacter*
153 *baumannii* clinical isolates (n = 58)
154

Clinical isolates	Antimicrobial agents	% Resistance (> 8 mg/L)	MIC_{50}	MIC_{90}	Range
All strains (n = 58)					
	Ceftazidime	**77.6 %**	**64**	**256**	**2 - 256**
	Cefepime	**75.9 %**	**32**	**256**	**1 - 256**
	Ceftobiprole	**70.7 %**	**64**	**64**	**0.06 - 64**
ISAmpC (+) (n = 31)					
	Ceftazidime	**100 %**	**256**	**256**	**16 – 256**
	Cefepime	**96.8 %**	**64**	**256**	**8 - 256**
	Ceftobiprole	**100 %**	**64**	**64**	**32 - 64**
ISAmpC (-) (n = 27)					
	Ceftazidime	**51.9 %**	**16**	**256**	**2 - 256**
	Cefepime	**51.9 %**	**16**	**32**	**1 - 256**
	Ceftobiprole	**37 %**	**8**	**64**	**0.06 - 64**

155



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B. RESISTANCE TO CARBAPENEMS

PAPER 4: **High prevalence of carbapenem-hydrolysing oxacillinases in epidemiologically related and unrelated *Acinetobacter baumannii* clinical isolates in Spain.**

PAPER 5: ***In vitro* activity of Doripenem against *Acinetobacter baumannii* clinical isolates.**

PAPER 6: **Characterization of the carbapenem-hydrolyzing oxacillinase OXA-58 in an *Acinetobacter* Genospecies 3 clinical isolate.**

PAPER 7: **Characterization of the carbapenem-hydrolyzing oxacillinase OXA-58 in an *Acinetobacter* phenon 6/ct13TU clinical isolate.**

PAPER 8: **Nosocomial bacteremia due to an as yet unclassified *Acinetobacter* genomic species 17-like strain.**

➢ PAPER 4:

High prevalence of carbapenem-hydrolysing oxacillinases in epidemiologically related and unrelated *Acinetobacter baumannii* clinical isolates in Spain.

M. Ruiz, **S. Martí**, F. Fernández-Cuenca, A. Pascual, J. Vila.

Clinical Microbiology and Infection 2007; 13(12):1192-1198

ORIGINAL ARTICLE 10.1111/j.1469-0691.2007.01825.x

High prevalence of carbapenem-hydrolysing oxacillinases in epidemiologically related and unrelated *Acinetobacter baumannii* clinical isolates in Spain

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ABSTRACT

Carbapenem-hydrolysing oxacillinases are reported increasingly in *Acinetobacter baumannii*. This study investigated the role of these β-lactamases in causing resistance to carbapenems in 83 epidemiologically related and unrelated imipenem-resistant *A. baumannii* clinical isolates. The isolates were also analysed for the presence of IS*Aba1* in the promoter region of the bla_{OXA-51}-like gene in order to investigate the role of IS*Aba1* in OXA-51 expression. All clinical isolates contained a bla_{OXA-51}-like gene, 20% contained a bla_{OXA-58}-like gene, and 42% contained a bla_{OXA-40}-like gene; bla_{OXA-23}-like, bla_{IMP} and bla_{VIM} genes were not detected in any of the isolates investigated. IS*Aba1* was found in 24 (82.7%) of 28 pulsetypes, and was located in the promoter region of the bla_{OXA-51}-like gene in five (20.8%) of these pulsetypes. Expression of bla_{OXA-51} was detected in the five isolates with IS*Aba1* located in the promoter region, but was not detected in an isogenic imipenem-susceptible *A. baumannii* isolate that did not have IS*Aba1* located in the promoter region. It was concluded that there is a high prevalence of oxacillinases with activity against carbapenems among genetically unrelated *A. baumannii* clinical isolates from Spain, and that concomitant expression of two carbapenemases (OXA-51-like and either OXA-40-like or OXA-58-like) may take place. Insertion of an IS*Aba1*-like element in the promoter of the bla_{OXA-51}-like gene promotes the expression of this gene, although this did not seem to play a major role in carbapenem resistance.

Keywords *Acinetobacter baumannii*, carbapenemase, expression, IS*Aba1*, oxacillinase, resistance

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INTRODUCTION

Acinetobacter baumannii is a nosocomial pathogen that is recognised as being responsible for a wide spectrum of infections, including bacteraemia, secondary meningitis, pneumonia and urinary tract infections [1]. It has been implicated increasingly in hospital-acquired infections, mostly affecting debilitated patients in intensive care units, in whom such infections are associated with high mortality rates [1]. Administration of appropriate antimicrobial therapy to these patients is therefore essential. Carbapenems usually have good potency against *A. baumannii*, with imipenem being the most active agent [1], but carbapenem resistance in *A. baumannii* has increasingly been reported worldwide during the last decade [2,3]. Several mechanisms responsible for resistance to carbapenems in *A. baumannii* have been described: (i) synthesis of carbapenemases [2]; (ii) decreased outer-membrane permeability caused by the loss or reduced expression of porins [4–8]; and (iii) alterations in penicillin-binding proteins [9,10].

Although IMP-type [11–14] and VIM-type [15] carbapenemases have been reported in *A. baumannii*, the carbapenemases found most frequently are those belonging to class D.

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To date, four groups of carbapenem-hydrolysing oxacillinases (Ambler class D β-lactamases) have been described in *A. baumannii* [16–20]. The first oxacillinase described in *A. baumannii* with activity against carbapenems was OXA-23 [21], and this enzyme is in a group that currently includes OXA-23, OXA-27 and OXA-49 [17,22]. A second group comprises OXA-24, OXA-25, OXA-26 and OXA-40 [16,22,23], and shares 60% amino-acid identity with the first group. The third and largest group comprises the OXA-51-like carbapenemases, which are encoded by chromosomally located genes that show 56% and <63% amino-acid identity with groups 1 and 2, respectively [24,25]. Finally, a new carbapenemase (OXA-58) has been characterised that shares <50% amino-acid identity with the other three groups.

Recently, a novel insertion sequence (IS), IS*Aba1* (www-is.biotoul.fr/is.html), which has 11-bp inverted repeat sequences flanked by 9-bp direct repeats of the target sequence, was identified in *A. baumannii* [26]. Many IS elements contain promoters that play a role in the expression of antibiotic resistance genes situated downstream from the site of insertion [27,28]. IS*Aba1* has been identified adjacent to a β-lactamase resistance gene (*ampC*) in *A. baumannii* [26,29], and primer extension studies showed that transcription of the *ampC* gene was dependent on promoter sequences within IS*Aba1* [27]. IS*Aba1* has also been found upstream from $bla_{OXA\text{-}51}$-like and, probably, $bla_{OXA\text{-}23}$-like genes [30,31].

The aim of the present study was to investigate the distribution of the different OXA-type enzymes, as well as VIM and IMP enzymes, in a collection of *A. baumannii* isolates from various locations in Spain. The presence of IS*Aba1* upstream of $bla_{OXA\text{-}51}$-like genes, together with its role in the expression of these genes, was also investigated.

MATERIALS AND METHODS

Bacterial isolates

Eighty-three isolates were selected from among 221 clinical isolates collected during November 2000 from 25 Spanish hospitals as part of a previous study of carbapenem resistance. All isolates were identified by amplified rDNA restriction analysis [32], and their epidemiological relationships were determined by pulsed-field gel electrophoresis, according to the method of Gautom [33].

Susceptibility testing

Microdilution assays according to CLSI guidelines [34] were used to determine MICs of the following antimicrobial agents: ampicillin, piperacillin, cephalothin, cefoxitin, gentamicin, amikacin, tobramycin, tetracycline, minocycline, doxycycline, rifampicin and colistin (Sigma, Madrid, Spain), ceftazidime (GlaxoSmithKline, Uxbridge, UK), cefepime (Bristol-Myers Squibb, Madrid, Spain), sulbactam and azithromycin (Pfizer, Sandwich, UK), imipenem (Merck, Hoddesdon, UK), meropenem (AstraZeneca, Macclesfield, UK), ciprofloxacin (Bayer, Leverkusen, Germany), and co-trimoxazole (Galloso, Madrid, Spain) [35]. Breakpoints used were those recommended by the CLSI for non-fermentative Gram-negative bacilli [34]; control strains used were those described previously [35].

PCR analysis

DNA was extracted by boiling a single colony in 25 μL of water for 10 min and then centrifuging in a microcentrifuge at maximum speed for 1 min. This was followed by the addition of 25 μL of a reaction mixture containing 0.5 μM of the relevant primers (Table 1), 200 μM dNTPs and 2.5 U of *Taq* DNA polymerase, to give a final volume of 50 μL. Initial

Table 1. Primers used in this study

Primers	Genes detected	Sequence (5′ → 3′)	Product size (bp)	Annealing temp.
OXA51 U	*oxa51*, *oxa69*, *oxa71*, *oxa75*, *oxa 78*	AACAAGCGCTATTTTTATTTCAG	641	53°C
OXA51 L		CCCATCCCCAACCACTTTT		
OXA58 U	*oxa58*	AGTATTGGGGCTTGTGCT	453	50°C
OXA58 L		AACTTCCGTGCCTATTTG		
OXA24 U	*oxa24*, *oxa25*, *oxa26*, *oxa33*, *oxa40*, *oxa72*	ATGAAAAAATTTATACTTCCTATATTCAGC	825	50°C
OXA24 L		TTAAATGATTCCAAGATTTTCTAGC		
OXA23 U	*oxa23*, *oxa27*, *oxa49*	GATGTGTCATAGTATTCGTCGT	641	52°C
OXA23 L		TCACAACAACTAAAAGCACTGT		
ISaba1U	IS*Aba1*	CATTGGCATTAAACTGAGGAGAAA	451	53°C
ISaba1L		TTGGAAATGGGGAAAACGAA		
VIM U	VIM-type	ATTGGTCTATTTGACCGCGTC	780	55°C
VIM L		TGCTACTCAACGACTGCGCG		
IMP U	IMP-type	CATGGTTTGGTGGTTCTTGT	488	55°C
IMP L		ATAATTTGGCGGACTTTGGC		

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denaturation (95°C for 5 min) was followed by 30 cycles of 95°C for 1 min, the optimal annealing temperature for each gene (Table 1) for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. PCR products were resolved in agarose 2% w/v gels in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Gels were stained with ethidium bromide and the DNA was visualised by UV light transillumination at 302 nm. When required, PCR products were recovered directly from the agarose gels and were purified with the Wizard SV gel and PCR system (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA sequencing was performed using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) and an automated DNA sequencer 3100 Genetic Analyzer (Applied Biosystems).

Quantification of mRNA by RT-PCR

One milliliter of bacterial culture (OD_{600} 0.6) was added rapidly to a solution comprising 125 μL of ethanol 95% v/v plus phenol 5% v/v, and was then centrifuged. The pellet was resuspended in 100 μL of lysozyme solution in water (0.1 mg/mL), vortexed, and then incubated for 30 min at room temperature, after which mRNA was extracted and purified using an RNAwiz kit (Ambion, Austin, TX, USA). RT-PCR was then performed using a SuperScrip One-step RT-PCR Kit with Platinum *Taq* (Invitrogen, Barcelona, Spain). Two sets of primers, OXA51 U and OXA51 L (Table 1), were used for the bla_{OXA-51}-like gene, with primers for the 16S rRNA gene as an internal control. Reaction mixtures comprised 1× reaction mix (SuperScrip One-step RT-PCR Kit), 0.5 μM each primer, 1 U of RT/platinum *Taq* MIX (SuperScrip One-step RT-PCR Kit), 500 ng of RNA template, and distilled water to 50 μL. Each reaction was performed with two initial steps, the first at 50°C for 30 min (reverse transcription), and the second at 95°C for 2 min to activate the *Taq* polymerase, followed by 19 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 1 min.

For quantification, it is important to stop the reaction, usually between cycles 10 and 25, to compare the expression of a gene in different isolates. After several trials, 19 cycles were used for each of the two genes. This low cycle number produces amplicons that are difficult to see in an agarose gel stained with ethidium bromide; the RT-PCR products obtained were therefore analysed in acrylamide gels (Amersham Biosciences, Barcelona, Spain) using a GenePhor apparatus (Pharmacia Biotech, Barcelona, Spain). The gel was then stained using a DNA silver-staining kit (Amersham Biosciences).

RESULTS

In total, 221 *A. baumannii* clinical isolates were collected from 25 Spanish hospitals [35]. All 83 imipenem-resistant *A. baumannii* isolates from this collection were chosen for the present study. The 83 selected isolates belonged to 28 different pulsetypes from 12 different hospitals. The number of isolates in each pulsetype ranged from one to 18. Table 2 summarises the presence of the genes encoding the main carbapenemases described to

Table 2. Distribution of different carbapenemase-encoding genes among isolates of *Acinetobacter baumannii* from various locations in Spain

Hospital	City	No. of isolates	Pulsetype P designation	Genes detected					Imipenem MIC (mg/L)
				OXA-51-like	OXA-58-like	OXA-40-like	IS*Aba1*	IS-OXA-51-like[a]	
H. Bellvitge	BCN	28	71	4	0	0	4	0	128
			72	18	0	1	2	0	64–128
			73	2	0	0	0	0	64
			74	1	0	0	0	0	64
			75	1	0	0	1	0	128
			78	1	0	0	1	0	128
			79	1	0	1	1	0	128
H. 12 Octubre	MD	4	44	4	0	4	4	0	64–128
H. C. Salamanca	SLM	1	11	1	0	0	0	0	64
H. La Princesa	MD	2	41	2	0	2	2	0	128
H. M. Valdecilla	SAN	2	32	2	2	0	2	0	16
H. Parapléjicos	TO	3	15	1	0	1	1	0	128
			22	2	0	2	2	0	128
H. Reina Sofia	CO	6	47	3	3	0	3	0	16–64
			48	3	2	0	3	0	32–64
H. Vall d'Hebron	BCN	1	45	1	0	0	1	1	128
H. V. Salud	TO	6	50	5	0	5	5	0	128
			52	1	0	1	1	0	128
H. V. Nieves	GR	7	53	6	5	0	6	5	16–32
			54	1	0	0	1	1	16
H. V. Rocio	SE	20	56	9	0	9	9	0	128
			57	2	0	2	2	0	128
			59	2	0	2	2	0	128
			61	3	3	0	3	3	16–32
			61.2	3	0	3	3	0	128
			65	1	1	0	0	0	16
H. Getafe	MD	3	34	2	0	2	2	0	128
			35	1	0	0	1	1	128
	Total	83	28	83	16	35	62	11	
	%			100	19.3	42.2	74.7	13.3	

City: BCN, Barcelona; MD, Madrid; SLM, Salamanca; SAN, Santander; TO, Toledo; CO, Córdoba; GR, Granada; SE, Sevilla.
P, number assigned to each pulsetype.
IS-OXA-51-like, isolates that contain IS*Aba1* inserted in the promoter region of the bla_{OXA-51}-like gene.

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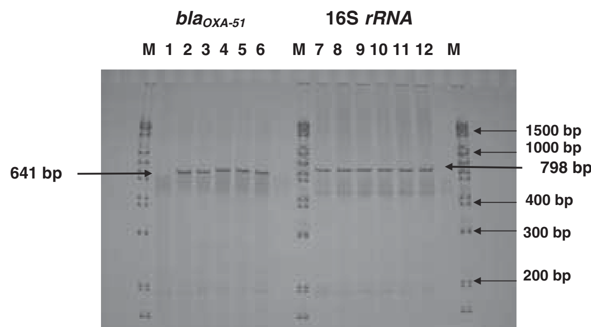

Fig. 1. Expression of bla_{OXA-51}-like genes (lanes 1–6) and, as controls, 16S rRNA genes (lanes 7–12). Lanes 1–6 and 7–12 show isolates belonging to pulsetypes 53, 35, 45, 53, 54 and 61, in that order. Lanes 1 and 7 contain an isolate belonging to pulsetype 53 that carries IS*Aba1*, but not in the promoter region of the bla_{OXA-51}-like gene. All other lanes contain isolates with IS*Aba1* located in the promoter region of the bla_{OXA-51}-like gene.

date in *A. baumannii*, including the four subgroups of oxacillinases having carbapenemase activity, as well as IMP- and VIM-type enzymes. All the isolates carried a gene encoding a β-lactamase belonging to the OXA-51-like group. In addition, 19% and 42% of the isolates carried a gene encoding an OXA-58-like or an OXA-40-like enzyme, respectively. The MICs of imipenem for isolates carrying an OXA-40-like or an OXA-58-like carbapenemase were ≥128 mg/L and 16–64 mg/L, respectively. No genes encoding β-lactamases of the OXA-23-like group, or VIM or IMP metallo-β-lactamases, were detected. Sixty-two (74.7%) of the 83 isolates contained the insertion sequence IS*Aba1*, which was located in the promoter region of the bla_{OXA-51}-like gene for 11 (17.7%) of the 62 isolates carrying this insertion element. Some degree of heterogeneity was observed within groups of isolates; thus, pulsetype 72 contained 18 isolates, but only one carried a bla_{OXA-40}-like gene, and pulsetype 53 contained six isolates, five of which carried a bla_{OXA-58}-like gene, with four of these overexpressing a bla_{OXA-51}-like gene, while the remaining isolate did not carry a bla_{OXA-58}-like gene and overexpressed a bla_{OXA-51}-like gene.

Expression of the bla_{OXA-51}-like gene was investigated in five isolates belonging to pulsetypes 35, 45, 53, 54 and 61, respectively, in which IS*Aba1* was located in the promoter region of the bla_{OXA-51}-like gene. In isolates from three pulsetypes (35, 45 and 54), the bla_{OXA-51}-like gene was the only carbapenemase, whereas the remaining two isolates (pulsetypes 53 and 61) also carried a bla_{OXA-58}-like carbapenemase. In addition, an isolate belonging to pulsetype 53, which also carried IS*Aba1*, but not in the promoter region of the bla_{OXA-51}-like gene, was included as a control. Fig. 1 shows that the bla_{OXA-51}-like gene was clearly expressed in all five isolates with IS*Aba1* in the promoter region of the gene (lanes 2–6), but not in the control isolate (lane 1). The PCR product obtained from the amplification was verified by sequencing using the IS*Aba1* forward primer and the bla_{OXA-51}-like gene reverse primer.

DISCUSSION

A. baumannii is associated frequently with nosocomial infections, with imipenem being the most active antimicrobial agent against these microorganisms. Acquisition of imipenem resistance means that colistin, and perhaps tigecycline, may be the only remaining therapeutic options for treating infections caused by multiresistant *A. baumannii*. The high level of resistance to imipenem in *A. baumannii* clinical isolates and the clinical risk-factors favouring the acquisition of imipenem-resistant *A. baumannii* have been reported previously [36]. Although carbapenem resistance may be caused, in part, by impaired permeability, resulting from decreased expression of porins, or by modifications in penicillin-binding proteins [4–10], most recent reports have indicated that carbapenem-hydrolysing β-lactamases play a more significant role [2]. In the present study, all isolates produced an OXA-51-like β-lactamase, with those that also produced an OXA-40-like β-lactamase having a higher imipenem MIC than isolates that also produced an OXA-58-like enzyme (Table 2). Other mechanisms of resistance to imipenem may be present, possibly including over-expression of the *adeABC* efflux pump [37].

Da Silva *et al*. [38] showed that a multiresistant epidemic clone of *A. baumannii* carrying the bla_{OXA-40} gene was disseminated widely in Portugal and Spain. In the present study, epidemiologically unrelated *A. baumannii* clinical isolates carrying bla_{OXA-40}-like genes were more prevalent than isolates carrying bla_{OXA-58}-like genes, suggesting that dissemination of a genetic element carrying a bla_{OXA-40}-like gene may have taken place. However, the spread of an *A. baumannii* clone or a genetic element carrying this gene

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seems, at present, to be limited to Spain and Portugal [38].

The OXA-58 carbapenemase described by Poirel *et al*. [20] shares <50% amino-acid homology with the three remaining OXA groups. OXA-58 was described originally in a carbapenem-resistant isolate of *A. baumannii* from France, but this carbapenemase has been found subsequently in isolates from Austria, Greece, Romania, Spain, Turkey and the UK [39–42], Argentina and Kuwait [39], and Venezuela (E. Salazar, personal communication). In the present study, an OXA-58-like enzyme was found in six of 28 *A. baumannii* clinical isolates belonging to different pulsetypes, again suggesting that a genetic element carrying a $bla_{\text{OXA-58}}$-like element may be disseminating.

The heterogeneity among the OXA-51-like group of enzymes is probably very high [24,41], and it is known that the OXA-51-like enzymes are intrinsic chromosomally-located β-lactamases in *A. baumannii* [43]. In the present study, eight PCR products from the amplified region of the $bla_{\text{OXA-51}}$-like gene were randomly chosen and sequenced, and were found to show 100% homology with either the $bla_{\text{OXA-66/76}}$ gene (PCR products from six different isolates) [25], the $bla_{\text{OXA-71}}$ gene (one isolate) [25], or the $bla_{\text{OXA-51}}$ gene (one isolate) [24]. Héritier *et al*. [43] showed that OXA-69, which is closely related to OXA-51, had only very weak catalytic efficiency for most β-lactam antibiotics, including carbapenems, and suggested that OXA-69 may not play a significant role in resistance to β-lactam antibiotics. The present study demonstrated over-expression of the $bla_{\text{OXA-51}}$-like gene in all isolates with IS*Aba1* located in the promoter region of the $bla_{\text{OXA-51}}$-like gene. Although another mechanism of resistance to carbapenems cannot be ruled out, the differing expression of the $bla_{\text{OXA-51}}$-like gene observed in the isogenic sensitive and resistant isolates belonging to pulsetype 53 suggests that insertion of IS*Aba1* in the promoter region of the $bla_{\text{OXA-51}}$-like gene may produce a slight increase in the MIC of imipenem. The end of the IS*Aba1* element found inserted in the promoter region of the $bla_{\text{OXA-51}}$ gene was located seven bases from the start codon of the $bla_{\text{OXA-51}}$-like gene, as described previously by Turton *et al*. [31].

In conclusion, although additional mechanisms of resistance to carbapenems cannot be ruled out, the present study demonstrated a high prevalence of oxacillinases with activity against carbapenems in genetically unrelated *A. baumannii* clinical isolates from Spain, and confirmed that insertion of an IS*Aba1*-like element in the promoter region of $bla_{\text{OXA-51}}$-like genes enhances the expression of such genes, and produces a small increase in the imipenem MIC.

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➢ PAPER 5:

In vitro activity of Doripenem against *Acinetobacter baumannii* clinical isolates.

S. Martí, J. Sánchez-Céspedes, V. Alba, J. Vila.

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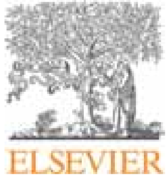

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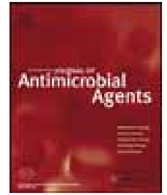

1 Short communication

2 In vitro activity of doripenem against *Acinetobacter baumannii* clinical isolates

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ABSTRACT

Doripenem is a carbapenem with activity against Gram-positive and Gram-negative pathogens. This study evaluated the in vitro activity of doripenem against a collection of 87 *Acinetobacter baumannii* clinical isolates, showing that the activity of doripenem was superior to imipenem and meropenem for strains carrying the bla_{OXA-58} gene. *A. baumannii* clinical isolates expressing the bla_{OXA-24} gene were resistant to doripenem, imipenem and meropenem. However, in clinical isolates expressing the bla_{OXA-58} gene, the percentage of isolates with a doripenem minimum inhibitory concentration >8 μg/mL was much lower than that of imipenem and meropenem. This study shows that the activity of doripenem was superior to imipenem and meropenem for strains carrying the bla_{OXA-58} gene.

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16 1. Introduction

17 *Acinetobacter baumannii* is a nosocomial pathogen causing infec-
18 tions mainly in patients in Intensive Care Units (ICUs). It has been
19 isolated from opportunistic infections especially in the respira-
20 tory tract, urinary tract and wounds from debilitated patients [1].
21 The extensive use of antimicrobial agents in ICUs facilitates the
22 selection of multiply resistant strains [2,3]. Carbapenems were the
23 first choice against these microorganisms, however resistance to
24 these antimicrobial agents has been reported worldwide [1]. This
25 increase has been associated with the production of β-lactamases,
26 changes in permeability, increases in efflux and modifications in
27 the affinity of penicillin-binding proteins [2–5].
28 Doripenem is a broad-spectrum carbapenem active against
29 Gram-positive bacteria, Enterobacteriaceae, anaerobes and non-
30 fermentative bacilli such as *Pseudomonas aeruginosa* and *A.*
31 *baumannii* [6]. In October 2007, the US Food and Drug Administra-
32 tion (FDA) approved doripenem as a new drug to treat complicated
33 urinary tract and intra-abdominal infections [7]. Initial studies
34 suggested that doripenem included the best qualities of the car-
35 bapenems by combining the exceptional activity of imipenem
36 against Gram-positive cocci with the activity of meropenem
37 against Gram-negative bacteria [6]. Similar to other carbapen-
38 ems, the structure of doripenem confers stability against most
39 β-lactamases; however, it remains unstable against metallo-β-
40 lactamases [6,8,9]. Previous reports have suggested that the activity
41 of doripenem against *A. baumannii* was equivalent to the activity
42 of imipenem and meropenem, especially in carbapenemase-
43 producing isolates that were resistant to all the carbapenems
44 [9,10]. The purpose of this study was to evaluate the in vitro
45 activity of doripenem in a collection of *A. baumannii* clinical iso-
46 lates.

47 2. Materials and methods

48 Eighty-seven epidemiologically non-related *A. baumannii* clin-
49 ical isolates were selected for this study. These isolates had
50 previously been characterised for the presence of different
51 carbapenemases (OXA-type, VIM and IMP-type) [2]. Minimum
52 inhibitory concentrations (MICs) were determined by the microdi-
53 lution method as described by the Clinical and Laboratory
54 Standards Institute [11]. Doripenem was obtained from Johnson
55 & Johnson Pharmaceutical Research and Development (Raritan,
56 NJ), imipenem was obtained from Merck (Madrid, Spain) and
57 meropenem was obtained from AstraZeneca (Madrid, Spain). All
58 drugs were prepared and dissolved as recommended by the man-
59 ufacturer.

60 3. Results and discussion

61 The isolates were separated into groups depending on the pro-
62 duction of OXA-58 or OXA-24 enzymes. The MIC_{50} and MIC_{90} values
63 (MIC for 50% and 90% of the organisms, respectively) of doripenem,
64 imipenem and meropenem are shown in Table 1. The results show
65 that the percentage of *A. baumannii* isolates with a doripenem MIC
66 >8 μg/mL (54%) is slightly lower than that of imipenem (57.5%) and
67 meropenem (64.4%) (Table 1; Fig. 1). However, the MIC_{50} and MIC_{90}

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Table 1
Activity of doripenem, imipenem and meropenem against *Acinetobacter baumannii* clinical isolates ($n = 87$).

Clinical isolates/ antimicrobial agent	% isolates with MIC >8 μg/mL	MIC (μg/mL)		
		MIC_{50}	MIC_{90}	Range
All strains ($n = 87$)				
Doripenem	54	8	256	0.06–256
Imipenem	57.5	16	128	0.1–128
Meropenem	64.4	16	64	1–64
OXA-58-positive ($n = 12$)				
Doripenem	33	4	8	2–128
Imipenem	100	16	64	16–64
Meropenem	100	16	64	8–64
OXA-24-positive ($n = 19$)				
Doripenem	100	256	256	128–256
Imipenem	100	128	128	16–128
Meropenem	100	64	64	8–64

MIC, minimum inhibitory concentration; $MIC_{50/90}$, MIC for 50% and 90% of the isolates, respectively.

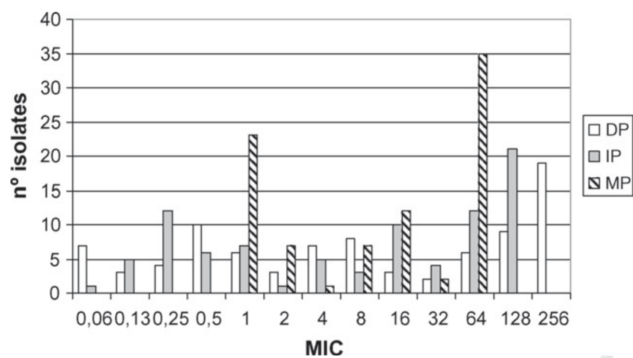

Fig. 1. Activity of doripenem (DP), imipenem (IP) and meropenem (MP) against 87 *Acinetobacter baumannii* clinical isolates, showing the number of isolates at each minimum inhibitory concentration (MIC).

values did not indicate great differences in the activity of the three antimicrobial agents.

Nineteen of the clinical isolates expressed the bla_{OXA-24} gene (Table 1). In this group, there were practically no differences among the activity of the three antibiotics. All the *A. baumannii* strains expressing this oxacillinase were resistant to doripenem, imipenem and meropenem.

Finally, 12 of the clinical isolates expressed the bla_{OXA-58} gene (Table 1). In this case, the percentage of isolates with a doripenem MIC >8 μg/mL (33%) was much lower than that of imipenem (100%) and meropenem (100%) to which all the isolates were resistant.

OXA-58 was first identified in France in 2003 and confers reduced susceptibility to carbapenems. Nowadays, this oxacillinase has been reported in hospitals from several European countries including Spain, Turkey, Romania, Greece, Austria, the UK and Italy; in addition, OXA-58 has also been detected in clinical isolates from Argentina, Kuwait and Australia and from injured USA military personnel returning from Iraq [4]. Previous experiments targeting the activity of doripenem against carbapenemase-producing *A. baumannii* ignored this oxacillinase, probably because of its relative newness. In our study, only 12 of the 87 clinical isolates produced this enzyme. Although the number of OXA-58-producing isolates in this study was very low, the results clearly suggest that the activity of doripenem against *A. baumannii* expressing the bla_{OXA-58} gene is higher than the activity of imipenem and meropenem. These results are in agreement with previously published studies on the activity of doripenem. Rice [10] has suggested that although doripenem has excellent activity against *P. aeruginosa* and *A. baumannii*, it lacks activity against isolates that are already resistant to other currently available carbapenems [10].

In summary, doripenem, imipenem and meropenem have equal activity against *A. baumannii* clinical isolates except when the bla_{OXA-58} gene is expressed; in isolates producing the OXA-58 carbapenamase, the activity of doripenem is clearly superior to that of imipenem and meropenem.

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Ethical approval: Not required.

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➢ PAPER 6:

Characterization of the carbapenem-hydrolyzing oxacillinase OXA-58 in an *Acinetobacter* Genospecies 3 clinical isolate.

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Characterization of the Carbapenem-Hydrolyzing Oxacillinase Oxa-58 in an *Acinetobacter* Genospecies 3 Clinical Isolate[▽]

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Based on imipenem resistance in an *Acinetobacter* genospecies 3 clinical isolate, we were able to identify, for the first time in this genomic species, a plasmid-encoded bla_{OXA-58} gene that was 100% homologous to the same gene in *Acinetobacter baumannii*.

Since 1986 members of the genus *Acinetobacter* are determined by DNA-DNA hybridization. Genospecies 1 (*Acinetobacter calcoaceticus*), 2 (*A. baumannii*), 3, and 13TU are genetically closely related and are commonly known as the *A. calcoaceticus-A. baumannii* complex. With the exception of genospecies 1, the other members of this complex have been involved in nosocomial infections and have the ability to spread in hospitals (3, 9, 19, 23, 25, 26). Treatment of these nosocomial infections is becoming a problem because increasing resistance to antibiotics, especially in the case of *A. baumannii*. In the last decade, carbapenem resistance in *Acinetobacter* spp. has been reported worldwide (3, 16, 23), mostly associated with the synthesis of carbapenem-hydrolyzing β-lactamases, reduced outer membrane permeability and, occasionally, modification of penicillin-binding proteins (7, 16, 22, 27). The most prevalent carbapenemases in *Acinetobacter* spp. are the carbapenem-hydrolyzing class D β-lactamases, which are divided into four phylogenetic subgroups: OXA-23, OXA-24, and OXA-58 with all their variants and the OXA-51 family, which is intrinsic to *A. baumannii* (16, 27).

OXA-58 confers reduced susceptibility to carbapenems, but it produces high-level resistance to carbapenems when additional efflux mechanisms are expressed (12, 15). It was first identified in France in 2003 and, at present, is found worldwide in *A. baumannii* isolates (13, 16, 20, 21), as well as in *A. junii* isolates from Romania and Australia (12, 14).

The clinical isolates Ac057 (*Acinetobacter* sp. strain G3) and Ac058 (*A. baumannii*) were obtained from the same hospital in November 2000 and were identified by amplified ribosomal DNA restriction analysis (ARDRA) (6, 24). The epidemiological difference was corroborated by pulsed-field gel electrophoresis (PFGE) with ApaI (Promega, Madrid, Spain) under conditions described elsewhere (11).

Antimicrobial susceptibility analysis was performed by Etest according to the manufacturer's instructions (AB Biodisk, Sölna, Sweden) and determined that both strains had an imipenem MIC of >32 μg/ml (Table 1). The breakpoints for imipenem were those proposed by the Clinical and Laboratory Standards Institute (5).

PCR analysis with specific primers for all class D β-lactamases (Table 2) determined the presence of the bla_{OXA-58} gene in both strains; *A. baumannii* strain Ac058 was also positive for the bla_{OXA-51} gene. Additional primers were designed at the beginning and end of the bla_{OXA-58} gene (Table 2) to amplify the whole fragment. This gene presented 100% homology with the bla_{OXA-58} gene from *A. baumannii* listed in GenBank.

Plasmid DNA identification was attempted by using genomic mapping with I-CeuI (10) and by digestion with the S1 nuclease (1). I-CeuI cuts a 26-bp site in the *rrl* gene (23S rRNA), shearing the bacterial genome into an analyzable number of fragments (10). The S1 nuclease transforms supercoiled plasmids into linear molecules (1). Digested genomic DNA and plasmids were sepa-

TABLE 1. MICs for the clinical isolates used in this study

Strain	MIC (μg/ml)[a]																
	AMP	PIP	CEF	FOX	CAZ	FEP	SAM	IMP	MEM	CIP	GEN	TOB	AMK	DOX	AZM	RIF	PMB
Ac057	256	512	256	256	8	8	8	>32	8	32	<1	0.25	0.5	<0.5	4	2	2
Ac058	256	512	256	256	256	64	64	>32	8	64	16	64	256	16	128	2	1

Abbreviations: AMP, ampicillin; PIP, piperacillin; CEF, cephalothin; FOX, cefoxitin; CAZ, ceftazidime; FEP, cefepime; SAM, ampicillin-sulbactam; IMP, imipenem; MEM, Meropenem; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; DOX, doxycycline; AZM, azithromycin; RIF, rifampin; PMB, polymyxin B.

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TABLE 2. Oligonucleotide sequences used in this study

Nucleotide	Sequence (5′–3′)	Size (bp)	Use
OXA51-1	AACAAGCGCTATTTTTATTTCAG	641	Detection *bla*$_{OXA-51}$ variants
OXA51-2	CCCATCCCCAACCACTTTT		
OXA23-1	GATGTGTCATAGTATTCGTCGT	641	Detection *bla*$_{OXA-23}$ variants
OXA23-2	TCACAACAACTAAAAGCACTGT		
OXA24-1	ATGAAAAAATTTATACTTCCTATATTCAGC	825	Detection *bla*$_{OXA-24}$ variants
OXA24-2	TTAAATGATTCCAAGATTTTCTAGC		
OXA58-1	AGTATTGGGGCTTGTGCT	453	Detection *bla*$_{OXA-58}$ variants
OXA58-2	AACTTCCGTGCCTATTTG		Southern blot probe[a]
OXA58-1TOT	ATGAAATTATTAAAAATATTG	843	Total gene amplification
OXA58-2TOT	TTATAAATAATGAAAAACACC		Total gene amplification
OXA58-inv1	GAGCGCAGAGGGGAGAATCGTC		Genetic surrounding
OXA58-inv2	CTCAGCACAAGCCCCAATACT		Genetic surrounding
OXA58-inv3	AAGCCATGCAAGCATCTACA		Genetic surrounding
OXA58-inv4	CATCTCTTTCACTTGTTGCTGAA		Genetic surrounding

[a] Primers OXA58-1 and OXA58-2 were used for detecting the *bla*$_{OXA-58}$ gene and also to generate the probe for Southern blot analysis.

rated by PFGE (Fig. 1). Probes were marked with the PCR DIG probe synthesis kit (Roche, Barcelona, Spain), and detection was performed with anti-digoxigenin antibody conjugated to alkaline phosphatase and the color substrates NBT/BCIP (Roche) according to the manufacturer's instructions. In Fig. 1a, the most intense bands would represent fragments of genomic DNA, and the faded bands represent plasmid DNA. Hybridization with probes for the *bla*$_{OXA-58}$ gene (Fig. 1c) and the 23S rRNA gene (Fig. 1b) suggest that in both isolates the *bla*$_{OXA-58}$ gene is present in a plasmid. With the S1 nuclease (Fig. 2a), the highest band would be the genomic DNA and the remaining bands would be linear plasmids. Hybridization with the probe for the OXA-58 gene (Fig. 2c) gives the same pattern as obtained with I-CeuI. The hybridization signal with the probe for the 23S rRNA gene was only observed in the undigested genomic DNA (Fig. 2b). Although conjugation experiments did not show any plasmid transfer between strains, Southern blot analysis suggests that the *bla*$_{OXA-58}$ gene could be present in a plasmid in both strains, and the plasmid from *A. baumannii* is possibly different from the plasmid in the *Acinetobacter* genospecies 3 isolate.

In order to determine the genetic structure surrounding of the *bla*$_{OXA-58}$ gene, DNA from both isolates was digested with MspI "C*CGG" (Promega). The fragments obtained were autoligated overnight at 16°C using a T4 DNA ligase (Promega).

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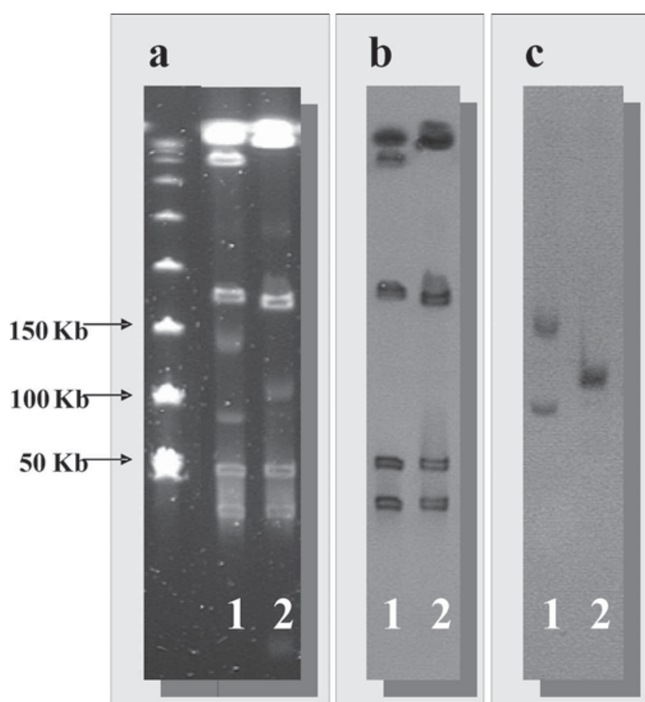

FIG. 1. Plasmid identification by genomic mapping with I-CeuI. (a) PFGE gel. (b) Hybridization with probe for the 23S rRNA gene. (c) Hybridization with probe for the OXA-58. Lane 1, *A. baumannii* strain Ac058; lane 2, *Acinetobacter* genospecies 3 strain Ac057.

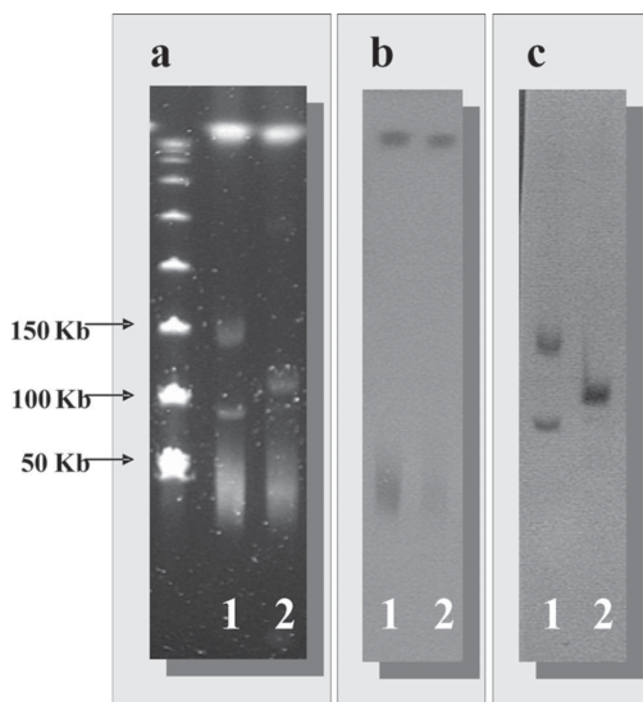

FIG. 2. Plasmid identification by digestion with S1 nuclease. (a) PFGE gel (b). Hybridization with probe for the 23S rRNA gene. (c) Hybridization with probe for the OXA-58. Lane 1, *A. baumannii* strain Ac058; lane 2. *Acinetobacter* genospecies 3 strain Ac057.

```
←ISABA3..........TTTCTTTATACTATCACTGAGGCAGGTTGGACAT
TTGATTGCTAGAGTTATTTGCATTTCTCTATTTTATCAAAATCCAA
TCGGCTTTTTCTTCAGCATACTTTTTGAAACACTACCAAATTTTAAA
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CAAAAACAAGTACAATTCCACAAGTGAATAACTCAATCATCGAT
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TCTACAGAAATATTTAAGTGGGATGGAAAGCCACGTTTTTTTAA
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CTACAGTGCCTGTATATCAAGAATTGGCACGTCGTATTGGTCCA
AGCTTAATGCAAAGTGAATTGCAACGTATTGGTTATGGCAATAT
GCAAATAGGCACGGAAGTTGATCAATTTTGGTTGAAAGGGCCTT
TGACAATTACACCTATACAAGAAGTAAAGTTTGTGTATGATTTAG
CCCAAGGGCAATTGCCTTTTAAACCTGAAGTTCAGCAACAAGTG
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ATGCTAAAAGTGGCTGGGGAATGGCTGTAGACCCGCAAGTGGG
TTGGTATGTGGGTTTTGTTGAAAAGGCAGATGGGCAAGTGGTG
GCATTTGCTTTAAATATGCAAATGAAAGCTGGTGATGATATTGC
TCTACGTAAACAATTGTCTTTAGATGTGCTAGATAAGTTGGGTG
TTTTTCATTATTTATAAGAATTAGAAGTTTGAGGTTAATCTATTTT
TGGTAGTGTTTCAAAAAGTATGCTGAAGAAAAAGCCGATTGGATTT
TGATAAAATAGAGAAATGCAAATAACTCTAGCAATCAAATGTCCA
ACCTGCCTCAGTGATAGTATAAAGAAAAATGGTATCAAAGTAGA
TGGGAAACAAAACTAT.......ISABA3→
```

TTTATC: -35 motif of the promoter

TTTCTT: -10 motif of the promoter

CATACTTTTTGAAACACTACCAAA – IRL ISAba3

FIG. 3. Structure of the genetic surrounding in *Acinetobacter* genospecies 3 strain Ac057, which is structurally identical to the one described by Poirel et al. (17).

The fragment of DNA containing the bla_{OXA-58} gene was used as a template for a PCR with inverse primers designed from the bla_{OXA-58} gene sequence (Table 2). All PCR fragments were sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Warrington, United Kingdom) and analyzed in an automatic DNA sequencer (3100 Genetic Analyzer; Applied Biosystems).

Analysis of the genetic surrounding confirms that both plasmids are different (Fig. 3). In Ac057, the bla_{OXA-58} gene is surrounded by two copies of the Insertion Sequence ISAba3; the copy downstream has the same direction as the bla_{OXA-58} gene, and the upstream copy has the opposite direction (Fig. 3). This structure has already been described in *A. baumannii* by Poirel et al. (17, 18).

The presence of the OXA-58 alone does not account for the level of resistance to imipenem of these isolates (MIC of >32 μg/ml). Further work is needed to determine whether additional efflux pumps or porin modifications are involved.

A. baumannii is certainly the most frequently isolated species in hospitals and also the microorganism of greatest clinical interest in this genus. However, *Acinetobacter* genospecies 3 and 13 are also nosocomial pathogens, and they should be considered in hospital settings. Previous studies in *Acinetobacter* genospecies 3 have revealed the presence of AmpC (2), IMP-4 (4), and bla_{VIM-2} (28). In addition to these previously described enzymes, we report here, for the first time, the presence of the bla_{OXA-58} in this microorganism. The main reason for the lack of interest on non-baumannii *Acinetobacter* isolates is probably their susceptibility to antimicrobial agents (9). However, as suggested by Horrevorts et al. (8), the clinical significance of genospecies 3 can be underestimated because the resistant strains can be erroneously classified as *A. baumannii*.

Nucleotide sequence accession number. The GenBank accession number for the bla_{OXA-58} in *Acinetobacter* genospecies 3 is EU642594.

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➢ PAPER 7:

Characterization of the carbapenem-hydrolyzing oxacillinase OXA-58 in an *Acinetobacter* phenon 6/ct13TU clinical isolate.

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Characterization of the carbapenem-hydrolyzing oxacillinase OXA-58 in an *Acinetobacter* phenon 6/ct13TU clinical isolate☆

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Abstract

The $bla_{OXA\text{-}58}$ gene identified in the *Acinetobacter* phenon 6/ct13TU clinical isolate presented 100% homology with the same gene in *Acinetobacter baumannii*. Its location in a plasmid suggests that these resistance genes may be transferred from 1 species to another.
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Keywords: *Acinetobacter* spp; Antibiotic resistance; OXA-58

The *Acinetobacter* genus has undergone important taxonomic changes since they were 1st identified. Nevertheless, since 1986, the members of this genus have been determined by DNA–DNA hybridization, and at least 32 different genospecies are now accepted in the *Acinetobacter* genus (Bergogne-Berezin and Towner, 1996; Van and Goossens, 2004; Vila et al., 2007; Weaver and Actis, 1994). The main nosocomial pathogen is *Acinetobacter baumannii*; however, other *Acinetobacter* spp. have been associated with nosocomial infections. Carbapenems usually retain good potency against these microorganisms, with imipenem being the most active (Bergogne-Berezin and Towner, 1996). Nevertheless, in the last decade, carbapenem resistance in *Acinetobacter* spp. has been reported worldwide (Poirel and Nordmann, 2006; Van and Goossens, 2004), mostly associated with the synthesis of carbapenem-hydrolyzing β-lactamases, reduced outer membrane permeability, and, occasionally, modification of penicillin-binding proteins (Poirel and Nordmann, 2006). The most prevalent carbapenemases in *Acinetobacter* spp. are the class D β-lactamases, which are divided into 4 different phylogenetic groups: OXA-23, OXA-24, OXA-58 with all their variants, and the OXA-51 family, which is intrinsic of *A. baumannii* (Poirel and Nordmann, 2006). The OXA-58 was 1st identified in France in 2003; at present, it has been found worldwide in *A. baumannii*, and it has also been described in *Acinetobacter junii* isolates from Romania and Australia, thereby demonstrating that resistance genes can disseminate from 1 genospecies to another (Marque et al., 2005; Peleg et al., 2006; Poirel and Nordmann, 2006). The OXA-58 confers reduced susceptibility to carbapenems, but in vitro experiments suggest that it can produce a high level of resistance when additional efflux mechanisms are expressed (Marque et al., 2005).

Acinetobacter spp. clinical isolates were collected from several Spanish hospitals in November 2000 and separated by amplified ribosomal DNA restriction analysis (ARDRA) into different clusters. The isolates were mostly classified as

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Table 1
Oligonucleotide sequences used in this work

Name	Nucleotide sequence (5′–3′)	Use
OXA51-1	AACAAGCGCTATTTTTATTTCAG	Detection *bla*$_{OXA-51}$ variants
OXA51-2	CCCATCCCCAACCACTTTT	
OXA23-1	GATGTGTCATAGTATTCGTCGT	Detection *bla*$_{OXA-23}$ variants
OXA23-2	TCACAACAACTAAAAGCACTGT	
OXA24-1	ATGAAAAAATTTATACTTCCTATATTCAGC	Detection *bla*$_{OXA-24}$ variants
OXA24-2	TTAAATGATTCCAAGATTTTCTAGC	
OXA58-1	AGTATTGGGGCTTGTGCT	Detection *bla*$_{OXA-58}$ variants
OXA58-2	AACTTCCGTGCCTATTTG	Southern blot probe/reverse transcriptase PCR
OXA58-1TOT	ATGAAATTATTAAAAATATTG	Gene amplification
OXA58-2TOT	TTATAAATAATGAAAAACACC	Gene amplification
OXA58-inv1	GAGCGCAGAGGGGAGAATCGTC	Genetic surrounding
OXA58-inv2	CTCAGCACAAGCCCCAATACT	Genetic surrounding
OXA58-inv3	AAGCCATGCAAGCATCTACA	Genetic surrounding
OXA58-inv4	CATCTCTTTCACTTGTTGCTGAA	Genetic surrounding

A. baumannii; however, there were also several *Acinetobacter* genospecies 3 and 13TU. A single clinical isolate was also identified by ARDRA as *Acinetobacter* phenon 6/ct13TU. This isolate was a cause of pneumonia in a 67-year-old patient admitted to the intensive care unit; it was resistant to ampicillin (256 μg/mL), piperacillin (256 μg/mL), cephalothin (256 μg/mL), cefoxitin (512 μg/mL), and rifampicin (4 μg/mL), intermediate to ceftazidime (16 μg/mL), and shows an MIC of imipenem of 6 μg/mL. Although this microorganism was susceptible to imipenem, an MIC of 6 μg/mL suggested a possible reduced susceptibility to this antimicrobial agent because it was higher than the usual MIC level generally encountered in imipenem-susceptible *Acinetobacter* spp. An isolate is considered resistant to imipenem with an MIC >16 μg/mL and sensitive with an MIC ≤4 μg/mL. Therefore, the aim of our study was to investigate the mechanism of reduced susceptibility to imipenem in this *Acinetobacter* phenon 6/ct13TU clinical isolate.

Detection of genes encoding for all class D β-lactamases was done by polymerase chain reaction (PCR) analysis using specific primers for each phylogenetic subgroup (Table 1). This analysis determined the presence of the *bla*$_{OXA-58}$ gene in the *Acinetobacter* phenon 6/ct13TU; the *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, and *bla*$_{OXA-51}$ genes were not present in this clinical isolate. Amplification of the total *bla*$_{OXA-58}$ gene was performed by PCR using the primers OXA58-T1 and OXA58-T2 (Table 1). All PCR fragments were sequenced using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Warrington, WA) and analyzed in an automatic DNA sequencer 3100 Genetic Analyzer (Applied Biosystems). The *bla*$_{OXA-58}$ gene presented 100% homology with the *bla*$_{OXA-58}$ gene from *A. baumannii* in the GenBank.

Plasmid DNA identification was attempted using genomic mapping with I-CeuI as described by Liu et al. (1993), followed by a Southern blot and a double hybridization with probes for the *bla*$_{OXA-58}$ and for the 23S rRNA (*rrl* gene) genes. The I-CeuI enzyme cuts a 26-base pair site in the *rrl* gene, shearing the bacterial genome into an analyzable number of fragments. Digested genomic DNA and plasmids were separated by pulsed-field gel electrophoresis (PFGE) (Fig. 1). Probes were marked with the PCR Dig Probe Synthesis Kit (Roche, Barcelona, Spain), and detection of the DIG-labeled DNA was performed with antidigoxigenin antibody conjugated to alkaline phosphatase and color substrates nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt (NBT/BCIP) (Roche) following the manufacturer's instructions. Hybridization with probes for the *bla*$_{OXA-58}$ gene and *rrl* gene demonstrate

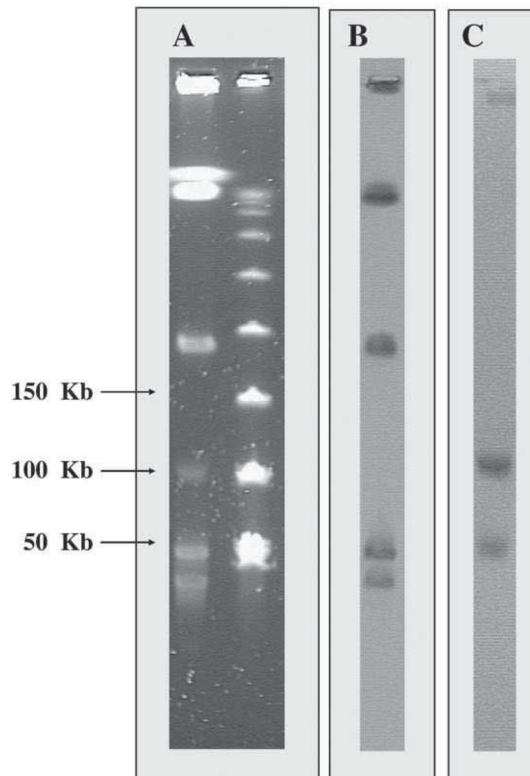

Fig. 1. Plasmid identification by genomic mapping with I-CeuI. (A) PFGE gel. (B) Hybridization with probe for the 23S rRNA gene. (C) Hybridization with probe for the *bla*$_{OXA-58}$.

that the *bla*$_{OXA-58}$ gene is present in a plasmid because the bands obtained for both hybridizations were different. The size of this plasmid was approximately 100 kb by comparison with the PFGE linear marker (Lambda Ladder PFG; New England Biolabs, Ipswich, MA).

To determine the genetic surrounding of the *bla*$_{OXA-58}$ gene, we digested the DNA with *Msp*I "C∗CGG" (Promega, Madrid, Spain) 4 h at 37 °C, and the enzyme was inactivated following the manufacturer's instructions. The digested fragments were ligated overnight at 16 °C using a T4 ligase (Promega), obtaining a mixture of circular small fragments of the total genome. The fragment of DNA containing the *bla*$_{OXA-58}$ gene was used as a template for a PCR with inverse primers designed from the *bla*$_{OXA-58}$ gene sequence (Table 1). The resulting bands were sequenced using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). This analysis showed that in the *Acinetobacter* phenon 6/ct13TU, the *bla*$_{OXA-58}$ gene is surrounded by 2 copies of the Insertion Sequence IS$_{Aba-3}$. The copy downstream has the same direction as the *bla*$_{OXA-58}$ gene, and the upstream copy has the opposite direction. A similar structure has already been described in *A. baumannii* by Poirel et al. (2005), and they suggested that this structure could correspond to a composite transposon and that the IS element upstream of the *bla*$_{OXA-58}$ gene could provide promoter sequences for the expression of this gene.

A .baumannii is certainly the main nosocomial pathogen within the genus *Acinetobacter*. Although the *Acinetobacter* phenon 6/ct13TU is not commonly identified as a causative agent of infection, this microorganism has been already isolated from blood, sputum, ulcer, and abscess. This strain belongs to a group of ARDRA profiles not yet classifiable by DNA–DNA hybridization (http://users.ugent.be/~mvaneech/ARDRA/Acinetobacter.html).

The *bla*$_{OXA-58}$ gene was 1st detected in France, and, currently, this carbapenemase has been identified in hospitals worldwide, not only in *A. baumannii* but also in other related microorganisms such as *A. junii* (Marque et al., 2005; Peleg et al., 2006; Poirel and Nordmann, 2006) or an *Acinetobacter* genospecies 3 clinical isolate (unpublished data). The *Acinetobacter* phenon 6/ct13TU may not be considered an important clinical microorganism; however, it is worthy of mention that a plasmid containing the *bla*$_{OXA-58}$ gene has been obtained from a clinical isolate of this microorganism causing pneumonia and that it can impair the potential treatment with carbapenems.

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➢ PAPER 8:

Nosocomial bacteremia due to an as yet unclassified *Acinetobacter* genomic species 17-like strain.

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Nosocomial Bacteremia Due to an As Yet Unclassified *Acinetobacter* Genomic Species 17-Like Strain

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We describe a case of bacteremia due to an as yet unclassified *Acinetobacter* genomic species 17-like strain. The recognition of this microorganism as non-*Acinetobacter baumannii* may have important epidemiological implications, as it relieves the hospital of the implementation of barrier precautions for patients infected or colonized as may be necessary with a multiresistant *A. baumannii* epidemic.

CASE REPORT

A 49-year-old man was admitted to a hospital in Barcelona (Spain) on 29 July 2000 because of L2-L3-L4 vertebral fractures as a consequence of a car accident. The patient was a heavy smoker (60 cigarettes a day) and consumed >20 g of alcohol daily. Several days later, renal insufficiency, ascites, and generalized edema developed. A diagnosis of hepatic insufficiency (probably related to alcoholic liver disease) with hepatorenal syndrome was made. On deterioration of the condition of the patient 1 month later, he was transferred to Hospital Bellvitge, also in Barcelona, where repeated paracenteses were performed and intravenous albumin administered, with some improvement. During hospitalization, the patient received piperacillin-tazobactam for primary bacteremia due to *Pseudomonas aeruginosa* over 15 days and ceftriaxone as empirical treatment for a low-grade fever, which was discontinued after negative blood cultures were obtained. Sixty days after admission, the patient presented an acute picture of fever with chills and hypotension, without focal signs or symptoms. Piperacillin-tazobactam was again started, a peripheral catheter (that had been in place for 8 days) was removed, and hemodialysis was begun due to worsened renal insufficiency. Two consecutive blood cultures were processed with an interval of 2 h. Both blood cultures yielded a gram-negative rod that was identified as *Acinetobacter baumannii* in the local laboratory and *Acinetobacter* genomic species 17 by amplified ribosomal DNA restriction analysis (ARDRA) (5) and 16S rRNA gene sequence analysis. However, the genomic fingerprint obtained by amplified fragment length polymorphism (AFLP) analysis could not be identified by comparison to those of >200 reference strains (see below). Ceftriaxone was substituted for piperacillin-tazobactam, as the organism was susceptible to the former. The bacteremic episode was resolved. Renal biopsy showed a diagnosis of immunoglobulin A mesangial glomerulonephritis with extracapillary reaction. The patient was discharged 5 months after admission. He died 2 months later as a consequence of liver failure with metabolic encephalopathy and gastrointestinal bleeding.

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The genus *Acinetobacter* has a complex taxonomy with up to 32 described named and unnamed genomic species (3, 14, 16). Reliable identification of *Acinetobacter* strains to species level is difficult and requires the use of molecular methods or a combination of genotypic and phenotypic methods (14). The species most frequently isolated from clinical samples are *A. baumannii* and *Acinetobacter* genomic species 3 and 13TU which, together with the environmental species *Acinetobacter calcoaceticus*, are combined in the so-called *A. calcoaceticus-A. baumannii* complex. The epidemiology and clinical relevance of *Acinetobacter baumannii* has been extensively studied (1), but there is scarce information about the epidemiology and clinical relevance of other, rarely isolated species. This is partly due to the lack of easy methods for species identification. Here we describe a case of bacteremia due to a strain identified by 16S rRNA gene sequence analysis as *Acinetobacter* genomic species 17-like but which could not be classified to any species by AFLP analysis.

The strain (Ac209, LUH8320) was sent to the reference laboratory (Hospital Clinic, Barcelona, Spain) for possible inclusion in the GEIH Ab-2000 project, a nationwide cohort study of all cases of *A. baumannii* colonization or infections in Spain performed during November 2000. The specific meth-

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odology of the study and some results have been published elsewhere (8, 17). In the Laboratory of Microbiology of the Hospital Clinic in Barcelona, Spain, and in the Department of Infectious Diseases of the Leiden University Medical Center, the strain was identified as an unnamed genomic species 17 using ARDRA. The profile, consisting of the combination of restriction patterns 1, 2, 1, 2, and 3 generated with the respective enzymes CfoI, AluI, MboI, RsaI, and MspI, together with BsmAI pattern 2 and with BfaI pattern 4, identified the organism as *Acinetobacter* genomic species 17 strains. With 16S rRNA gene sequence analysis, the microorganisms had a sequence similarity of 99.09% to *Acinetobacter* genomic species 17. With the high-resolution genomic fingerprinting analysis using AFLP, the isolate did not cluster at or above the 50% level with the reference strains of any described (genomic) species and was thus considered an as yet undescribed species (15). Further phenotypic studies were performed according to the method of Bouvet and Grimont (4) with minor modifications (14). The strain showed hemolytic activity and produced gelatinase, while it did not acidify Hugh-Leifson medium with D-glucose. It grew at 37°C but not at 44°C in brain heart infusion broth. Its carbon source utilization profile was different from those of all of the 32 *Acinetobacter* strains described (5, 6, 8, 14). The most notable feature was its ability to grow on histamine, a feature reported so far only in strains of the nonproteolytic genomic species 10 and 11 (4), proteolytic genomic species 14TU (9), and an as yet unclassified proteolytic strain (5). In addition, it did not utilize DL-lactate and 4-aminobutyrate (5), thereby distinguishing it from genomic species 17.

Antimicrobial susceptibility testing was performed by microdilution following the guidelines established by the CLSI (formerly NCCLS) (13). The antimicrobial agents used were ampicillin, piperacillin, cefoxitin, cephalothin, ceftazidime, cefepime, sulbactam, ampicillin plus sulbactam, imipenem, meropenem, ciprofloxacin, gentamicin, tobramycin, amikacin, doxycycline, and polymyxin B. *Pseudomonas aeruginosa* ATCC 27853 was used as a control strain.

Most *Acinetobacter* bacteremias are caused by *A. baumannii*. However, some cases may be caused by other species. Bacteremia due to *Acinetobacter* genomic species 13TU (12), *Acinetobacter junii* (10), and *Acinetobacter ursingii* (11) (identified by molecular methods) have been described. In the present study, a severe clinical case of an as yet unclassified hemolytic *Acinetobacter* strain is described; this case presented as a primary bacteremia with clinical evidence of sepsis in a predisposed patient. The infection responded to antibiotic therapy and removal of a venous catheter (which was the suspected origin of the bacteremia).

Correct identification of *Acinetobacter* species is relevant for therapeutic reasons, as species other than *A. baumannii* are generally susceptible to more antimicrobial agents. The microorganism of the present study was susceptible to all the antimicrobial agents tested except for cefoxitin and cephalothin, while a slightly elevated MIC of polymyxin B (2 mg/liter) was observed. Moreover, the recognition of this microorganism as non-*A. baumannii* may have important epidemiological implications, as it relieves the hospital from implementation of barrier precautions that might be necessary with a multiresistant *A. baumannii* epidemic.

The organism was identified by the widely used commercial API20NE system as *A. baumannii*, while 16S rRNA gene analysis identified the organism as similar to the unnamed genomic species 17. This emphasizes that correct identification of *Acinetobacter* species, according to the current taxonomy, is problematic. The problems of phenotypic identification of acinetobacters has been documented (2, 9), but the usefulness of 16S rRNA gene sequence analysis has not been evaluated yet. In addition, ARDRA profiles, which are essentially based on the 16S sequence, are not always conclusive, since some profiles may occur in different species (7). AFLP fingerprint analysis, using the Leiden University AFLP library of 200 reference strains of all described species, did not identify the strain as any of the 32 species of *Acinetobacter* described. These problems underscore the problem of correct identification of *Acinetobacter* species in the diagnostic laboratory.

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C. EFFLUX PUMP MEDIATED RESISTANCE

PAPER 9: Prevalence of the *tetA* and *tetB* genes as mechanisms of resistance to tetracycline and minocycline in *Acinetobacter baumannii* clinical isolates.

ADDITIONAL RESULTS I:

Detection and sequencing of a *mdfA*-like gene encoding an efflux pump in an *Acinetobacter baumannii* clinical isolate.

(Results presented as a poster in the 17th European Congress of Clinical Microbiology and Infectious Diseases, Munich, Germany, 2007).

PAPER 10: Proteomic analysis of a fraction enriched in cell enveloped proteins of *Acinetobacter baumannii.*

ADDITIONAL RESULTS II:

Proteomic analysis of a fraction enriched in cell envelope proteins in a quinolone-resistant *Acinetobacter baumannii* mutant.

(Results presented as a poster in the II Congress of the Spanish Proteomics Society, Valencia, Spain, 2007).

➢ PAPER 9:

Prevalence of the *tetA* and *tetB* genes as mechanisms of resistance to tetracycline and minocycline in *Acinetobacter baumannii* clinical isolates.

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Enfermedades Infecciosas y Microbiología Clínica 2006; 24(2): 77-80

Prevalencia de los genes *tetA* y *tetB* como mecanismo de resistencia a tetraciclina y minociclina en aislamientos clínicos de *Acinetobacter baumannii*

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INTRODUCCIÓN. Doscientas veintiuna cepas clínicas de *Acinetobacter baumannii* fueron recogidas de 25 hospitales en España. El objetivo de estudiar este grupo de cepas era ver el predominio de los genes *tetA* y *tetB* en una colección de cepas de *A. baumannii* no relacionadas epidemiológicamente.

MÉTODOS. Las cepas fueron distribuidas en 79 clones por análisis del ADN cromosómico mediante digestión con *SmaI* y electroforesis de campo pulsado. La concentración inhibitoria mínima (CIM) a tetraciclina y minociclina se determinó por E-test. Una cepa en representación de cada clon resistente a tetraciclina fue estudiada mediante reacción en cadena de la polimerasa (PCR) con cebadores específicos para *tetA* y *tetB*.

RESULTADOS. Cincuenta y nueve (74,7%) de los 79 clones eran resistentes a tetraciclina (CIM ≥16 mg/l), 40 de los cuales (50,6% del total) eran resistentes además a minociclina (CIM > 1 mg/l). Se tomó una cepa en representación de cada clon resistente a tetraciclina para estudiar la prevalencia de los genes *tetA* y *tetB*. El análisis por PCR de las cepas dio como resultado que un total de 39 cepas representativas de otros tantos clones (66%) poseían el gen *tetB*, mientras que sólo ocho (13,6%) fueron positivos para el gen *tetA*. Doce cepas no tenían ninguno de estos dos genes. Ninguna de las cepas analizadas presentaba ambos genes.

CONCLUSIÓN. Aunque la resistencia a tetraciclina es superior a minociclina en aislamientos clínicos de *A. baumannii*, el gen *tetB* que afecta ambos agentes antimicrobianos es más prevalente que el gen *tetA* que sólo afecta a tetraciclina.

***Palabras clave: A. baumannii*. Tetraciclina. Minociclina. *tetA*. *tetB*.**

Prevalence of the *tetA* and *tetB* genes as mechanisms of resistance to tetracycline and minocycline in *Acinetobacter baumannii* clinical isolates

INTRODUCTION. Two hundred twenty-one *Acinetobacter baumannii* clinical strains were collected from 25 hospitals in Spain. The aim of this study was to analyze the prevalence of the *tetA* and *tetB* genes in a collection of *A. baumannii* strains that were not epidemiologically related.

METHODS. The strains were distributed in 79 clones by genomic DNA analysis with low frequency restriction enzymes and pulsed-field gel electrophoresis. The MICs for tetracycline and minocycline were determined by the E-test. One strain representing each of the tetracycline-resistant clones was analyzed by polymerase chain reaction (PCR) with specific primers for the *tetA* and *tetB* genes.

RESULTS. Fifty-nine (74.7%) out of the 79 clones were tetracycline-resistant (MIC ≥ 16 mg/l) and 40 (50.6% of the total) were also minocycline-resistant (MIC > 1 mg/l). One strain representative of each tetracycline-resistant clone was taken to study the prevalence of the *tetA* and *tetB* genes. The PCR analysis showed that 39 strains representing the same number of clones (66%) had the *tetB* gene, while only 8 (13.6%) were positive for the *tetA* gene. Twelve strains did not have any of these genes. None of the analyzed strains had both genes.

CONCLUSION. Although resistance to tetracycline in *Acinetobacter baumannii* clinical isolates is greater than that to minocycline, the *tetB* gene, which affects both antimicrobial agents, has a higher prevalence than the *tetA* gene, which affects only tetracycline.

***Key words: A. baumannii*. Tetracycline. Minocycline. *tetA*. *tetB*.**

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*Al final del artículo se ofrece la relación de los miembros del Grupo de Estudio de Infección Hospitalaria (GEIH).

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Introducción

Acinetobacter baumannii es un bacilo gramnegativo que se encuentra frecuentemente colonizando y/o infectando pacientes ingresados en hospitales españoles[1]. Aunque se han comunicado casos de infecciones adquiridas en la comunidad, *A. baumannii* está generalmente asociado a brotes de infecciones nosocomiales en pacientes debilitados. En los últimos años, ha habido un incremento en el número de infecciones intrahospitalarias causadas por este microorganismo[1]. La capacidad de *A. baumannii* para causar infecciones nosocomiales se relaciona con su habilidad para desarrollar rápidamente resistencia a los antibióticos, junto con la capacidad que tiene este organismo para sobrevivir durante mucho tiempo en el hábitat hospitalario[2].

El elevado nivel de resistencia a los agentes antimicrobianos ocasiona frecuentemente un problema para elegir el antimicrobiano adecuado para el tratamiento de infecciones ocasionadas por este microorganismo. De hecho, en estos momentos, la mayoría de las cepas de *A. baumannii* que causan infecciones nosocomiales son resistentes a múltiples antibióticos, incluyendo en ocasiones a los carbapenemes, considerados de elección para el tratamiento de infecciones graves ocasionadas por patógenos multirresistentes[3]. Por este motivo, es interesante investigar todas las potenciales alternativas terapéuticas. Recientemente, Wood et al[4] han descrito que la doxiciclina y minociclina podrían ser una posible opción terapéutica para tratar infecciones por *A. baumannii*.

Las tetraciclinas son antibióticos de amplio espectro que presentan un efecto bacteriostático debido a la asociación reversible entre el antibiótico y el ribosoma[5]. Estos antibióticos actúan uniéndose a la subunidad 30S del ribosoma de la bacteria durante la fase inicial de la síntesis de proteínas; de este modo, evitan la unión del aminoacyl-tRNA a su lugar de unión en el ribosoma[6].

Esta clase de antibióticos ha sido ampliamente usada, pero después de su introducción en la agricultura y para uso veterinario, la resistencia a estos antibióticos aumentó considerablemente. Uno de los problemas ha sido el uso de tetraciclinas como promotores de crecimiento y en 1969, el *Swann report* sugirió que el uso de dosis subterapéuticas de antibióticos podría contribuir al incremento de bacterias patógenas en humanos resistentes a estos antibióticos[5].

TABLA 1. **Resultados del análisis mediante PCR de 59 clones; presencia de los genes *tetA*/*tetB* en las cepas analizadas**

	tetA⁺	***tetB***⁺	***tetA***⁻/***tetB***⁻
Número de clones *tet*® (n = 59)	8 (13,6%)	39 (66%)	12 (20,3%)
CIM (mg/l) Tet			
Límites	64-128	128	16-128
CIM 50	64	128	16
CIM 90	128	128	128
CIM (mg/l) Mino			
Límites	0,250-1	2-64	0,125-2
CIM 50	0,380	8	0,380
CIM 90	1	16	0,750

PCR: reacción en cadena de la polimerasa; CIM: concentración inhibitoria mínima; Tet: tetraciclina; Mino: minociclina.

Los principales mecanismos de resistencia a tetraciclinas son: *a)* la protección ribosomal, y *b)* la expulsión activa[6,7]. La protección ribosomal es debida a la unión de una proteína citoplasmática al ribosoma que impedirá que la tetraciclina se una a esta diana[6]. Uno de los genes relacionados con la protección ribosomal es *tetM*; este gen se ha descrito en un aislamiento clínico de *A. baumannii,* sin embargo tiene una baja prevalencia en aislamientos clínicos[8]. El mecanismo de resistencia mejor estudiado es la expulsión activa de las tetraciclinas. Los genes *tet* codifican una proteína de membrana citoplasmática (Tet) que actúa como un transportador activo; esta proteína intercambia un protón por un complejo tetraciclina-catión[6]. El proceso de resistencia está regulado por 2 genes: uno codifica una bomba de expulsión activa y el otro una proteína represora; la actividad de estos genes está regulada por la presencia o ausencia de tetraciclina[5]. En bacterias gramnegativas, los genes que más comúnmente producen resistencia a tetraciclina son *tetA*-*tetE*, *tetG* y *tetH*. El gen *tetA,* junto con el gen *tetR* (codifica la proteína reguladora de *tetA*) se han localizado en un transposón en aislamientos clínicos de *A. baumannii*[9], lo que indica la posibilidad de adquisición por transferencia horizontal entre microorganismos. *TetA* y *tetB* son los determinantes de resistencia a tetraciclinas más ampliamente distribuidos en *Enterobacteriaceae*[7].

El objetivo de este estudio fue estudiar el predominio de los genes *tetA* y *tetB* en una colección de cepas de *A. baumannii* no relacionadas epidemiológicamente, aisladas de diversos hospitales españoles.

Métodos

Cepas

En este estudio se analizaron un total de 79 cepas representantes de cada uno de los 79 clones no relacionados entre ellos por los criterios y métodos anteriormente referidos[1]. Brevemente: las cepas de *A. baumannii* habían sido recogidas en 25 hospitales españoles participantes en el estudio GEIH Ab 2000. Estas cepas fueron identificadas como *A. baumannii* mediante ARDRA (análisis del patrón de restricción resultante de la digestión con enzimas de restricción de elevada frecuencia de corte del producto de amplificación del ADNr 16S). La relación epidemiológica se determinó mediante análisis del cromosoma bacteriano digerido con una enzima de restricción de baja frecuencia de corte *SmaI* y electroforesis de campo pulsado (PFGE).

PCR de los genes *tetA* y *tetB*

La detección de los genes *tetA* y *tetB* se hizo mediante reacción en cadena de la polimerasa (PCR) con cebadores específicos para *tetA* (5'-GTA ATT CTG AGC ACT GTC GC-3' y 5'-CTG CCT GGA CAA CAT TGC TT-3') y para *tetB* (5'-TTG GTT AGG GGC AAG TTT TG-3' y 5'-GTA ATG GGC CAA TAA CAC CG-3'). Los controles utilizados fueron cepas de *Salmonella* sp. procedentes del Hospital Clínic, Barcelona, que contenían los genes *tetA* y *tetB*. Una colonia de cada aislamiento clínico fue resuspendida en 25 µl de agua y hervida durante 10 min. La amplificación se realizó usando 200 µM dNTPs, 0,5 µM de cada cebador y 2,5 unidades de *Taq* polimerasa en un volumen final de 50 µl. Las condiciones de amplificación fueron de 30 ciclos de 94 °C durante 1 min, 55 °C durante 1 min y 72 °C durante 1 min, con una extensión final de 72 °C durante 5 min. El producto de PCR fue separado en un gel de agarosa al 1,5%. Se tomó una cepa en representación de cada uno de los 59 clones resistentes a tetraciclina (40 de estos clones también eran resistentes a minociclina) para estudiar el predominio de los genes *tetA* y *tetB* (tabla 1).

Determinación de la sensibilidad a los antimicrobianos

La sensibilidad a tetraciclina y minociclina fue determinada por E-test siguiendo las instrucciones de los productores (AB Biodisk®, Sölna, Sweden). Se tomó como punto de corte para tetraciclina una CIM de 16 mg/l (las cepas con una CIM de 16 mg/l se consideraron resistentes) y para minociclina una CIM > 1 mg/l (las cepas con una CIM de 1 mg/l se consideraron sensibles).

Resultados y discusión

De 221 cepas estudiadas, 131 (59,3%) fueron resistentes a tetraciclina y minociclina, 40 (18%) fueron resistentes a tetraciclina, pero sensibles a minociclina, y el resto (22,6%) sensibles. Agrupadas por clones, 59 (74,7%) de los 79 clones eran resistentes a tetraciclina, de los cuales 40 (50,6% del total de clones) eran también resistentes a minociclina. No había ninguna cepa sensible a tetraciclina y resistente a minociclina.

Las cepas de *A. baumannii* elegidas para este estudio fueron analizadas mediante PCR con cebadores específicos para los genes *tetA* y *tetB*. El tamaño de los productos de PCR era de 950 pb para el gen *tetA* y 650 pb para *tetB* y se correspondía con el tamaño de los controles positivos usados para estos 2 genes (fig. 1). El análisis por PCR de las cepas dio como resultado que un total de 39 clones (66%) poseían el gen *tetB*, mientras que sólo 8 clones (13,6%) fueron positivos para el gen *tetA*. El análisis de la cepa representativa de cada uno de los 12 clones restantes no mostró la presencia de *tetA* ni *tetB*, pese a presentar un fenotipo propio de las *tetA*⁺ (tabla 1). La CIM de las cepas con el gen *tetB* eran siempre de 128 mg/l, mientras que la mitad de las cepas que tenían el gen *tetA* tenían una CIM de 64 mg/l.

Los resultados también demostraron que hay una relación entre la presencia del gen *tetB* y la resistencia a tetraciclina y minociclina. Las cepas resistentes a tetraciclina presentaron esta resistencia por adquisición del gen *tetA*, *tetB* o posiblemente otro gen *tet*. Treinta y nueve de las 40 cepas resistentes a tetraciclina y minociclina adquirieron esta resistencia debido a la presencia del gen *tetB*; se encontró sólo una cepa que no tenía el gen *tetB* y que, sin embargo, era resistente a tetraciclina y minociclina (en este caso podría haber otro gen que afectara la resistencia a la minociclina). Esta cepa tiene una CIM a tetraciclina y minociclina que es similar a otras cepas (CIM tetraciclina = 128 mg/l y CIM minociclina = 2 mg/l); todas las cepas que tenían esta CIM eran positivas para el gen *tetB*. Estos resultados confirman la conclusión de Guardabassi et al[10] cuando afirmaba que los genes *tetA* y *tetB* estaban presentes frecuentemente en aislamientos clínicos de *A. baumannii* resistentes a tetraciclina, mientras que eran infrecuentes en cepas acuáticas de *Acinetobacter* spp.[10].

Las tetraciclinas no han sido generalmente usadas para tratar infecciones causadas por *A. baumannii*, y aun así, la resistencia de este microorganismo a las tetraciclinas es muy alta. Los genes *tetA* y *tetB* están ampliamente diseminados entre las bacterias gramnegativas y se encuentran normalmente localizados en transposones que a su vez están insertados dentro de plásmidos, lo que les proporciona movilidad y les facilita la diseminación entre diferentes tipos de bacteria. *A. baumannii* puede colonizar el tracto intestinal de pacientes hospitalizados fundamentalmente en hospitales con una endemia elevada[11]. Este nicho ecológico presenta las condiciones óptimas para que *A. baumannii* adquiera los genes *tetA* y *tetB* mediante transferencia horizontal a partir de otras bacterias gramnegativas presentes en él.

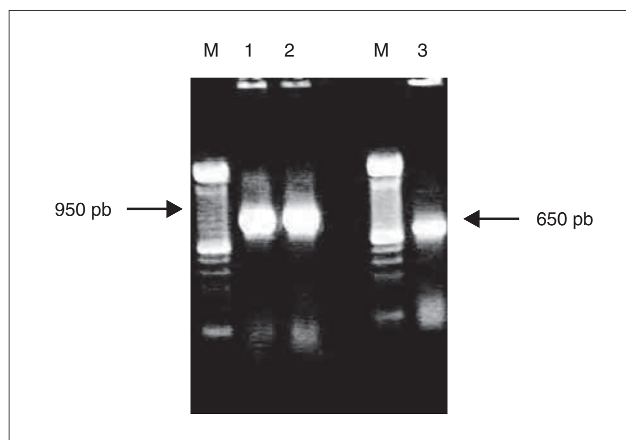

Figura 1. Amplificación mediante PCR de los genes *tetA* y *tetB*. Carriles M, marcador de ADN de 100 pb; carriles 1 y 2, gen *tetA*; carril 3, gen *tetB*.

El gen *tetB* codifica la bomba de expulsión más eficaz y de esta manera proporciona resistencia a tetraciclina y también a minociclina[7]. Los resultados obtenidos en este trabajo están de acuerdo con esta descripción del gen *tetB* ya que el 95% de las cepas resistentes a minociclina tenían el gen *tetB*, en contraposición a las cepas resistentes únicamente a tetraciclina, en las cuales diferentes genes *tet* podían estar involucrados en esta resistencia.

En un estudio piloto reciente realizado en Estados Unidos, Wood et al[4], sugieren que la minociclina o la doxicilina podrían ser efectivas en el tratamiento de neumonías causadas por cepas de *A. baumannii* multirresistentes. Según este estudio, la terapia con minociclina o doxicilina fue efectiva en 6 de los 7 pacientes incluidos. Sin embargo, en España la utilización de esta clase de antibióticos queda invalidada por la elevada frecuencia de resistencias[12].

Como conclusión se puede decir que las cepas clínicas de *A. baumannii* aisladas en España son frecuentemente resistentes a tetraciclina debido a la adquisición de los genes *tetB* y, en menor medida *tetA*. El menor número de cepas resistentes a minociclina es debido a que hay menos genes que proporcionan resistencia a este antimicrobiano; en este estudio, únicamente TetB proporcionaba resistencia a minociclina.

Agradecimiento

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➢ ADDITIONAL RESULTS I:

Detection and sequencing of a *mdfA*-like gene encoding an efflux pump in an *Acinetobacter baumannii* clinical isolate.

Acinetobacter baumannii **is currently an important cause of nosocomial infection over the world due to the increasing multiresistance of this microorganism. The overexpression of antimicrobial efflux pumps is an important way of acquiring this multidrug-resistance because they may confer resistance to different types of antimicrobial agents. The objective of this work was to detect and characterize new efflux pumps in** ***Acinetobacter baumannii.***

A small portion of a ***mdfA*****-like gene was amplified with degenerate primers obtained from homologous regions of the** ***mdfA*** **gene in other microorganisms. The whole gene was obtained by reverse PCR analysis and sequenced to determine the complete gene sequence. The** ***mdfA*****-like gene in** ***A. baumannii*** **showed a 34 % homology with the** ***mdfA*** **gene in** ***Escherichia coli*** **and a 70 % homology with** ***Acinetobacter baylyi*** **ADP1. This gene was also present in all the strains from a collection of** ***A. baumannii*** **obtained from Spanish hospitals; this high prevalence suggests a chromosomal location of the** ***mdfA*****-like gene in these species.**

Acinetobacter baumannii is an opportunistic pathogen which causes infections mainly in patients in intensive care units. The extensive use of antimicrobial agents in ICUs can select for the emergence of multiresistant strains. In fact, *A. baumannii* clinical isolates resistant to all antimicrobial agents have already been reported (228). The multiresistance of this microorganism is due in part to decreased outer membrane permeability, the presence of efflux pumps or to the interplay between both mechanisms of resistance. Efflux pumps can export structurally-distinct classes of antimicrobial agents out of the cell by an energy dependent mechanism; overexpression of these transporters has often been associated with bacterial multidrug resistance (228). *A. baumannii* has a high intrinsic resistance to antimicrobial agents; however, only three multidrug efflux pumps have been described in this microorganism: AdeABC, AdeIJK and AbeM (38,160,228). The objective of this study was to detect and characterize new efflux pumps in *A. baumannii.*

The search for efflux pumps was done by designing primers in homologous regions of the genes encoding for efflux pumps in other microorganisms. The *mdfA* gene in *Escherichia coli* and the ORF57 in *Acinetobacter baylyi* ADP1 (presumably *mdfA*) were compared, and degenerate primers were designed in the fragments with a higher nucleotide homology (Table 1). The mismatch nucleotides between both sequences were substituted with inosines. A 300 bp portion of the *mdfA* gene was amplified using the primer combination O57F1 / O57R2 (Table 1) and sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). This initial fragment had 53 % amino acid homology with the MdfA efflux pump in *E. coli*. In order to obtain the whole gene (Fig.1), the genomic DNA from *A. baumannii* strain RUH134 was digested with *Msp*I (Promega), a high frequency cutting restriction enzyme that recognizes and digests the DNA when it detects the restriction site "C*CGG". The fragments obtained were autoligated overnight at

16 ºC using a T4 DNA ligase (Promega). The portion of DNA containing the *mdfA* gene was detected by PCR and confirmed by sequencing. Afterwards, this fragment was used as template for an inverse PCR with inverse primers designed from the initial 300 bp *mdfA* amplified portion (Table 1). Although, there were no restriction sites for *Msp*I in the *mdfA* gene from *E. coli* and *Acinetobacter baylyi*, this enzyme was cutting the gene in *A. baumannii*; we had sequenced only 775 bp of the gene. All the high frequency cutting restriction enzymes were cutting inside the gene, therefore, the rest of the experiment was carried out with the restriction enzyme *Hinc*II (Promega) which recognizes the restriction site "GT(T/C)*(A/G)AC". The whole *mdfA* gene of *A. baumannii* strain RUH134 was sequenced and it was 1,230 nucleotides long. This gene had a 34 % homology with the *mdfA* gene in *E. coli*, and a 70 % homology with the same gene in *A. baylyi* ADP1 (Table 2). The prevalence of this *mdfA*-like gene was analysed in a group of sixty-four epidemiologically unrelated strains collected from different Spanish hospitals in the year 2000. All the clinical isolates were positive for this gene, suggesting a chromosomal location of the *mdfA* gene in *A. baumannii.*

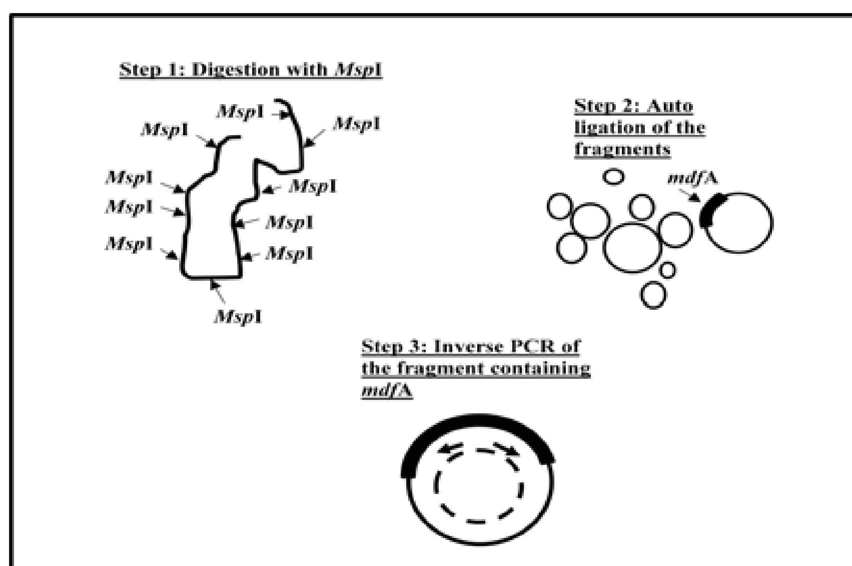

Fig.1: Schematic representation of the methodology used in this study.

Table 1: Primers used in this study.

PRIMERS	SEQUENCE (5' → 3')	FUNCTION
O57-F1	**ATCGGIAACGATITIATTCA**	**Detection**
O57-F2	**TTGTCIGATCGTITTGG**	**Detection**
O57-R1	**CCAAGTAITGGIGCAA**	**Detection**
O57-R2	**AAGGITTCCTGAATTGC**	**Detection**
I57-F	**TAACGCGACAAATTGAACAC**	**Inverse PCR**
I57-R	**CACAAGGCAAAAAATAAAAC**	**Inverse PCR**

Table 2: Homology between the *mdfA* gene in *A. baumannii* RUH134 and the same gene in *E. coli* and *A. baylyi* ADP1.

	NUCLEOTIDES	AMINO ACIDS
Acinetobacter baylyi ADP1	70 %	78 %
Escherichia coli	34 %	40 %

➢ PAPER 10:

Proteomic analysis of a fraction enriched in cell enveloped proteins of *Acinetobacter baumannii*.

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SHORT COMMUNICATION

Proteomic analysis of a fraction enriched in cell envelope proteins of *Acinetobacter baumannii*

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Acinetobacter baumannii is a multiresistant opportunistic nosocomial pathogen. A protein fraction was purified and analyzed by 2-DE. Twenty-nine major protein spots were selected for protein identification using trypsin digestion and MS analysis. As the *A. baumannii* genome has not yet been described, protein identification was performed by homology with other *Acinetobacter* species in the NCBi database. We identified ribosomal proteins, chaperones, elongation factors and outer membrane proteins (Omp), such as OmpA and the 33–36-kDa OMP. Proteomic analysis of *A. baumannii* provides a platform for further studies in antimicrobial resistance.

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Keywords:
Acinetobacter baumannii / 2-DE / Cell envelope proteins

Acinetobacter baumannii are gram-negative, non-motile coccobacilli that have been associated with outbreaks of nosocomial infections in hospitals all over the world [1]. The main infection caused by this microorganism is nosocomial pneumonia; but *A. baumannii* has also been associated with bacteremia, urinary tract infection and secondary meningitis. Treatment of these nosocomial infections is becoming a problem because the level of resistance to antibiotics is rising [2]. Nowadays, multidrug resistance is common among *A. baumannii* isolates; there have been cases of microorganisms resistant to almost all the commercially available antimicrobials [3]. *A. baumannii* is intrinsically resistant to many common antibiotics; in addition, it also develops antibiotic resistance extremely rapid, and it has a rapid response to the challenge with antimicrobials in the hospital environment. The use of antibiotics selects the overgrowth of intrinsically resistant bacteria such as *A. baumannii* [4].

The multiresistance of this microorganism is due in part to a decreased permeability, to an increased efflux pump or to the interplay between both mechanisms. There is a synergy between the effect of efflux pumps and the permeability of the outer membrane (OM) [5]. As all the gram-negative microorganisms, *A. baumannii* has an OM surrounding the peptidoglycan that acts as a permeation barrier. This membrane contains large OM proteins (Omp) called porins that allow the entrance of nutrients and possibly the efflux of waste products [6]. The characteristics of the constitutively expressed porins are important to determine the intrinsic level of antibiotic resistance in gram-negative bacteria. The presence of slow porins as the major transport channel produces a higher intrinsic resistance due to their low permeability. A slow porin from the OmpA family (HMP-AB) [7] would explain the high intrinsic antibiotic resistance of *A. baumannii*.

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Abbreviations: OM, outermembrane; **Omp**, OM protein

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Currently, fewer antibiotics are effective for treating infections caused by multiple resistant bacteria, therefore, it is necessary to develop new antimicrobial agents. The traditional method for developing new antibiotics was to try compounds analogous to existing drugs, and look for a better therapeutic activity and a broader spectrum of action [8]. With this method, the antimicrobial agents available act against a limited number of targets, which favors cross-resistance. Nowadays, the major goal is to find antimicrobial agents with other modes of action [8]. New technologies are coming up and they will have a positive impact on antimicrobial drug discovery; among them, proteomics can help to determine new bacterial targets for the antibiotics because, with this technology, it is possible to characterize the gene products depending on the biological and environmental conditions [9]. Proteomics has been described by Paine *et al.* [10] as "the study of a particular species' complete protein repertoire encoded for by its genome".

The characterization of membrane proteins is necessary because of the importance of the Omp in antibiotic resistance, together with the transport of the nutrients, the cell-cell signaling, the attachment to host cells and the virulence in pathogenic strains [9]. The proteomic analysis of a fraction enriched in cell envelope proteins will provide a platform for further studies in the field of antimicrobial resistance.

The proteomic study was done with the *A. baumannii* strain A15-43; a previously characterized clinical strain obtained from the Hospital Clínic of Barcelona collection [11]. *A. baumannii* A15-43 was grown overnight in nutrient broth (Oxoid, Basingstoke, UK) at 37°C with constant shaking. Fresh nutrient broth media (500 mL) was inoculated with a 1:50 dilution of the overnight culture and grown to an OD_{600} = 0.4–0.6 at 37°C with shaking. The cells were harvested by centrifugation (Hermle Z400K) at 3500 × g for 30 min and washed twice with 10 mL 0.9% NaCl. The pellet was dissolved in 5 mL disintegration buffer (7.8 g/L NaH_2PO_4, 7.1 g/L Na_2HPO_4, 0.247 g/L $MgSO_4$ 7 H_2O + protease inhibitors mix, Amersham Biosciences) and sonicated on ice for a total of 5 min with intervals of 1 min (Branson Sonifier 250). The unbroken cells were separated by centrifugation at 1500 × g for 10 min at 4°C. The supernatant was collected and centrifuged at 100 000 × g for 45 min at 4°C using a Beckman Coulter Optima™ L-90K ultracentrifuge. The pellet containing the cell envelope proteins was dissolved in 100 μL dH_2O, and treated with the 2-D Clean-Up Kit (Amersham Biosciences).

The analysis of the protein mixture was done by 2-DE. A protein sample of 50 μL was solubilized in 200 μL of rehydration solution (8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue) containing 0.5 mg DTT. The sample was briefly centrifuged and added to a 13-cm, pH 3–10 IPG strip (Amersham Biosciences). After 12 h of gel rehydration at 20°C, the IEF was performed using an Ettan IPGphor IEF System (Amersham Biosciences) in three steps: 500 V for 1 h, 1000 V for 1 h and 8000 V for 2 h.

The first dimension strips were equilibrated in 10 mL of SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) for 30 min at room temperature. The strips were placed on the top of a 10% acrylamide vertical gel and sealed with 0.5% agarose dissolved in running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS). The running conditions for the second dimension were 15 mA/gel for 30 min and 30 mA/gel for 4 h; the voltage was left free.

The polyacrylamide gels were stained using a silver staining kit from Amersham Biosciences. A selection of proteins was made according to the intensity and sharpness of the dots (Fig. 1). Twenty-nine major protein spots were selected for identification via enzymatic digestion followed by MS analysis; even if there were more proteins, only the major ones were selected and analyzed.

Proteins excised from the 2-DE gel were in-gel digested with trypsin (sequencing grade modified, Promega) in the automatic Investigator ProGest robot of Genomic Solutions. Briefly, excised gel spots were washed sequentially with ammonium bicarbonate buffer and ACN. Proteins were reduced and alkylated, respectively, by treatment with 10 mM DTT solution for 30 min, and treatment with 100 mM iodoacetamide. After sequential washings with buffer and ACN, proteins were digested overnight, at 37°C with 0.27 nmol trypsin. Tryptic peptides were extracted from the gel matrix with 10% formic acid and ACN; the extracts were pooled and dried in a vacuum centrifuge.

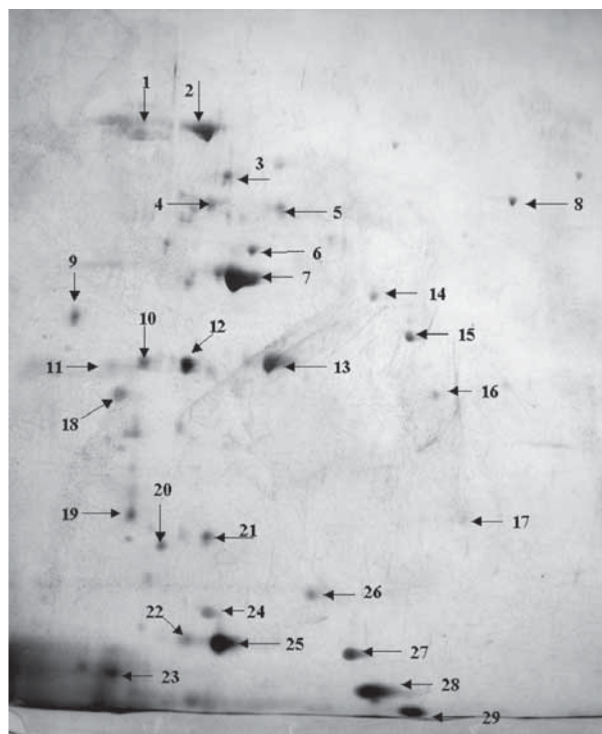

Figure 1. 2-DE of a fraction enriched in cell envelope proteins of *A. baumannii* A15-43.

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Table 1. Proteomic analysis of a fraction enriched in cell envelope proteins of *A. baumannii*

Spot no.	Mol. wt.	Sequence[a)]	Score[b)]/% of coverage	Protein	Organism	Accession no.[c)]	Mass spectrometer	PSORT Analysis[d)]
A) High homology with members of the *Acinetobacter sp.*								
1	**57 193**	AAVEEGVVAGGGVALVR	**69/3**	Chaperone Hsp60	***Acinetobacter sp***	gi|50085881	Q-TOF	Cytoplasmic
2	**19 790**	ISNIRELISVLEAVAK QDTLTAELENPFILLVDR TGKPLLIIAEDVEGEALA EGVITVEEGSGFEDALDV	**71/48**	Chaperone Cpn60	*Acinetobacter junii*	gi|22121790	MALDI-TOF/TOF	Cytoplasmic
3	**50 337**	VGLFGGAGVGK DVLLFVDNIYR YTLAGTEVSALLGR QLDPLVVGQEHYEIAR	**205/11**	β-subunit ATP synthase	*Acinetobacter sp ADP1*	gi|50083469	Q-TOF	Multiple localization sites
5	**43 213**	TTDVTGAIQLK FDAEVYVLSK TTLTAAIATICAK ELLSTYDFPGDDTPVIR	**184/12**	Elongation factor EF-Tu	*Acinetobacter sp ADP1*	gi|50083572	Q-TOF	Cytoplasmic
6	**37 251**	VILEPLER QGPGDITAADLR FPEGETRPVGR LFDQNEAYLTLEK EEVDPILLRPVDDLELTVR	**241/18**	α-Subunit RNA polymerase	*Acinetobacter sp ADP1*	gi|50086190	Q-TOF	Multiple localization sites
7	**37 793**	VFATITGSR LSEYPNATAR IEGHTDNTGPR SALVNEYNVDASR QINGNFYVTSDLITK LSTQGFAWDQPIADNK YEIPDLSYHNDEEGTLGN	**198/13**	Outer membrane protein HMP	*Acinetobacter baumannii*	gi|21666310	MALDI-TOF/TOF	Outer membrane
8	**51 163**	**SQQFDLVVIGGGP GGYEAAIR**	**86/4**	Dihydrolipoamide dehydrogenase	*Acinetobacter sp. ADP1*	gi|50085914	Q-TOF	Cytoplasmic
10	**31 473**	AVGESVAI **AVGESVAIDEKQDAVT ARDEK** GPLAEAAFLNQASNV SVAYNYIK	**190/13**	33–36-kDa outer membrane protein	*Acinetobacter baumannii*	gi|57336104	Q-TOF	Unknown (non-cytoplasmic)
11	**23 996**	LGTILGPR QNVETLVADLK	**109/8**	50S ribosomal protein L1	*Acinetobacter sp ADP1*	gi|50083576	Q-TOF	Unknown
12	**31 579**	VLDPLADK YVLAASTTTGK RPIYAGNAIATVGSDEAI IVGTVR	**164/13**	α-Subunit electron transfer flavoprotein	*Acinetobacter sp ADP1*	gi|50085723	Q-TOF	Unknown
13	**30 977**	FEVGEGIEK AIESGKPANIVEK	**93/7**	Elongation factor EF-Ts	*Acinetobacter sp ADP1*	gi|50085371	Q-TOF	Cytoplasmic
14	**37 441**	YAGAPFLR INIDTDLR KINIDTDLR FTRPPTGDILAIDR	**219/8**	Aldolase	*Acinetobacter sp ADP1*	gi|50085065	Q-TOF	Multiple location sites

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Table 1. Proteomic analysis of a fraction enriched in cell envelope proteins of *A. baumannii*

Spot no.	Mol. wt.	Sequence[a)]	Score[b)]/% of coverage	Protein	Organism	Accession no.[c)]	Mass spectrometer	PSORT Analysis[d)]
15	**30 804**	VVGGVTPGK VLVQGFTGK SGTLTYEAVAQTTK MGHAGAIISGGKGTAEEK	**203/16**	Succinyl-CoA synthetase α-chain	*Acinetobacter sp ADP1*	gi|50085912	Q-TOF	Unknown
16	**31 641**	ASLEAGVR AAKPLLQAR VNAISAGPIR LSIAYGIAQALHR EGAELAFTYPNEK	**221/18**	Reductase	*Acinetobacter sp ADP1*	gi|50086120	Q-TOF	Unknown
17	**22 139**	YQSLTLKDDLYR	**58/5**	Xanthine phosphoribosyl transferase	*Acinetobacter sp ADP1*	gi|50086165	Q-TOF	Unknown
20	**19 331**	DSDALFVDR	**44/5**	Inorganic pyrophosphatase	*Acinetobacter sp ADP1*	gi|50083517	Q-TOF	Cytoplasmic
21	**20 829**	**IQIVEINAGGIGR TFVIDPE GKEGEATLAPSIDLVGKI**	**175/20**	Thioredoxin-like	*Acinetobacter sp. ADP1*	gi|50085223	Q-TOF	Cytoplasmic
	20 803	**DASELLR** **DASELLRK** GTFVIDPEGK IQIVEINAGGIGR	**160/16**	Peroxiredoxin	*Acinetobacter radioresistens*	gi|45826512		Cytoplasmic
26	**18 536**	SFPQVELNTNK DGFYDGVIFHR GYHADVPLENVVIESAK	**149/23**	Rotamase	*Acinetobacter sp ADP1*	gi|50085061	Q-TOF	Cytoplasmic
28	**18 058**	LFEEFAK ALLIEDKK	**66/8**	50S ribosomal protein L10	*Acinetobacter sp. ADP1*	gi|61214999	Q-TOF	Multiple localization sites
	15 438	EILGATNPK	**37/6**	Nucleoside diphosphate kinase	*Acinetobacter sp ADP1*	gi|50083793		Cytoplasmic
29	**15 775**	MDVILLQR AVAATEANTAAFEAR	**172/15**	50S ribosomal protein L9	*Acinetobacter*sp ADP1	gi|50085517	Q-TOF	Cytoplasmic
	15 112	YNDAIIR	**49/5**	30S ribosomal protein S6	*Acinetobacter sp. ADP1*	gi|50085515		Cytoplasmic
B) Match with proteins from other bacterial species								
22	**17 572**	ISVNSPIAR EQQGFCEAR	**54/11**	Transcription elongation factor	*Psychrobacter sp*	gi|41689214		Multiple localization sites

a) Peptides in bold were not sequenced.
b) Score gave by MASCOT search engine.
c) Acession number of NCBI database.
d) Prediction of protein localization was performed using the public software PSORT-B v.1.1.2 (http://psort.nibb.ac.jp/) [17].

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Tryptic peptides were either analyzed by MALDI-TOF/TOF MS (4700 Proteomics Analyzer, Applied Biosystems) or nanoESI-MS/MS (Q-TOF Global, Micromass-Waters). In the first case, peptides were redissolved in 5 µL 0.1% TFA in 50% ACN. Typically, a 0.5-µL aliquot was mixed with the same volume of a matrix solution, 5 mg/mL CHCA (Aldrich) in 0.1% TFA in 50% ACN. Three major peaks were selected to be characterized further by MS/MS analysis. Spectra were submitted for database searching in a generic MASCOT format. The samples that were not identified by MALDI-TOF were analyzed by on-line LC-MS/MS (Cap-LC-nano-ESI-Q-TOF; CapLC, Micromass-Waters). In these cases, samples were resuspended in 10 µL 10% formic acid solution and 4 µL were inject to chromatographic separation in reverse-phase capillary C_{18} column (75 µm id and 15 cm length, PepMap column, LC Packings). The eluted peptides were ionized via coated nano-ES needles (PicoTip™, New Objective). A capillary voltage of 1800–2500 V was applied together with a cone voltage of 80 V. The collision in the CID was 20–35 eV and argon was employed as collision gas. Data were generated in PKL file format, which were submitted for database searching in MASCOT server. NCBI database was employed by default. Database search were performed using NCBI database without species specification. Since the nucleotide sequence of the *A. baumannii* genome is not yet available, protein identification was achieved by homology with other *Acinetobacter* species such as *A. calcoaceticus* ADP1.

Table 1 describes the proteins identified in this study. According to the results obtained, the proteins could be grouped into three distinct categories. The first group included 20 out of the 29 analyzed proteins; these proteins had a high homology with members of the *Acinetobacter* sp. Generally, this homology was obtained with *A. calcoaceticus* but some proteins also showed homology with *A. junii*, *A. radioresistens* and *A. baumannii* (Table 1). The second group included the proteins that had a possible match with proteins belonging to other bacterial species. There was an unique protein classified in this group, which showed homology with the *Psychrobacter* spp. In this group, the percentage of homology was lower; this is normal because the amino acid sequence between different species has a higher variation (Table 1). The third group included not identified proteins. It could be attributed to a low homology with proteins of other *Acinetobacter* species described in databases. Moreover, these proteins could be specific for *A. baumannii* and, therefore, they may not have any significantly homologous protein in other species. On the other hand, these proteins can be included in the group of proteins that are not identified for some other reason. However, only 8 of the 29 proteins sequenced were left in this third group (data not shown).

In addition to several enzymes and ribosomal proteins, we have obtained two chaperones (spots 1 and 2), three elongation factors (spots 5, 13 and 22), and two Omps (spots 7 and 10).

The chaperones Hsp60 and Cpn60 form part of a family of molecular chaperones called GroEL and they are involved in the folding of several substrates. The final objective of this folding is to achieve the correct tertiary structure of proteins and, indirectly, also the correct quaternary structure [12, 13]

The elongation factors Tu and Ts have always been associated to the cytoplasm of the cell where they are involved in protein synthesis. Recently, Granato *et al*, suggested that the elongation factor Tu (EF-Tu) protein could be found on the cell envelope of *Lactobacillus johnsonii* acting as an adhesion factor [14]. This molecule has also been found associated to the membrane of *Escherichia coli* [14, 15]. The elongation factor Ts (EF-Ts) has also been found in the cell envelope of *Streptococcus oralis*; it was suggested that the EF-Tu and EF-Ts were expressed at the cell surface [16].

The Omp HMP (spot 7) is a monomeric porin similar to the OmpA of enterobacteria and the outer membrane protein F (OprF) of *Pseudomonas aeruginosa*. This porin has already been described by Gribun *et al.* [7] as a porin with a large pore size. Despite having a large pore size, these proteins are called "slow porins" because they have a slower penetration rate than the normal trimeric porins. This could be the reason why the microorganisms presenting this kind of porins as the major channel, also have a higher intrinsic resistance to the antibiotics.

The rest of proteins sequenced are enzymes involved in metabolic pathways and ribosomal proteins. Looking at these results, it seems that many of the proteins are cytoplasmic; therefore, we used a software for prediction of protein subcellular localization (PSORT). The PSORT predicts that 63% of known proteins are cytoplasmic (Table 1). However, this software predicts that proteins such as the EF-Tu and EF-Ts are cytoplasmic but, as mentioned above, there are several publications suggesting that these elongation factors can be present in the cell envelope.

To summarize, among the proteins identified, there are chaperones, elongation factors, Omps, ribosomal proteins and different enzymes. However, we have to highlight two proteins: first, the HMP, an Omp similar to OprF described in *Pseudomonas aeruginosa*. In fact, both microorganisms share some similar characteristics concerning the permeability to antimicrobial agents. Second, the 33–36-kDa Omp that could be involved in antibiotic resistance (data not shown).

The reference map of proteins from *A. baumannii* is now the basis for determining cell envelope molecules associated with antibiotic resistance, as well as cell-cell signaling and pathogen-host interactions.

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➢ ADDITIONAL RESULTS II:

Proteomic analysis of a fraction enriched in cell enveloped proteins in a quinolone-resistant *Acinetobacter baumannii* mutant.

Quinolone resistance in *A. baumannii* isolates is acquired by mutations in the *gyr*A and *par*C genes, decreased permeability of the outer membrane and presence of efflux pumps. The objective of this study was to perform a comparative proteomic analysis of wild-type *A. baumannii* clinical isolates and their isogenic quinolone-resistant mutants.

A protein fraction enriched in cell envelope proteins was prepared for two sets of isogenic quinolone mutants. These extracts were purified and analysed by two-dimensional gel electrophoresis and the overexpressed proteins were characterised using trypsin digestion and mass spectrometry analysis (MALDI TOF-TOF).

Fifteen proteins presented changes in their expression between the wild-type strain and its isogenic quinolone-resistant mutant. Characterisation of these proteins showed that most of them were metabolic proteins; however, some outer membrane proteins with an increased expression in the resistant mutant strain were also observed. These proteins were an OmpA-like protein and a HMP-like protein of 38 KDa. Moreover, two other OMPs were overexpressed in one of the quinolone-resistant mutants: an OMP of 35 KDa and a possible ABC-transporter.

Decrease in membrane permeability together with the presence of efflux pumps has been described as a mechanism of resistance to different antimicrobial agents. Changes in antibiotic resistance should be related to membrane proteins. An increased expression of porins linked to hypothetical efflux pumps may be involved in the increased resistance of these clinical strains.

Acinetobacter baumannii is a multiresistant opportunistic nosocomial pathogen affecting mainly patients in intensive care units. This microorganism develops quinolone resistance through mutations in the *gyr*A and *par*C genes of the DNA gyrase or topoisomerase IV, decreased permeability of the outer membrane and overexpression of efflux pumps (160). Porins may play a significant role in the acquisition of resistance to antimicrobial agents. In Gram-negative bacteria, regulation of porin expression in response to the presence of antimicrobial agents is a common survival strategy in front of these toxic substances. Nevertheless, in *A. baumannii* there is still a lack of information concerning the function of the OMPs and the permeability properties of the outer membrane (228). Efflux transporters are expressed in all living cells, protecting them from the toxic effects of organic chemicals; overexpression of these efflux pumps has often been associated with multidrug resistance. Many quinolones are substrates for multidrug efflux pumps; at present, in *A. baumannii* the only ones described are the RND-type pumps AdeABC and AdeIJK, and the MATE efflux pump AdeM (38,160,228). The objective of this study was to perform a comparative proteomic analysis of wild-type *A. baumannii* clinical isolates and their isogenic quinolone-resistant mutants in order to identify outer membrane modifications which could be related to the acquisition of quinolone resistance.

Two clinical isolates (A15-43 and 77) were selected for this study: these isolates were susceptible to clinafloxacin and ciprofloxacin, respectively. Quinolone-resistant mutants (A15-43M and 77M) were obtained by submitting these isolates to repeated *in vitro* subinhibitory concentrations of the respective antimicrobial agent. Strains A15-43 wt and A15-43M showed a MIC of clinafloxacin of 3 mg/L and >32 mg/L, respectively; strains 77 wt and 77M had a MIC of ciprofloxacin of 1.5 mg/L and >32 mg/L, respectively. All these mutations were stable after fifteen successive passages on non-selective medium. The mutations in the *gyr*A and *par*C genes were studied by sequencing

and comparing the appropriate fragments of these two genes (the quinolone resistance-determining region "QRDR"); there were no additional mutations in these two genes between the wild type strains and their isogenic quinolone-resistant mutants. These results imply that changes in quinolone resistance would be due to other mechanisms of resistance such as changes in membrane permeability as a result of a modification in porin expression or active efflux of the antimicrobial agent.

Proteomic analysis was performed on a protein fraction enriched in cell envelope proteins prepared after growing the strains at the same OD_{600}. These extracts were purified and analysed by two-dimensional gel electrophoresis; the overexpressed proteins were characterised using trypsin digestion and mass spectrometry analysis (MALDI TOF-TOF or nanoESI-MS/MS) as previously described in paper 9 (131). Changes in protein expression were observed in both sets of isogenic mutants. Characterisation of these proteins showed that most of them were metabolic proteins (Table 1); this is due in part to the contamination of the membrane extraction with cytoplasmic proteins, and to the presence in the cellular membrane of some proteins which are also involved in these metabolic processes. When looking at the outer membrane proteins, there was no change in the abundance of the HMP protein; this OMP is the main structural protein in *A. baumannii* and has a high homology with the OmpA of Enterobacteriaceae. There were no changes in the 33-36 KDa OMP either, a protein that has been involved in the acquisition of resistance to imipenem (228). However, some outer membrane proteins with an increased expression in the resistant mutant strain were found (Table 2). These proteins were an OmpA-like protein of 27 KDa and a HMP-like protein of 24 KDa (Fig 1 & 2). Moreover, two other OMPs appeared more intense in the gel for the resistant mutant 77M (Fig 2): a 35 KDa OMP and a possible ABC-transporter. To date, these proteins have not been reported in *A. baumannii* and their function is still unknown; further studies are needed to characterise all

these proteins. In addition, 2D analysis of new protein extractions has to be performed to confirm that all this differences are indeed related to the acquisition of quinolone resistance.

Decrease in membrane permeability together with the overexpression of efflux pumps has been described as a mechanism of resistance to different antimicrobial agents. Changes in antibiotic resistance should be related to membrane proteins; an overexpression of porins linked to hypothetical efflux pumps may be involved in the increased resistance of these clinical isolates.

Table 1: Modifications in metabolic proteins

PROTEINS	A15-43 wt	A15-43 M	77 wt	77 M
1. Chaperone Hsp60	+			
2. Chaperone Cpn60		+		
3. EF-Tu		+		
4. 50S Ribosomal protein	+			
5. CsuA/B	+		+	
6. EF-Tu		+		+
7. Inorganic pyrophosphatase		+		
8. Dihydrolipoamide dehydrogenase		+		+
9. Rotamase		+		
10. Transcriptional Regulator		+		+
11. Aspartate aminotransferase				+

Table 2: Modifications in outer membrane proteins

PROTEINS	A15-43 wt	A15-43 M	77 wt	77 M
a. HMP	+	+	+	+
b. OMP (38 KDa)	+	+	+	+
c. 33-36 KDa protein	+	+	+	+
d. Omp A-like (27 KDa)		+		+
e. HMP-like (24 KDa)		+		+
f. OMP (35 KDa)				+
g. ABC-transport system				+

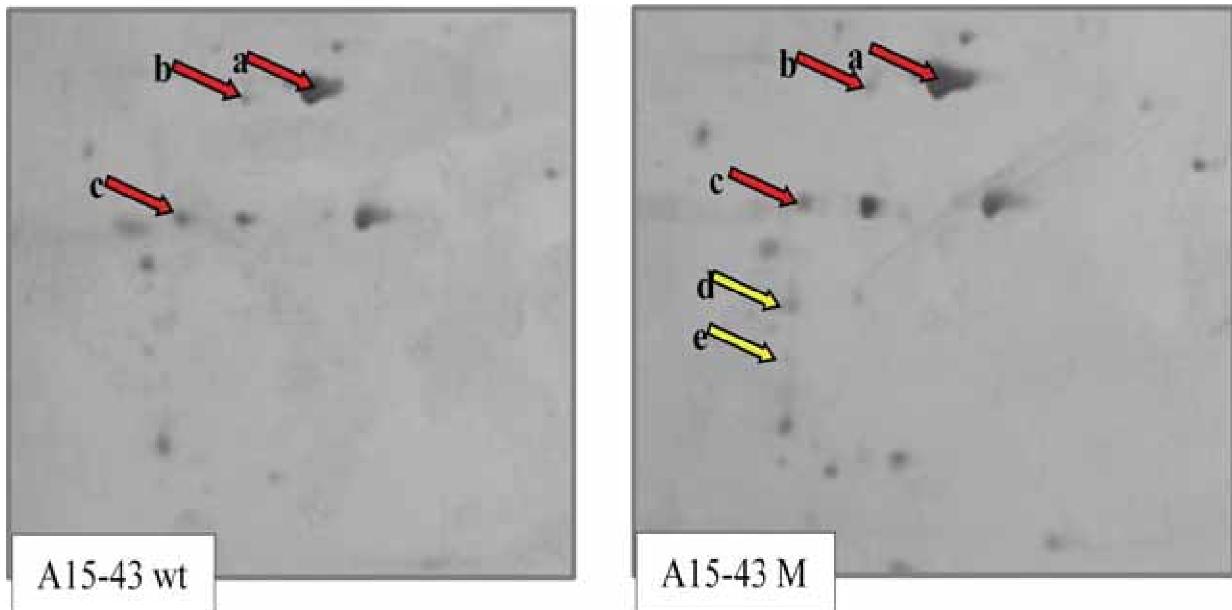

Fig 1: 2D gel electrophoresis comparing a clinafloxacin susceptible wild type strain and the isogenic resistant mutant.

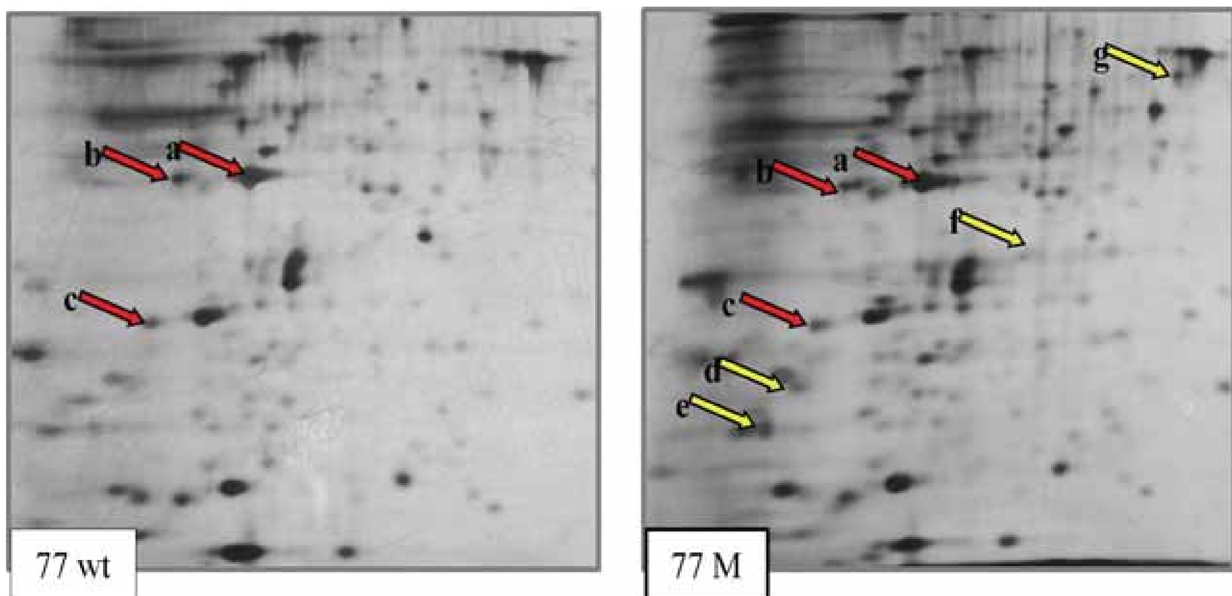

Fig 2: 2D gel electrophoresis comparing a ciprofloxacin susceptible wild type strain and the isogenic resistant mutant.

D. BIOFILM FORMATION

PAPER 11: Biofilm formation in *Acinetobacter baumannii*: associated features and clinical implications.

ADDITIONAL RESULTS III:

Effect of the acquisition of quinolone resistance in biofilm formation by *Acinetobacter baumannii* clinical isolates.

(Results presented as a poster in the 16th European Congress of Clinical Microbiology and Infectious Diseases, Nice, France, 2006 and in an oral communication in the RECAM 2006, Barcelona, Spain).

➢ PAPER 11:

Biofilm formation in *Acinetobacter baumannii*: associated features and clinical implications.

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RESEARCH NOTE

Biofilm formation in *Acinetobacter baumannii*: associated features and clinical implications

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ABSTRACT

Biofilm formation in 92 unrelated strains of *Acinetobacter baumannii* isolated in a multicentre cohort study was investigated using a microtitre plate assay. Fifty-six (63%) isolates formed biofilm. These isolates were less frequently resistant to imipenem or ciprofloxacin than were non-biofilm-forming isolates (25% vs. 47%, p 0.04; and 66% vs. 94%, p 0.004, respectively). All catheter-related urinary or bloodstream infections and the sole case of shunt-related meningitis were caused by biofilm-forming strains. Multivariate analysis revealed that treatment in an intensive care unit, ciprofloxacin resistance and isolation from a respiratory sample were associated with non-biofilm-forming isolates, while previous aminoglycoside use was associated with biofilm-forming isolates.

Keywords *Acinetobacter baumannii*, biofilm formation, ciprofloxacin resistance, imipenem resistance, infections, risk-factors

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Acinetobacter baumannii is a significant worldwide nosocomial pathogen with a particular ability to develop antimicrobial resistance and cause nosocomial outbreaks of infection [1]. This organism frequently causes infections associated with medical devices, e.g., vascular catheters, cerebrospinal fluid shunts or Foley catheters [1–3]. Biofilm formation is a well-known pathogenic mechanism in such infections [4]. In addition, the environmental survival of some microorganisms may be facilitated by biofilm formation on abiotic surfaces. Little is known concerning biofilm formation in *A. baumannii* [5–8]. Therefore, the present study investigated the frequency of biofilm formation and the associated clinical correlations and variables for 92 clonally unrelated isolates selected from among 221 isolates of *A. baumannii* collected during the GEIH-Ab 2000 project [2], which was a multicentre prospective cohort study performed in 28 Spanish hospitals. The methods and general clinical, epidemiological and microbiological results of this study have been reported in detail elsewhere [2,9,10]. For the purpose of the present analysis, if an isolate included in this study was clonally related to at least one other isolate from the original collection, it was considered to be epidemic [2]. The study was approved by the local ethics committees of the participating hospitals.

Biofilm formation was determined in the Hospital Clinic, Barcelona, Spain, using an overnight culture, diluted 1:100 in fresh Luria–Bertoni broth in 96-well plates and incubated without shaking at 37°C for 48 h. Of the 96 wells, four were left uninoculated and used as negative controls. Biofilm was stained with crystal violet 1% w/v and quantified at 570 nm after solubilisation with ethanol–acetone. The experiment was performed in duplicate in two 96-well plates. Isolates were classified as biofilm-forming if they yielded OD_{570} values that were at least twice those of the negative controls. When an isolate was clearly positive for biofilm formation in the assay and the duplicate assay was borderline, the isolate was considered to be biofilm-positive. When an isolate was clearly positive in the first assay and the duplicate assay was clearly negative, the isolate was considered to be non-evaluable and was excluded. Susceptibility to antimicrobial agents was determined by microdilution according to CLSI recommendations [11].

The epidemiological and clinical features of patients colonised or infected with biofilm-

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forming and non-biofilm-forming *A. baumannii* isolates were compared. Continuous variables were compared using the Mann–Whitney *U*-test and categorical variables were compared using the chi-square test (Fisher's exact test, if required). Multivariate analysis was performed by logistic regression analysis. Statistical analyses were performed using SPSS v.12.0 (SPSS Inc., Chicago, IL, USA).

Of the 92 isolates studied, 56 (63%) formed biofilm *in vitro*, 33 (36%) did not form biofilm, and three (3%) were non-evaluable. Thus, 89 isolates were used in the final analysis. Although one representative isolate of each pulsed-field gel electrophoresis type was initially analysed, the results for biofilm formation always agreed with the representative isolate when other isolates belonging to the same pulsed-field gel electrophoresis type from the original collection ('epidemic strains') were tested. Biofilm-forming isolates were less frequently imipenem-resistant (25% vs. 47%, p 0.04), ciprofloxacin-resistant (66% vs. 94%, p 0.004) and epidemic (31% vs. 53%, p 0.04) than were non-biofilm-forming isolates. No significant differences in susceptibility to doxycycline (65% vs. 60%), ceftazidime (73% vs. 83%), sulbactam (39% vs. 27%), gentamicin (80% vs. 77%), tobramycin (76% vs. 73%) or rifampicin (0 vs. 3%) were observed (p >0.1).

Complete epidemiological and clinical data were available for 78 patients and were included in the analysis of factors associated with biofilm formation. Univariate analyses are shown in Table 1. ORs (95% CI) for the variables selected in multivariate analysis were: treatment in an intensive care unit, 0.1 (0.004–0.8); respiratory tract sample, 0.2 (0.005–0.4); ciprofloxacin resistance, 0.06 (0.009–0.4); and previous receipt of aminoglycosides, 13.1 (2.3–74.9). When CDC criteria were used [12], the frequencies of infection caused by biofilm-forming and non-biofilm-forming isolates were similar (20/49 (41%) vs. 13/29 (45%), p 0.1). Types of infections are shown in Table 2. Infections caused by non-biofilm-forming isolates showed a non-significant trend toward the presence of sepsis and a higher mortality rate when compared with infections caused by biofilm-forming isolates (92% vs. 70%, p 0.1, and 23% vs. 14%, p 0.6, respectively).

There is very limited information concerning the ability of *A. baumannii* to form biofilm [5–7]. In a collection of clinical isolates of *A. baumannii*, Sechi *et al.* [8] found that 16 (80%) of 20 isolates formed biofilm, perhaps because of a dominant *A. baumannii* clone. In the present study, 63% of 92 clonally unrelated *A. baumannii* clinical isolates formed biofilm. Interestingly, all clonally related isolates shared either an ability or an inability to form a biofilm, which suggests that this is a clone-specific feature and that its expression does not vary substantially under different conditions; however, further studies are needed to investigate this hypothesis.

Table 1. Univariate analysis of factors associated with biofilm-forming isolates of *Acinetobacter baumannii* (data expressed as a percentage of cases unless otherwise specified)

	Biofilm-forming (n = 49)	Non-biofilm-forming (n = 29)	OR (95% CI)	p value[a]
Mean age, years (SD)[b]	55 (21)	62 (14)	–	0.08[c]
Male gender	72	78	0.7 (0.2–2.0)	0.5
Underlying disease				
Non-fatal	74	62	–	0.5
Ultimately fatal	24	32		
Rapidly fatal	4	6		
Diabetes mellitus	10	22	0.4 (0.1–1.4)	0.1
Neoplasia	17	28	0.5 (0.1–1.5)	0.2
Chronic pulmonary disease	15	28	0.5 (0.1–1.5)	0.2
ICU treatment	26	53	0.3 (0.1–0.7)	0.01
Mean days of hospital stay (SD)	29 (37)	22 (25)	–	0.3[c]
Central venous catheter	58	61	0.8 (0.3–2.2)	0.7
Mechanical ventilation	44	52	0.7 (0.2–1.8)	0.4
Urinary catheter	77	77	0.9 (0.3–2.8)	0.9
Previous antimicrobial agents	86	84	1.3 (0.3–4.8)	0.7
Aminoglycosides	43	20	3.0 (0.9–10.3)	0.06
Fluoroquinolones	21	10	2.4 (0.5–12.3)	0.2
Cephalosporins	46	27	2.3 (0.8–6.2)	0.09
Carbapenems	13	17	0.7 (0.1–3.2)	0.1
Type of sample				
Respiratory tract	25	53	0.3 (0.1–0.8)	0.01
Blood	10	0	–	0.07[d]
Urine	32	14	3.0 (0.9–10.1)	0.06
Wound	27	27	0.9 (0.3–3.0)	0.8
Others	6	6	0.9 (0.1–8.1)	0.8[d]

ICU, intensive care unit; SD, standard deviation.
[a]Chi-square test except where specified.
[b]There were only three paediatric patients, all of whom yielded a biofilm-forming isolate.
[c]Mann–Whitney *U*-test.
[d]Fisher's exact test.

Table 2. Types of infections caused by biofilm-forming and non-biofilm-forming isolates of *Acinetobacter baumannii* (data expressed as absolute numbers of infections)

	Biofilm-forming (n = 20)	Non-biofilm forming (n = 13)
IV catheter-related infection	3	0
Foley-related UTI	6	0
CSF shunt infection	1	0
VA respiratory tract infection	5	8
Non-VA respiratory tract infection	1	0
Skin and soft-tissue infection	4	5

IV, intravascular; UTI, urinary tract infection; CSF, cerebrospinal fluid; VA, ventilator-associated.

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Although limited by the low number of cases, the present results suggest that biofilm plays a role in the pathogenesis of some device-associated *A. baumannii* infections (e.g., those involving Foley catheters, venous catheters and cerebrospinal fluid shunts); in contrast, ventilator-associated pneumonia was not caused predominantly by biofilm-forming isolates. These results suggest the hypothesis that infections caused by biofilm-forming isolates might be associated with a diminished frequency of systemic response or mortality; however, this association was not statistically significant and further studies would be necessary to investigate this possibility.

Biofilm-forming isolates were less frequently resistant to imipenem and ciprofloxacin, and seemed to be less epidemic. A possible explanation is that biofilm-forming isolates are not as dependent as their non-biofilm-forming counterparts on antimicrobial resistance and epidemic characteristics to survive in the hospital environment. Sechi *et al.* [8] have previously reported no relationship between biofilm formation and the production of PER-1 β-lactamase. However, patients who had previously received aminoglycosides were at an increased risk of being colonised or infected by biofilm-forming *A. baumannii*. Previous aminoglycoside use may exert a different selection pressure on biofilm formation, irrespective of the in-vitro susceptibility.

In summary, >60% of unrelated *A. baumannii* isolates from clinical samples formed biofilm, and these isolates were associated mainly with device-associated infections. These isolates were less frequently resistant to imipenem and ciprofloxacin.

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➢ ADDITIONAL RESULTS III:

Effect of the acquisition of quinolone resistance in biofilm formation by *Acinetobacter baumannii* clinical isolates.

The ability of *Acinetobacter baumannii* to resist desiccation and disinfection is a well known quality which could be explained by its capacity to form biofilm on solid surfaces. Biofilm formation could be implicated in the long survival of this microorganism in hospitals and it could be responsible for the endemic occurrence and posterior epidemic outbreaks of *A. baumannii* in certain hospitals. The main objective of this study was to analyse the potential relationship between the acquisition of quinolone resistance and biofilm formation in *A. baumannii* clinical isolates.

***A. baumannii* quinolone-resistant mutants were prepared by serial passages in media with increasing antibiotic concentrations. Biofilm analysis confirmed that the quinolone resistant mutants had reduced their ability to form biofilm. Comparative 2D gel electrophoresis between the wild-type and mutant strains showed a clear difference in expression of CsuA/B. This protein, which is involved in type 1 pili formation, was expressed in the wild-type strains but it disappeared in their isogenic mutants.**

These initial results suggest that quinolone resistant *A. baumannii* strains are less prone to produce biofilm than their susceptible counterparts. This association is linked to a decreased expression of type 1 fimbrae, the first step in biofilm formation.

Acinetobacter baumannii is an important human pathogen that is capable of surviving on nutrient-limited surfaces during long periods of time. This multiresistant opportunistic pathogen can survive for several days in the hospital environment, even in dry conditions on dust particles. The ability of *Acinetobacter baumannii* to resist desiccation and disinfection is a well known quality which could be explained by its capacity to form biofilm on solid surfaces (214). Biofilm is an association of microbial cells which are surrounded by a matrix of polysaccharide material; this structure is an optimal environment for genetic material exchange between the different microorganisms. Biofilm formation by nosocomial pathogens is associated with certain infectious diseases and it has an important role in a variety of device-related infections (49); it could also be responsible for the endemic occurrence and posterior epidemic outbreaks of *A. baumannii* in certain hospitals. In the previously presented work on biofilm formation by *A. baumannii* (Paper 10), we concluded that biofilm-forming isolates were less frequently resistant to imipenem and ciprofloxacin (184). With these results, the next step was to determine the differences in protein expression between quinolone susceptible and resistant clinical isolates. Therefore, the main objective of this study was to analyse the potential relationship between the acquisition of quinolone resistance and biofilm formation in *A. baumannii* clinical isolates.

Two *A. baumannii* clinical isolates and their respective quinolone-resistant mutants were used in this study (A15-43 and 77). These clinical isolates had been submitted to increasing *in vitro* concentrations of clinafloxacin and ciprofloxacin, respectively (174). Strains A15-43 wt and A15-43M had a MIC of clinafloxacin of 3 mg/L and >32 mg/L; strains 77 wt and 77M had a MIC of ciprofloxacin of 1.5 mg/L and >32 mg/L. There were no changes in *gyr*A and *par*C genes between the wild type strains and their isogenic mutants.

Biofilm formation was studied as described in paper 10 (184). Both sets of mutants showed the same pattern for biofilm formation (Table 1); the susceptible isolates were able to form biofilm while the quinolone-resistant mutants had this ability reduced (Fig 1). These results corroborated the initial hypothesis that acquisition of quinolone resistance is associated with a reduction in the ability to form biofilm.

A proteomic analysis of these isolates was performed to identify the mechanism involved in the reduced capability to form biofilm observed in the quinolone resistant mutants. A protein fraction enriched in cell envelope proteins was prepared as described in paper 9 (131) and analysed by two-dimensional gel electrophoresis. Over-expressed proteins were characterised using trypsin digestion and mass spectrometry analysis (MALDI TOF-TOF or nanoESI-MS/MS) (131). The proteomic analysis was done at least three times with different protein extractions. Comparative analysis between the wild type and mutant strains showed differences in the expression of several proteins. One of them had a high homology with CsuA/B, a protein involved in type 1 pili formation, and was only expressed in the susceptible wild type strains (Fig 2). The *csuA/B* gene forms part of an operon which includes the genes *csuA* to *csuE* and encodes for the type 1 pili subunit CsuA/B protein (214). Scanning Electron Microscopy (SEM) analyses were performed as described by Tomaras *et al.* (214), to determine the differences between susceptible and resistant isolates (Fig 3, 4, & 5). Biofilm formation in *A. baumannii* is characterized by the formation of solid aggregates at the liquid-air interface and growing upwards onto the walls of the tube (214). For this reason, the electron microscopy was performed at the three phases: above, at and below the liquid-air interface. As expected, bacteria within the liquid media were either in their planktonic state or forming small aggregates; moreover, most of the attached cells were found at the liquid-air interface or above. No clear differences were observed between the *A. baumannii* strain 77 and its isogenic mutant (Fig 3 & 5). Both

strains seem to aggregate and form biofilm at the liquid-air interface; however, above this surface, the susceptible strain seems to form a thicker biofilm than the resistant strain. On the other hand, there are clear differences in the biofilm formation of the strain A15-43 wt and its isogenic mutant (Fig 4 & 5). Above the liquid surface, the susceptible strain forms a thick biofilm structure, while the clinafloxacin resistant mutant is found in a planktonic state of growth. In Fig 5.C, the susceptible bacteria are attached together with filaments that resemble pili structures, while the resistant strain is missing these structures (Fig 5.D).

These experiments suggest a relationship between the acquisition of quinolone resistance and the inability to form a compact biofilm structure. Nevertheless, in order to demonstrate this relationship, it will be necessary to study the expression of all the genes that form part of the Csu operon, and to complement the resistant strain with the CsuA/B gene to see if this strain recovers the ability to form biofilm.

Quinolone resistant *A. baumannii* isolates are less prone to produce biofilm than their susceptible counterparts. This association is linked to a decreased expression of type 1 fimbrae, the first step in biofilm formation. Therefore, the results obtained suggest that there is a relationship between the biofilm formation and the resistance to quinolones.

Table 1: Relationship between biofilm formation and quinolone resistance in *A. baumannii* isogenic mutants.

STRAINS	OD_{570}		BIOFILM FORMATION	
A15-43 wt	0.132	0.156	+	+
A15-43 M	0.096	0.081	-	-
77 wt	0.193	0.204	+	+
77 M	0.077	0.101	-	-
Negative Control	0.052	0.053	-	-

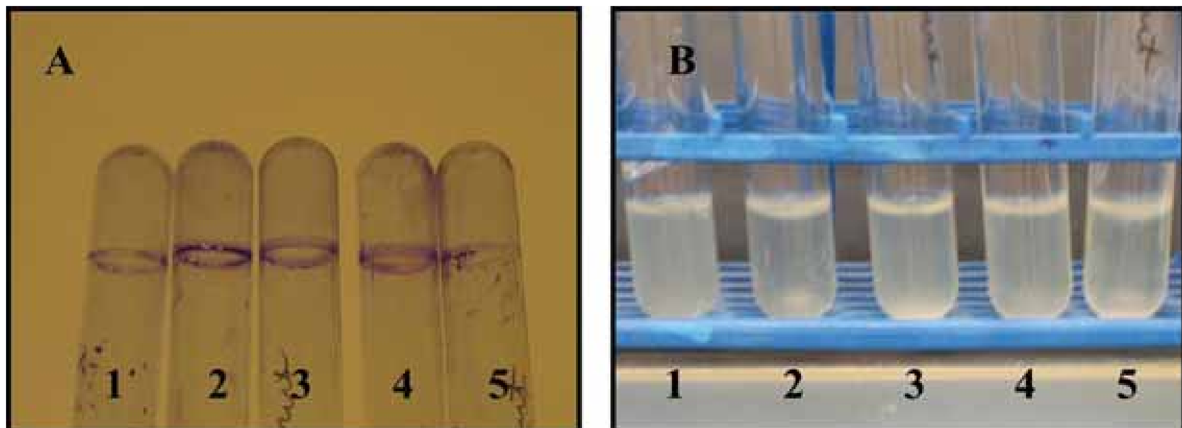

Fig 1: Detection of biofilm formed in polystyrene tubes after overnight incubation at 37 ºC without shaking. A) Crystal violet staining. B) Growth on LB broth before staining. Strains: 1] *A. baumannii* ATCC 19606; 2] 77 wt; 3] 77 M; 4] A15-43 wt; 5] A15-43 M

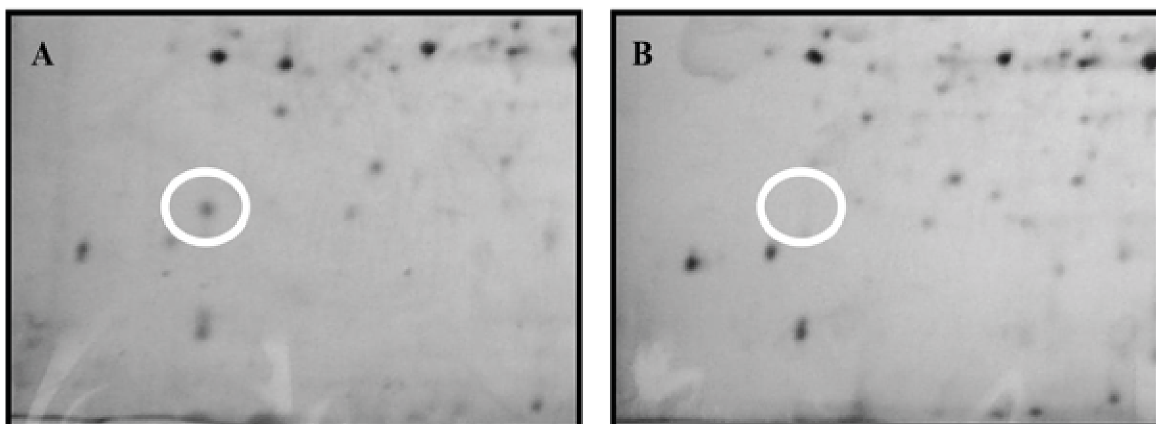

Fig 2: Two-dimensional gel electrophoresis of the strain (A) 77 wt and its quinolone resistant isogenic mutant (B) 77 M. The outlined protein is CsuA/B which appears only in the wild type strain. Strain A15-43 had an equal behaviour (Data not shown).

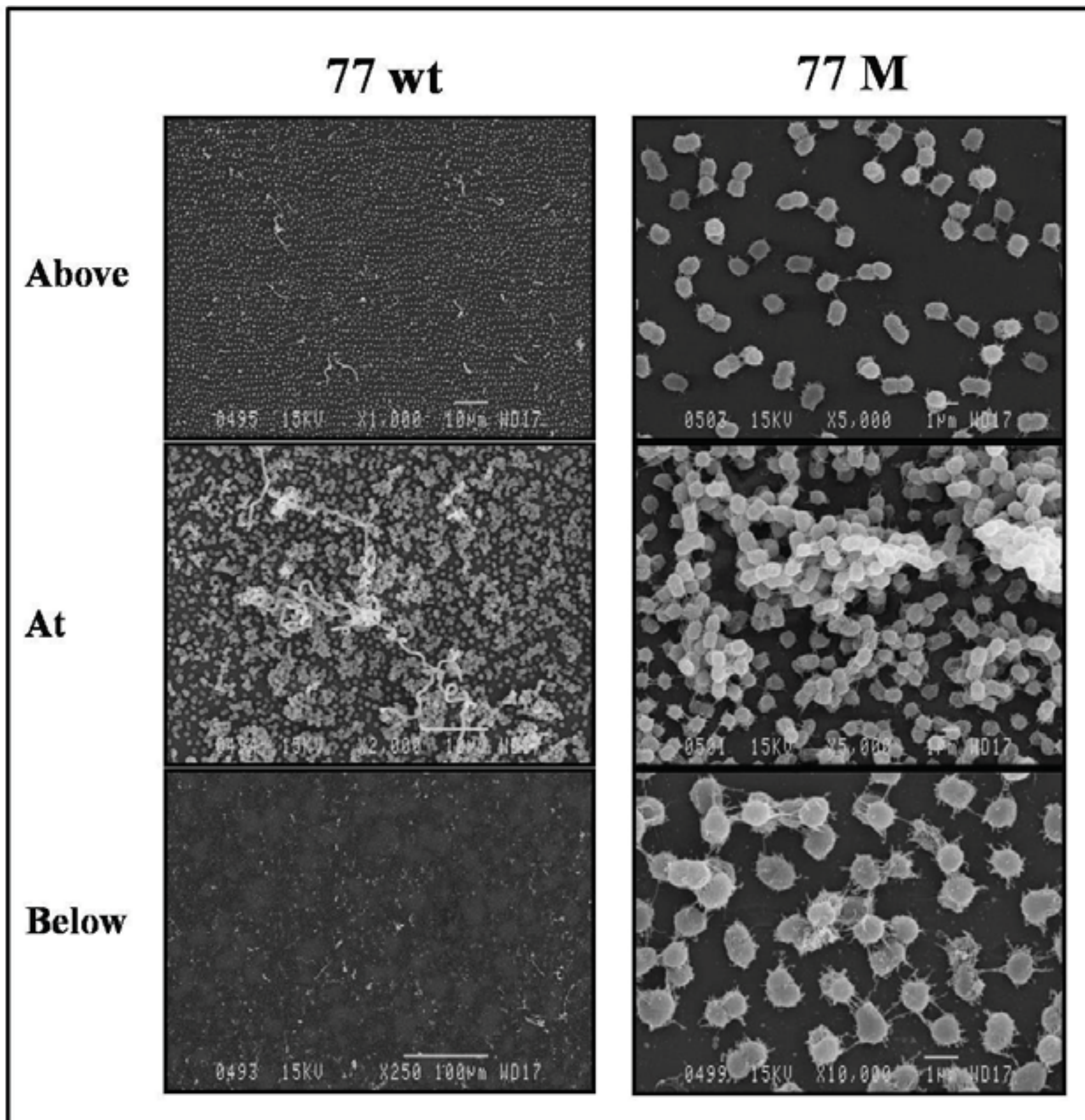

Fig 3: Scanning Electron Microscopy of *A. baumannii* strain 77 and the ciprofloxacin resistant isogenic mutant. Biofilm formation above, at and below the liquid-air interface. (Electron Microscopy performed by Luis A Actis)

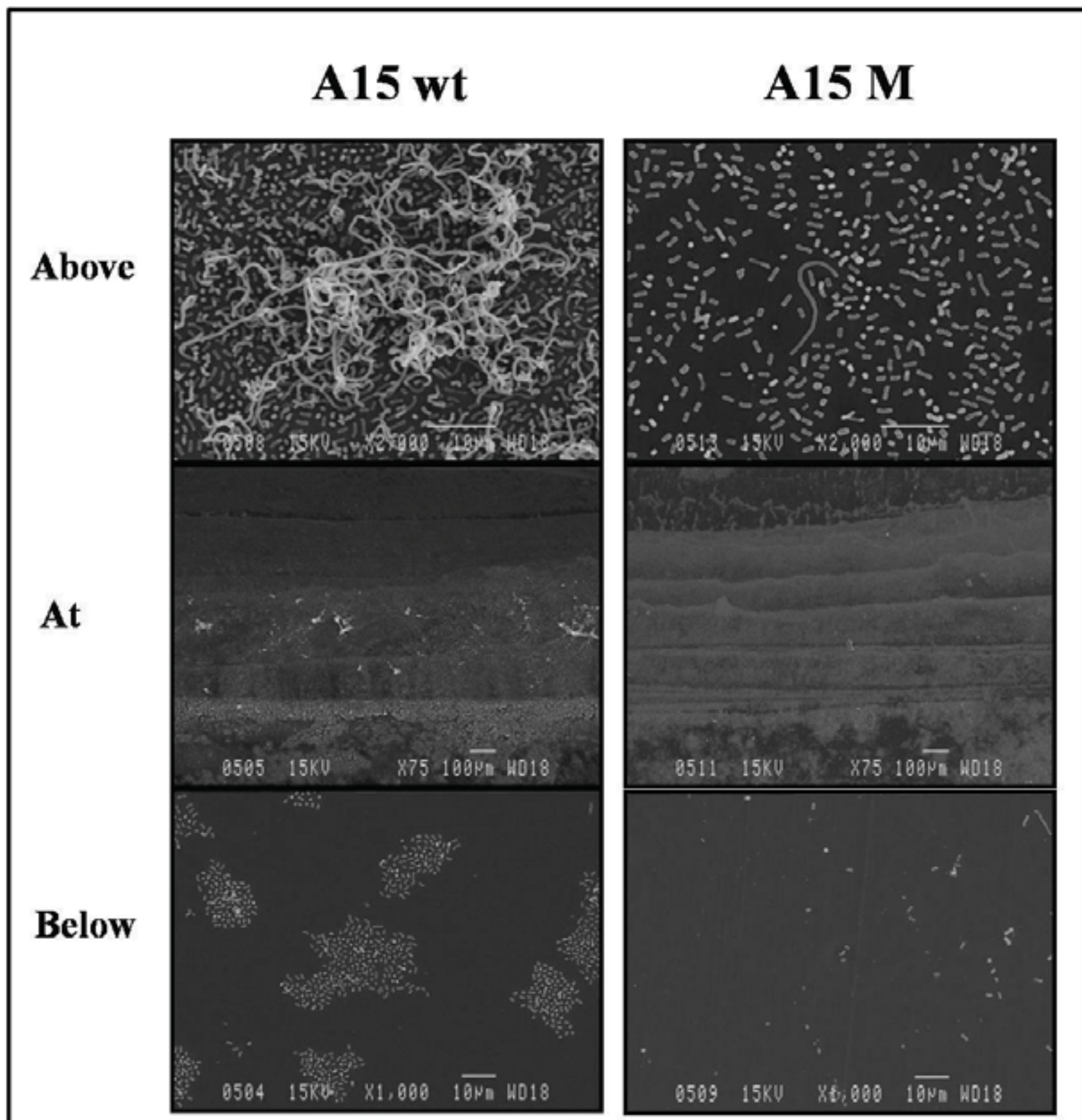

Fig 4: Scanning Electron Microscopy of *A. baumannii* strain A15-43 and the clinafloxacin resistant isogenic mutant. Biofilm formation above, at and below the liquid-air interface. (Electron Microscopy performed by Luis A Actis)

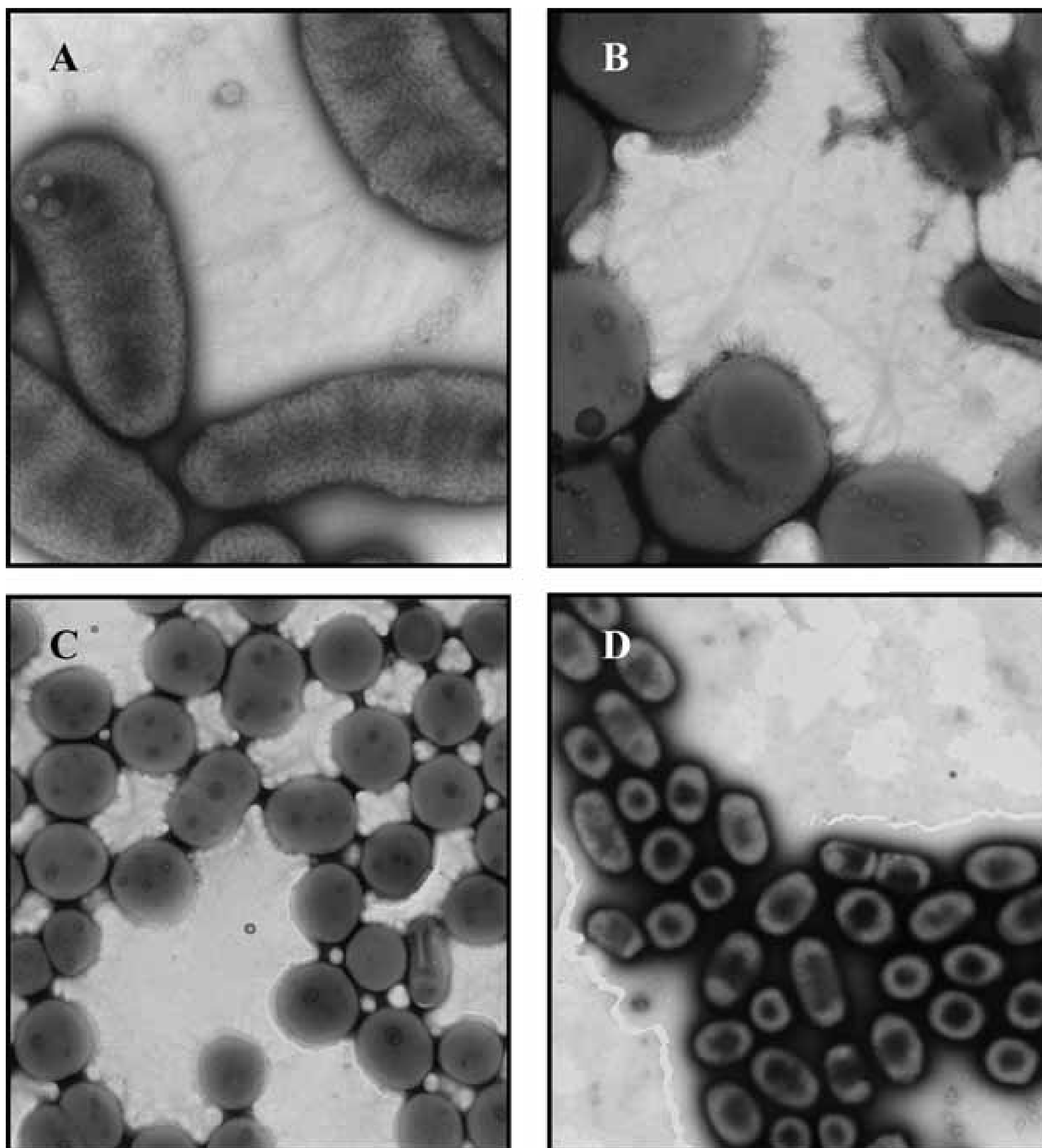

Fig 5: Scanning Electron Microscopy of A) 77 wt; B) 77 M; C) A15-43 wt; D) A15-43 M. (Electron Microscopy performed by Luis A Actis)

E. MECHANISMS OF RESISTANCE TO COLISTIN

ADDITIONAL RESULTS IV:

LPS modification and changes in membrane proteins are associated to increased colistin resistance in *Acinetobacter baumannii*, *Acinetobacter* Genospecies 3 & *Acinetobacter* Genospecies 13 clinical isolates.

(Results presented as a slide presentation in the 48th annual Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, USA, 2008).

➢ ADDITIONAL RESULTS IV:

LPS modification and changes in membrane proteins are associated to increased colistin resistance in *Acinetobacter baumannii*, *Acinetobacter* Genospecies 3 & *Acinetobacter* Genospecies 13 clinical isolates.

Acinetobacter baumannii **is a multiresistant pathogen causing infections mainly in patients in ICUs. Colistin remains as the last solution to treat infections caused by these MDR strains. The objective of this work was to determine the mechanisms of resistance to colistin in clinically relevant members of the genus** ***Acinetobacter*****.**

Clinical isolates of the ABC complex were subjected to increased colistin concentrations; the mutant strains were stable to passages in non-selective media. Changes in protein expression were analysed by SDS-PAGE electrophoresis of cell envelope protein extracts. LPS were obtained after proteinase K digestion of whole cells and were separated by SDS-PAGE.

Cell envelope analysis showed a difference in expression in two proteins; OmpW was expressed in susceptible strains while a signal peptide was expressed in the resistant isolates. LPS profile in SDS-PAGE from the different strains was similar in the susceptible and resistant strains. However, the LPS amount stained on the gel was largely increased in the colistin resistant ***A. baumannii*** **strains.**

The loss of the Omp W, together with an increment in LPS production, may play an important role in membrane permeability in the members of the genus ***Acinetobacter*****; this decreased permeability could be responsible for the resistance to colistin.**

Acinetobacter baumannii is an opportunistic pathogen causing infections mainly in patients in intensive care units, where the extensive use of antimicrobial agents can select for the emergence of multidrug resistant strains. In fact, clinical isolates resistant to all commercialized antimicrobial agents have been reported (228). The emergence of MDR bacteria has forced the reassessment of polymyxins as therapeutic agents. The polymyxins are bactericidal agents with a detergent-like mechanism of action over the bacterial cell membrane. The only clinically valuable members of this antimicrobial group are polymyxin B and especially polymyxin E (colistin), two structurally similar compounds which differ in a single aminoacid. Colistin became available for clinical use in the 1960s but shortly after, it was replaced by other antimicrobial agents which were considered less toxic. Despite its toxicity, colistin remains as a last resort antimicrobial therapy to treat infections caused by these multiresistant pathogens (120,121,242). Unfortunately, the increment in the use of polymyxins has resulted in the emergence of colistin resistant pathogens; among them, polymyxin resistance has been described in 5 % to 28 % of *A. baumannii* clinical isolates, including multiresistant strains, from Brazil, the USA and South Korea (115). The objective of this work was to determine the mechanisms of resistance to colistin in clinically relevant members of the genus *Acinetobacter*.

Acinetobacter baumannii (77778), *Acinetobacter* Genospecies 3 (Ac057) and *Acinetobacter* Genospecies 13TU (152569) clinical isolates, together with the *A. baumannii* type strain ATCC 19606, were subjected to increasing colistin concentrations. *Acinetobacter* Genospecies 3 and 13TU had an initial MIC < 1 mg/L and rapidly acquired resistance to colistin. By contrast, *A. baumannii* clinical isolates were very difficult to mutate; therefore, the initial studies were performed with the type strain ATCC 19606. Fortunately, we were able to obtain a clinical isolate from Asturias (Hospital Central de Asturias) which had a reduced susceptibility to colistin. This isolate was recovered from a

central catheter of a 73-year-old female and had a MIC of colistin of 2 mg/L. The production of colistin mutants with this strain was straightforward and it followed the same pattern already observed with the other *Acinetobacter* spp. The final mutant strains were able to grow in media with 256 mg/L of colistin, with exception of the *Acinetobacter* Genospecies 3 strain Ac057 which grew in media with 32 mg/L of colistin. Fifteen consecutive passages in non-selective media were performed to prove the stability of this resistant phenotype. In addition, REP-PCR analyses were performed with all the series of mutants to confirm that resistance was due to a structural modification and not to a contamination with another pathogen intrinsically resistant to colistin. The REP-PCR was performed with a combination of the forward primer of the ERIC-PCR (ERIC-1: 5' ATG TAA GCT CCT GGG GAT TCA C 3') and the forward primer of the REP-PCR (REP-1: 5' III GCG CCG ICA TCA GGC 3'). PCR conditions were 40 cycles of 94 ºC for 1 minute, 40 ºC for 1 minute and 65 ºC for 7 minutes.

During the mutant production, there was an inflection point were the isolates showed a change in their colony morphology; the concentration of colistin required for arriving to this morphological change was different for the four strains used (Table 1 & 2). Nevertheless, this modification produced similar results in all the tested strains. The strains recovered after the initial passes on plates with colistin were tolerant to colistin; they grew on increasing concentrations of this antibiotic but, their initial MIC was not modified or it suffered minimal variations. At a given point, two different morphologies were observed which corresponded to a colistin susceptible and a colistin resistant strain (Fig 1). After this inflection point, the strains were not able to grow on selective MacConkey media and the MIC for the resistant strain went up to $\geq$ 128 mg/L.

A proteomic analysis of these isolates was performed to identify the mechanism involved in the acquisition of colistin resistance. SDS-PAGE electrophoresis with cell

envelope protein extracts was performed as described in paper 9 (131) to study changes in protein expression between the wild type and the mutant strains. Characterisation of over-expressed proteins was done by trypsin digestion and mass spectrometry analysis (MALDI TOF-TOF or nanoESI-MS/MS) (131). Cell envelope analysis showed that expression of two proteins was altered between susceptible and resistant strains; these modifications were observed in the four sets of mutants tested (Fig 2). The expression of a 22 KDa protein present in the susceptible strains was downregulated in the resistant mutant strains: this protein was the outer membrane protein W (OmpW). In addition, differences in a 10 KDa protein were also observed; in this case, the protein was only present in the resistant mutants and was characterized as a signal peptide. When the OmpW was expressed, the signal peptide was not expressed; the point of change between the expression of the OmpW and the signal peptide corresponds to the same inflection point where the mutant strains become resistant to colistin and stop growing on selective MacConkey media.

In other clinical pathogens, colistin resistance has been associated to lipid A modifications, as well as to changes in protein expression. Entrance of colistin inside the bacterial cell is due to an interaction between the positively charged antimicrobial agent and the negatively charged lipopolysaccharides (242). LPS analysis was performed on the susceptible and resistant strains after growing them in TSB (20 mM Tris-acetate [pH 7.5], 0.2 M potassium glutamate, 2 mM magnesium chloride, 20% glycerol, 0.05 mM phenylmethylsulfonyl fluoride) at 37 ºC. In addition, for screening purposes, LPS were obtained after proteinase K digestion of whole cells as described by Darveau *et al.* (39); afterwards, LPS were separated by SDS-PAGE and visualised by silver staining (Fig 3). As it can be observed in Fig 3, the LPS profile in SDS-PAGE between the susceptible and resistant strains is similar; however, the LPS amount stained on the gel is largely increased in the resistant strains. It is important to point out that the real amount of cells used for LPS

isolation by the Darveau and Hancock method (39) is similar in all the cases (approximately 10^6 viable cells).

In *Acinetobacter* Genospecies 13TU, the LPS analysis has been done on the wild type strain and four of the mutant strains recovered from plates with colistin at 2, 16, 32 and 512 mg/L. For this strain, the inflection point for all the morphological changes was 16 mg/L. All the mutants growing in the plates with ≤ 16 mg/L had a MIC of colistin of ≤ 1 mg/L, were able to grow on selective MacConkey media, had the same amount of LPS and the OmpW was expressed in all of them. On the other hand, the mutants growing above 16 mg/L had a MIC of colistin ≥ 128 mg/L, did not grow on selective MacConkey media, had a down-regulated OmpW expression and finally, the amount of LPS stained in the gel was clearly superior to the susceptible wild type strain. The other *Acinetobacter* spp. show a similar behaviour.

Acinetobacter spp. resistant to colistin are sporadically isolated from hospitals worldwide, confirming the great ability of these microorganisms to acquire resistance to antimicrobial agents. Our work suggests that the mechanism of resistance to colistin involves protein and LPS modification. In addition, *in vitro* analysis suggests that *Acinetobacter* spp. have an initial tolerance to colistin before they become completely resistant. Unfortunately, several reports have already reported an increasing number of colistin heteroresistance and tolerance in the clinical environment (88,89,112,122); it is of special concern a report by Li *et al.* (122) demonstrating for first time heteroresistance in *A. baumannii* clinical isolates which were apparently susceptible to colistin on the basis of MICs (122). If the *Acinetobacter* spp. have the same behaviour *in vivo* than *in vitro*, this could be the previous step before becoming completely resistant to colistin.

Table 1: MIC of colistin and growth on MacConkey plates for all the *Acinetobacter baumannii* mutants. * Colistin in plate represents the colistin concentration used in the agar plate to select for colistin resistance.

STRAINS	COLISTIN IN PLATE* (mg/L)	MIC (mg/L)	GROWTH ON MacConkey
A. baumannii ATCC 19606	0	≤ 1	YES
	0.125	≤ 1	YES
	2 S	≤ 1	YES
	2 R	64	NO
	4	64	NO
	8	256	NO
	16	256	NO
	32	256	NO
	64	256	NO
	128	256	NO
	256	256	NO
A. baumannii 77778 clinical isolate	0	2	YES
	2	2	YES
	4	2	YES
	8	2	YES
	16	8	YES
	32	8	YES
	64	8	YES
	128 S	8	YES
	128 R	128	NO
	256	512	NO

Table 2: MIC of colistin and growth on MacConkey plates for the *Acinetobacter* Genospecies 3 and 13TU mutants. * Colistin in plate represents the colistin concentration used in the agar plate to select for colistin resistance.

STRAINS	COLISTIN IN PLATE* (mg/L)	MIC (mg/L)	GROWTH ON MacConkey
Acinetobacter Genospecie 3 Strain Ac057	0	≤ 1	YES
	0.250	≤ 1	YES
	0.500	≤ 1	YES
	1	≤ 1	YES
	2 S	≤ 1	YES
	2 R	8	NO
	4	32	NO
	8	32	NO
	16	32	NO
	32	64	NO
Acinetobacter Genospecie 13TU Strain 152569	0	≤ 1	YES
	0.250	≤ 1	YES
	0.500	≤ 1	YES
	1	≤ 1	YES
	2	≤ 1	YES
	4	≤ 1	YES
	8	≤ 1	YES
	16	≤ 1	YES
	32	128	NO
	64	256	NO
	128	512	NO
	256	512	NO

Fig 1: Morphological changes observed when the *Acinetobacter* Genospecies 13TU became resistant to colistin. 1) MH with 16 mg/L of colistin sulphate. 2) MacConkey media. Before getting resistant to colistin the strain grows in both plates. Afterwards, it does not grow on MacConkey media.

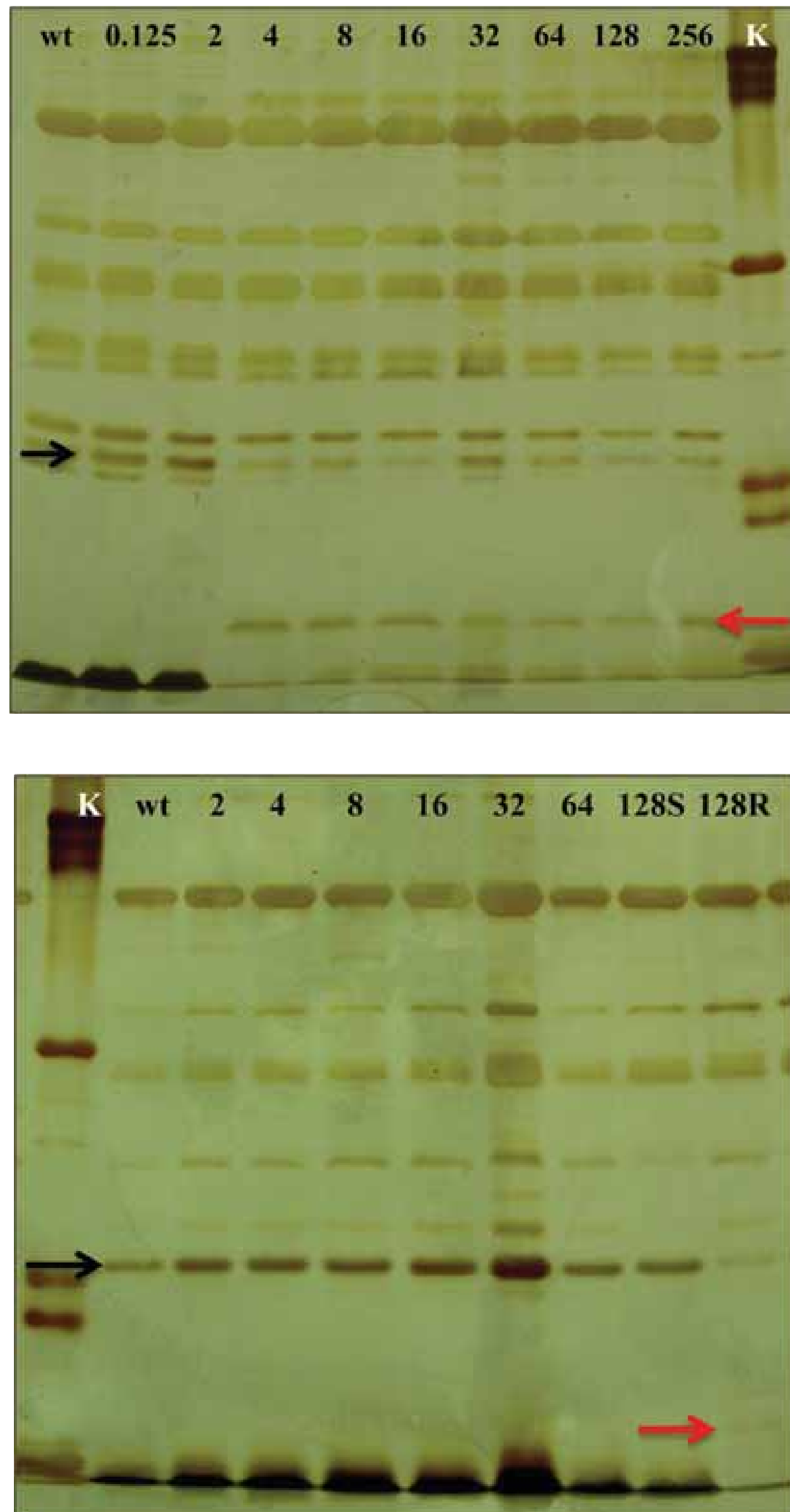

Fig 2: SDS-PAGE gel electrophoresis of a protein extraction of *A. baumannii* ATCC 19606 (above) and *A. baumannii* 77778 clinical isolate. Black arrows represent the OmpW protein and red arrows represent the signal peptide.

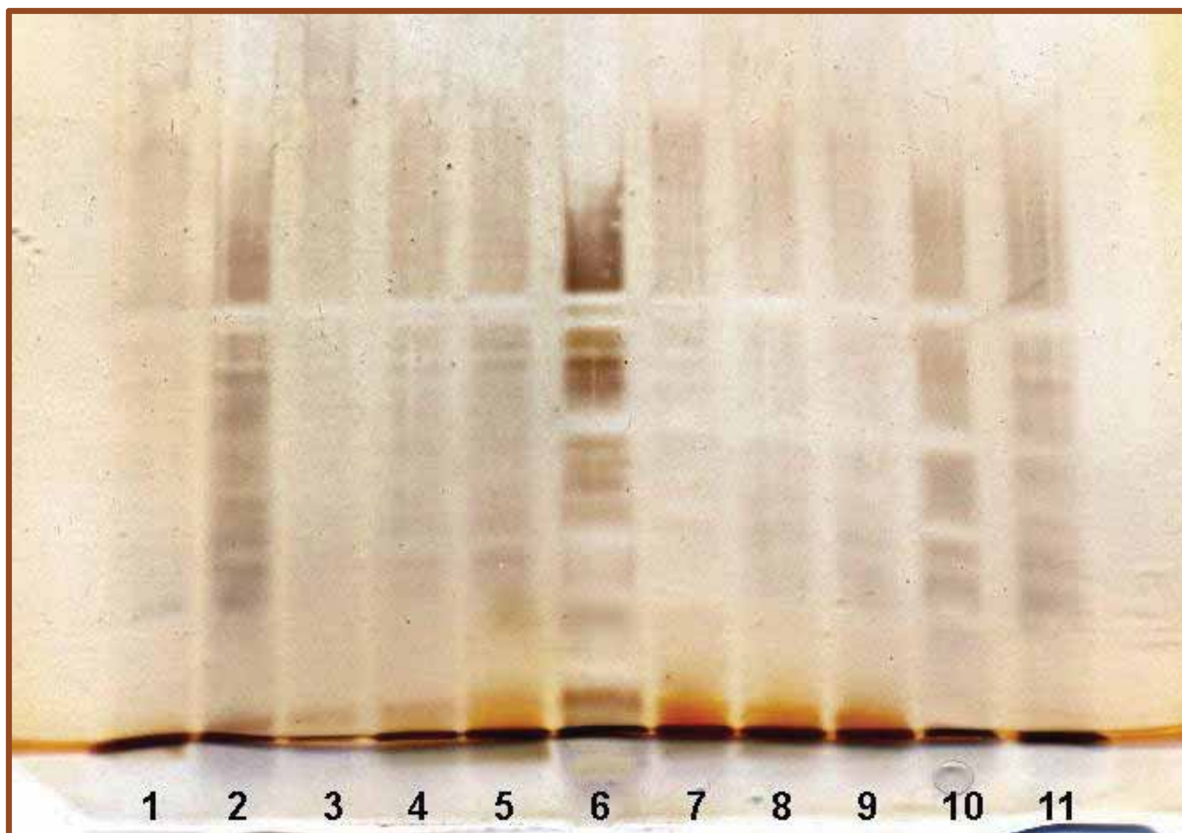

Fig 3: LPS profile in SDS-PAGE from the different colistin mutants obtained in this study. 1- Ac057 wt; 2- Ac057 (16); 3- Ac057 (2S); 4- Ac057 (2R); 5- Ab ATCC 19606 wt; 6- Ab ATCC 19606 (256); 7- 152569 wt; 8- 152569 (2); 9- 152569 (16); 10- 152569 (32); 11- 152569 (512)

V. DISCUSSION

- **Mechanisms of resistance to β-lactam antibiotics in *Acinetobacter* spp. clinical isolates due to the production of β-lactamase enzymes and clinical importance of non-baumannii *Acinetobacter* spp. (Papers 2, 4, 6, 7 & 8).**

Acinetobacter baumannii has emerged as an important nosocomial pathogen within hospitals worldwide. The Infectious Diseases Society of America reported this microorganism as one of the "red alert" pathogens which threat the efficacy of all the currently known antimicrobial agents (160,211). Before the 1970s, infections caused by members of the genus *Acinetobacter* could be treated with a wide range of antimicrobial agents, including aminoglycosides, tetracyclines and β-lactam antibiotics. Initially, they became resistant to penicillins and cephalosporins, while imipenem remained as the most active drug against these microorganisms. Unfortunately, during the last decade, carbapenem resistant *A. baumannii* isolates have been increasingly isolated from hospitals worldwide (11,160). Acquisition of resistance to cephalosporins and carbapenems means that colistin, and perhaps tigecycline, may be the only remaining therapeutic options for treating infections caused by multiresistant *A. baumannii* isolates. The most prevalent mechanism of resistance to β-lactam antibiotics in *A. baumannii* is the synthesis of chromosomal and plasmid-mediated β-lactamases (160).

The aim of these articles was to study the mechanisms of resistance to β-lactam antibiotics in *Acinetobacter* spp. due to the synthesis and overexpression of β-lactamase enzymes. The initial objective was to study the prevalence of β-lactamases in a collection of epidemiologically unrelated *A. baumannii* clinical isolates and to determine the role of the Insertion Sequence IS_{Aba1} in the expression of these β-lactamase enzymes **(Papers 2 & 4)**. Additionally, a second objective was to determine the mechanism of resistance to carbapenems in non-baumannii clinical isolates **(Papers 6 & 7)**.

In the **Paper 2**, a selection of 75 epidemiologically unrelated, ceftazidime resistant *A. baumannii* clinical isolates were analysed for the presence of the bla_{ADC}-like gene (*Acinetobacter*-derived cephalosporinase); a ceftazidime susceptible strain was also included in the analysis as a negative control. The bla_{ADC}-like gene was present in 74 out of the 76 tested strains (97.3 %); in addition, 40 of the 76 tested strains (54 %) also had the Insertion Sequence IS_{Aba1} located in the promoter region of the bla_{ADC}-like gene. Previous studies had established that the transcription of the gene encoding for the AmpC β-lactamase was dependent on the presence of an IS element inserted in the upstream region of this gene, which increased AmpC expression and resistance to extended-spectrum cephalosporins (34,198). In the study presented in paper 2, the prevalence of the IS_{Aba1} in this collection of epidemiologically unrelated clinical isolates was 69 % and it was found in the promoter region of the bla_{ADC}-like gene in 54 % of the cases. These data are in agreement with the results obtained by Corvec *et al*. (34), who found the IS element located in the upstream region of the bla_{ADC}-like gene in 52.4 % of the 42 tested strains (34). All these clinical isolates were resistant to ceftazidime due to the overexpression of the AmpC cephalosporinase, and had a MIC of ceftazidime ≥ 32 mg/L (MIC_{50} = 256 mg/L). By contrast, most of the isolates with the Insertion Sequence elsewhere than this promoter region or with no IS_{Aba1} at all, were susceptible or had an intermediate resistance to ceftazidime (MIC_{50} = 4 mg/L); only 7 out of 34 clinical isolates (20.6 %) were resistant, and this resistance would be due to additional mechanisms of β-lactam resistance other than the overexpression of the bla_{ADC}-like gene. The ceftazidime susceptible strain included in this study was also analysed; this clinical isolate was positive for the bla_{ADC}-like gene but it did not have the Insertion Sequence IS_{Aba1}. All these results suggest that the presence of the IS_{Aba1} in the promoter region of the bla_{ADC}-like gene confers resistance to ceftazidime; this mechanism of resistance to cephalosporins is commonly found in the

isolates from Spanish hospitals. In addition, analysis of two strains from the same pulse type, one positive for the IS element and the other negative, suggest that these genetic elements can be acquired or lost within the hospital environment.

Insertion Sequences contain strong promoters that play a role in the expression of the antibiotic resistance genes which are located downstream from the site of insertion of these mobile elements. In *A. baumannii*, IS elements have been associated with overexpression of other resistance genes such as the genes encoding for the OXA-23, OXA-58 and OXA-51 oxacillinases, members of the Ambler Class D β-lactamases which confer resistance to carbapenems (160). Although carbapenem resistance can be caused by impaired permeability due to decreased expression of porins or by modifications in penicillin-binding proteins, recent reports suggest that presence of carbapenem-hydrolysing β-lactamases play an important role in the acquisition of resistance to this group of antimicrobial agents (165). In the **Paper 4**, a selection of 83 epidemiologically unrelated, imipenem resistant *A. baumannii* clinical isolates were analysed for the presence and overexpression of the different types of carbapenemases. All the isolates carried a gene encoding a β-lactamase belonging to the OXA-51 group. In addition, the bla_{OXA-58} and bla_{OXA-40} genes were present in 19 % and 42 % of the clinical isolates, respectively; β-lactamases of the OXA-23 group, VIM and IMP metallo-β-lactamases were not detected. In this study, epidemiologically unrelated *A. baumannii* clinical isolates carrying the bla_{OXA-40} gene were more prevalent than isolates with the bla_{OXA-58} gene, suggesting the dissemination of a genetic element containing the bla_{OXA-40} gene may have taken place. These results corroborate the data previously presented by Da Silva *et al.* (37), who showed that a multiresistant epidemic clone of *A. baumannii* carrying the bla_{OXA-40} gene was disseminated widely in Portugal and Spain (37); indeed, almost half of the clinical isolates analysed were positive for the presence of this carbapenemase. On the other hand,

the *bla*$_{OXA-58}$ gene was only found in 6 of the 28 epidemiologically unrelated *A. baumannii* clinical isolates, suggesting that a genetic element carrying a *bla*$_{OXA-58}$ gene may be starting the dissemination process throughout Spain. The OXA-58 was first identified in France in 2003 and, at present, is found worldwide in *A. baumannii* isolates, as well as in *A. junii* isolates from Romania and Australia; this carbapenemase has recently been detected in *Acinetobacter* Genospecies 3 and phenon 6/ct13TU (Papers 6 & 7). The clinical isolates analysed in this paper were collected as part of a multicenter study from 25 Spanish hospitals in the year 2000. Da Silva reported the dissemination of the *bla*$_{OXA-40}$ gene in the Iberian Peninsula in the year 2004 from a collection of isolates obtained between 1998 and 2003 (37); probably the incidence of this oxacillinase in Spain is nowadays higher. Similarly, if the results from the collection obtained in the year 2000 suggest a dissemination of the *bla*$_{OXA-58}$ gene, the current detection of this gene should be higher. Indeed, a report by Coelho *et al.* (32), suggests that the *bla*$_{OXA-58}$ gene was introduced into the genus *Acinetobacter* at least ten years ago and nowadays, this oxacillinase is globally scattered among these microorganisms (32). None of the isolates analysed contained a copy of the *bla*$_{OXA-58}$ and *bla*$_{OXA-40}$ genes. The MIC of imipenem of the clinical isolates carrying the OXA-58 ranged between 16 and 64 mg/L (MIC_{50} = 16); by contrast, the isolates producing the OXA-40 had a MIC of imipenem ranging between 16 and 128 mg/L and a MIC_{50} = 128 mg/L. Although some additional mechanisms of resistance to carbapenems, such as the presence of efflux pumps, could be present on these isolates, these results indicate that the *bla*$_{OXA-40}$ gene would be responsible for a higher MIC of imipenem than the *bla*$_{OXA-58}$ gene. Similar results were reported by Heritier *et al.* (92) after cloning and expressing these genes in susceptible *A. baumannii* strains. They reported that the OXA-58 confers only weak levels of resistance to imipenem in contrast with the expression of the OXA-40 β-lactamase (92).

The *bla*$_{OXA-51}$ is a chromosomally encoded gene intrinsic of *A. baumannii* isolates, and as such, it was present in all the analysed strains. Nevertheless, the objective was to analyse the role of the Insertion Sequence IS$_{Aba1}$ in the expression of this gene. This IS element was present in 74.7 % of the isolates included in this experiment; the difference in percentage with respect to the results in Paper 2 is due to the inclusion of several isolates belonging to the same pulse type. On the other hand, only 11 isolates (17.7 %) had this Insertion Sequence located in the promoter region of the *bla*$_{OXA-51}$ gene. Expression analysis by RT-PCR was performed on five epidemiologically unrelated isolates, in which the IS$_{Aba1}$ was located in the promoter region of the *bla*$_{OXA-51}$ gene, and on one strain carrying this IS element elsewhere than this promoter region. The present study demonstrated over-expression of the *bla*$_{OXA-51}$ gene in all the isolates which had the Insertion Sequence IS$_{Aba1}$ in the promoter region of the gene. Although another mechanism of resistance to carbapenems cannot be ruled out, the difference in expression of the *bla*$_{OXA-51}$ gene observed in the isogenic susceptible and resistant isolates belonging to the same pulse type suggests that the insertion of the IS element in the promoter region of this gene may produce a slight increase in the MIC of imipenem. All the isolates tested in this study produced an OXA-51-like β-lactamase; however the clinical isolates that also produced an OXA-40-like oxacillinase had a higher imipenem MIC than the ones producing an OXA-58 enzyme.

Most of the studies to determine the mechanisms of resistance to antimicrobial agents are performed on *A. baumannii* isolates; indeed, this microorganism is the major representative of the genus *Acinetobacter.* Nevertheless, with the exception of *A. calcoaceticus* (Genospecies 1), the other members of the ABC complex are also involved in nosocomial infection, and other *Acinetobacter* spp. have occasionally been associated with infection. The objective of the next two papers was to determine the mechanism of

resistance to carbapenems in non-baumannii clinical isolates **(Papers 6 & 7)**. In these papers, the *bla*$_{OXA58}$ gene was detected for first time in an *Acinetobacter* Genospecies 3 clinical isolate **(Paper 6)** and in an *Acinetobacter* phenon 6/ct13TU **(Paper 7)**.

In November 2000, eight clinical isolates were gathered from the same hospital; one of them was initially mistaken for an *A. baumannii* strain due to the high levels of resistance to several antimicrobial agents. Identification by ARDRA determined that this isolate was an *Acinetobacter* Genospecies 3. An imipenem-resistant *A. baumannii* clinical isolate from the same hospital was selected to work in parallel. Both isolates had a MIC of imipenem >32 mg/L which suggested the presence of a carbapenem-hydrolyzing oxacillinase. The *bla*$_{OXA58}$ gene was detected by PCR methodology in both clinical isolates, and presented 100 % homology with the same gene from *A. baumannii*, suggesting that these resistance genes may be transferred from one species to another. Southern blot analysis with specific probes for the *bla*$_{OXA58}$ gene and the 23S rRNA gene determined that this β-lactamase was present in *Acinetobacter* Genospecies 3 within a 100 Kb plasmid. Analysis of the genetic surrounding determined that the *bla*$_{OXA58}$ gene in the Genospecies 3 was surrounded by two copies of the Insertion Sequence IS$_{Aba3}$; the copy downstream had the same direction than the gene and the upstream copy had the opposite direction. This structure has already been described in *A. baumannii* by Poirel *et al.* (164,166) and it has also been found in the *Acinetobacter* phenon 6/ct13TU (Paper 7).

In **Paper 7**, the same experimental procedure was performed on an *Acinetobacter* phenon 6/ct13TU (ct = “close to”) clinical isolate. This isolate was a cause of pneumonia in a 67-year-old patient admitted to the intensive care unit. Although it was susceptible to imipenem, a MIC of 6 mg/L suggested a possible reduced susceptibility to this antimicrobial agent because it was higher than the usual value encountered in imipenem-susceptible *Acinetobacter* spp. The *bla*$_{OXA58}$ gene was also present in a 100 Kb plasmid,

with two copies of the Insertion Sequence IS$_{Aba3}$ surrounding the gene, as it has been previously described in the *Acinetobacter* Genospecies 3. Poirel *et al.* (164) suggested that this structure could correspond to a composite transposon and that the IS element upstream the *bla*$_{OXA58}$ gene could provide promoter sequences for the expression of this gene (164).

The *Acinetobacter* phenon 6/ct13TU showed a reduced susceptibility to imipenem which could be completely explained by the presence of the *bla*$_{OXA58}$ gene. On the other hand, in the Genospecies 3 isolate, the presence of this gene alone does not account for the high level of resistance to imipenem. A recent report by Bertini *et al.* (16) implies that the presence of more than one copy of the *bla*$_{OXA58}$ gene could confer high level resistance to carbapenems. They suggest that the region containing this gene can be duplicated by the presence of Insertion Sequences, increasing the level of resistance to these antibiotics in the clinical isolate (16). However, additional work would be needed to determine whether this high level of resistance is due to the presence of several copies of the *bla*$_{OXA58}$ gene as suggested by Bertini *et al.*, to the expression of additional efflux mechanisms or to porin modifications.

Acinetobacter baumannii certainly is the most frequently isolated species in hospitals and also the species of greatest clinical interest in this genus. The studies performed in papers 2 & 4 show the high level of resistance to cephalosporins and carbapenems in *A. baumannii* clinical isolates. However, other *Acinetobacter* spp. should be taken into account in hospitals as putative causative agents of nosocomial infection. Previous studies in *Acinetobacter* Genospecies 3 have revealed the presence of AmpC (8), IMP-4 (27), and *bla*$_{VIM-2}$ (240). As suggested by Horrevorts *et al.* (96), the clinical significance of the Genospecies 3 can be underestimated because the resistant strains can be erroneously classified as *A. baumannii.* On the other hand, although the *Acinetobacter* phenon 6/ct13TU is not commonly identified as a causative agent of infection, this microorganism

has been already isolated from blood, sputum, ulcer, and abscess. This microorganism may not be considered an important clinical pathogen; however, it is worthy of mention that a plasmid containing the bla_{OXA58} gene has been obtained from a clinical isolate causing pneumonia and that it can impair the potential treatment with carbapenems.

Infections caused by members of the genus *Acinetobacter* are frequently attributed to *A. baumannii* due to the difficulty to identify these microorganisms to the genomic species level. Nevertheless, as it has been shown in **Papers 6, 7 & 8**, other non-baumannii genospecies are increasingly identified as causative agents of nosocomial infection. In **Paper 8,** we have reported a severe clinical case of bacteraemia due to an unclassified *Acinetobacter* genomic species 17-like strain, which had been initially identified by the widely used commercial API20NE system as *A. baumannii.* Further analysis with molecular methodology and genomic fingerprinting proved that this isolate had a high homology (99.09 %) with the *Acinetobacter* genomic species 17. Further phenotypic studies showed that this isolate had haemolytic activity and produced gelatinase; in addition, its carbon source utilization profile was different from those of all of the *Acinetobacter* strains described so far. Antimicrobial susceptibility tests determined that this microorganism was susceptible to all the antimicrobial agents tested except for cefoxitin and cephalotin; on the other hand, it showed a slightly elevated MIC of polymyxin B (2 mg/L). Although this isolate was clearly susceptible and probably did not produce any β-lactamase enzyme, we considered important to introduce this study in the present section in order to prove that *Acinetobacter* spp. other than *A. baumannii* are increasingly being identified as the causative agents of severe nosocomial infections. The initially erroneous identification of this isolate, together with the clinical isolates previously reported in **Papers 6 & 7** which had also been identified as *A. baumannii*, proves that correct identification of the *Acinetobacter* species is problematic. The main

identification systems used in hospitals, such as the API20NE, are able to identify these microorganisms to the genus level, but fail to obtain a correct identification to the genomic species level. Certainly, most *Acinetobacter* bacteraemias are caused by *A. baumannii*; however, bacteraemia due to *Acinetobacter* genomic species 13TU (137), *Acinetobacter junii* (124), and *A. ursingii* (126) have already been described in the literature. Although this *Acinetobacter* genomic species 7 isolate was highly susceptible to most antimicrobial agents, it is important to note that it was responsible for a case of bacteraemia and the patient had to remain five months hospitalised. A correct identification of the *Acinetobacter* genomic species is important for therapeutic reasons, as species other than *A. baumannii* are generally susceptible to more antimicrobial agents. Moreover, the recognition of this microorganism as non-baumannii may have important epidemiological implications, as it relieves the hospital from the implementation of barrier precautions that might be necessary for controlling multiresistant *A. baumannii* epidemics.

- **Evaluation of the antimicrobial activity of two novel β-lactam antibiotics against *A. baumannii* clinical isolates (Papers 3 & 5).**

Acinetobacter baumannii is becoming more resistant each day, and as it has been shown in the previous papers, other *Acinetobacter* spp. are also acquiring new mechanisms of resistance. Cephalosporins were a good option to treat infections caused by these microorganisms. However, the increased overexpression of class C β-lactamases has resulted in the acquisition of resistance to extended-spectrum cephalosporins (160); even when the isolates are considered susceptible after *in vitro* analysis, their MIC values are usually close to the clinical breakpoint (44). Unable to use cephalosporins, carbapenems and especially imipenem, took over and became the preferred treatment for serious *A. baumannii* infections. Unfortunately, in the last decade, resistance to this group of antimicrobial agents has been rising, owed to a great extent to the synthesis of class D β-lactamases, together with membrane modifications that decrease the antimicrobial uptake (223). Currently, new antimicrobial agents with activity against Gram-negative microorganisms are needed. By contrast, a great number of pharmaceutical companies have abandoned research on drug discovery and development driven by the risks of poor financial outcome; the only new agents coming out are modifications of existing ones (160).

The main objective of the following two papers was to evaluate the *in vitro* activity of ceftobiprole **(Paper 3)** and doripenem **(Paper 5)** in a collection of *A. baumannii* clinical isolates.

Ceftobiprole is a broad spectrum cephalosporin which has successfully completed phase III clinical trials and is currently waiting for approval in both the USA and Europe. The drug has recently obtained the regulatory approval from Health Canada; however is

still awaiting a decision from the FDA (204,243). Previous studies have proposed that the activity of ceftobiprole against Gram-negative bacteria as *Pseudomonas aeruginosa* is comparable to the activity of other known antimicrobial agents such as ceftazidime or cefepime (24,42,241). In **Paper 3**, *in vitro* activity of ceftobiprole, ceftazidime and cefepime was compared on fifty-eight epidemiologically unrelated *A. baumannii* clinical isolates which had been separated into two groups depending on the presence or absence of the IS_{Aba1} in front of the bla_{ADC}-like gene. The overall resistance of the isolates to ceftobiprole (70.7 %) is slightly lower than the resistance to ceftazidime (77.6 %) or cefepime (75.9 %). When the bla_{ADC}-like gene was overexpressed due to the presence of the Insertion Sequence in the promoter region of this gene, all but one isolate were resistant to the cephalosporins, including ceftobiprole; the isolates had MICs ≥ 16 mg/L except one strain that had a MIC of cefepime of 8 mg/L. This results corroborate the findings of Queenan *et al.* (171), who suggested that in *Pseudomonas aeruginosa* isolates producing high levels of AmpC, the MIC for all the cephalosporins tested was over 16 mg/L (171). However, when the bla_{ADC}-like gene was not expressed, the activity of ceftobiprole against *A. baumannii* was better than the activity of ceftazidime and cefepime. Over 60 % of the isolates had a MIC ≤ 8 mg/L for ceftobiprole which is a good percentage in a multiresistant microorganism such as *A. baumannii*.

Doripenem is a broad-spectrum carbapenem with activity against *A. baumannii* that was approved by the Food and Drug Administration (FDA) in 2007 as a new drug to treat complicated urinary tract and intra-abdominal infections (65,68). Previous reports suggested that the activity of doripenem against *A. baumannii* was equivalent to the activity of imipenem and meropenem, especially in carbapenemase-producer isolates which were resistant to all the carbapenems (145,178). In **Paper 5**, *in vitro* activity of doripenem, imipenem and meropenem was compared on eighty-seven epidemiologically

unrelated *A. baumannii* clinical isolates which had been previously characterized for the presence of different carbapenemases. The overall resistance of the isolates to doripenem (54 %) is slightly lower than the resistance to imipenem (57.5 %) or meropenem (64.4 %). In spite of this, the MIC_{50} and MIC_{90} did not indicate great differences in the activity of the three antimicrobial agents. With respect to the activity of doripenem in front of class D β-lactamases, the results are variable. All the *A. baumannii* clinical isolates expressing the *bla*$_{OXA24}$ gene were resistant to doripenem, imipenem and meropenem. On the other hand, doripenem had a better activity than imipenem and meropenem when the *bla*$_{OXA58}$ gene was expressed; the percentage of isolates resistant to doripenem (MIC > 8 mg/L) was much lower (33 %) than for imipenem or meropenem to which all the isolates were resistant. Previous studies targeting the activity of doripenem against carbapenemase-producing *A. baumannii* ignored the OXA-58 oxacillinase, probably because the presence of this enzyme in *Acinetobacter* spp. is relatively new. The results described in **Paper 4** corroborate the low presence of this enzyme in a collection of epidemiologically unrelated *A. baumannii* clinical isolates collected in the year 2000. In the present study, only twelve of the eighty-seven isolates synthesized the OXA-58 enzyme. Nevertheless, although this number was very low, the results clearly suggest that the activity of doripenem against *A. baumannii* expressing the *bla*$_{OXA58}$ gene is higher than the activity of imipenem and meropenem.

Initial studies suggested that doripenem included the best qualities of the carbapenems by combining the exceptional activity of imipenem against Gram-positive cocci with the activity of meropenem against Gram-negative bacteria (68). In front of *A. baumannii*, this antimicrobial agent offers a slight improvement when compared to imipenem and meropenem in clinical isolates expressing the OXA-58 β-lactamase.

- **Membrane-based mechanisms of resistance in *A. baumannii* clinical isolates (Papers 9, 10 & Additional Results I & II).**

The clinical utility of the β-lactam antibiotics is being jeopardized by the emergence of *A. baumannii* isolates producing a wide range of β-lactamase enzymes. Nevertheless, with regard to resistance, the major threat involves the emergence of membrane-based mechanisms that reduce the permeability of the membrane to certain antibiotics or actively expulse them outside the bacterial cell. Mutations affecting the expression of porins or efflux pumps may decrease bacterial susceptibility to a broad spectrum of unrelated antimicrobial agents (158,228). The outer membrane in *A. baumannii* is intrinsically less permeable to antimicrobial agents than that of other microorganisms such as *Escherichia coli*; this could be attributed to the small number of porins as well as their small pore size (228). Besides this intrinsic high impermeability, the decreased expression of some OMPs and overexpression of efflux pumps transforms the *A. baumannii* membrane in a wall difficult to cross.

The main objective of the following papers was to study the membrane-based mechanisms of resistance in *A. baumannii* isolates. The initial objective was to determine the percentages of tetracycline and minocycline resistance in a collection of *A. baumannii* clinical isolates, followed by a more in depth study of the mechanisms underlying this resistance **(Paper 9)**. A second objective was to characterize the main *A. baumannii* membrane proteins and to identify new putative membrane-based mechanisms of resistance following genetic and proteomic approaches **(Paper 10 & Additional Results I & II)**.

In **Paper 9**, a selection of 79 epidemiologically unrelated *A. baumannii* clinical isolates were analysed for resistance to tetracycline and minocycline. A total of 59 isolates

(74.7 %) were resistant to tetracycline; from them, 40 isolates (50.6 %) were also resistant to minocycline. PCR analysis determined the presence of the *tetA* and *tetB* resistance genes in 8 (13.6 %) and 39 (66 %) of the 59 tetracycline resistant isolates, respectively. The remaining 12 clones did not present any of these two genes; however, these isolates were phenotypically identical to those containing the *tetA* gene. Tetracycline resistance was obtained by acquisition of the *tetA, tetB* or another mechanism of resistance not identified in this study; on the other hand, in thirty-nine out of the forty minocycline resistant isolates, this resistance was due to the presence of the *tetB* gene. There was one minocycline resistant isolate lacking the *tetB* gene which possibly acquired this resistance by other mechanisms of resistance, either another *tet* gene or the presence of additional multidrug efflux pumps. Therefore, there is a clear relationship between the presence of the *tetB* gene and resistance to both antimicrobial agents because all the isolates with a MIC of tetracycline of 128 mg/L and a MIC of minocycline ≥ 2 mg/L were positive for the *tetB* efflux pump. All these results agree with a previously published study by Guardabassi *et al.* (84), who suggested that these two efflux pumps were frequently found in tetracycline resistant *A. baumannii* clinical isolates, while they were unusual among *Acinetobacter* spp. from aquatic environment (84). The *tetB* gene was defined by Roberts (181), as the gene encoding the most efficient tetracycline efflux pump because it confers resistance to tetracycline and minocycline, in contrast to the TetA efflux pump which only has activity against tetracycline (181). Our results corroborate this description of the *tetB* gene because 95 % of the minocycline resistant isolates contained a TetB efflux pump. Although tetracyclines have not been generally used to treat *A. baumannii* infections, the level of resistance to this group of antimicrobial agents is very high. The *tetA* and *tetB* genes are generally plasmid encoded and present within transposon structures which favours their mobility and dissemination among different types of pathogenic bacteria; indeed, these two

genes are widely distributed within Gram-negative pathogens. Colonization of the intestinal tract of hospitalised patients by *A. baumannii* clinical isolates becomes a perfect environment for the acquisition by horizontal transfer of these tetracycline resistance genes from other Gram-negative pathogens (33). Wood *et al.* (237) have suggested that minocycline and doxycycline could be an effective treatment for pneumonia caused by multidrug-resistant *A. baumannii* (237); however, the use of these antimicrobial agents in Spain is not recommended due to the high level of resistance observed in *A. baumannii* clinical isolates. Although resistance to tetracycline in *A. baumannii* is greater than that to minocycline, the *tetB* gene, which affects both antimicrobial agents, has a higher prevalence than the *tetA* gene which affects only tetracycline.

TetA and TetB are specific efflux pumps conferring resistance to antibiotics from the tetracycline family. Certainly, this mechanism of resistance is important in front of tetracyclines; however, the real importance of efflux pumps involves those efflux systems which are able to actively expel unrelated antimicrobial agents. Multidrug efflux pumps recognize a broad range of structural and chemically different substrates. Efflux transporters are expressed in all living cells to protect them from the toxic effects of organic chemicals; nevertheless, bacterial multidrug resistance has often been associated with overexpression of these transporters (228). Although *A. baumannii* has a high intrinsic resistance to antimicrobial agents, only three multidrug efflux pumps have been described in this organism: AdeABC, AdeIJK and AbeM. Identification of novel efflux pumps has been attempted following genomic and proteomic approaches. Description of a novel MdfA-like efflux pump has been reported in the **Additional Results I**. The *mdfA* gene was detected by PCR with degenerate primers designed from highly homologous fragments from the same gene in *Escherichia coli* and the ORF57 in *A. baylyi* ADP1 (presumably *mdfA*). The whole *mdfA* gene of *A. baumannii* strain RUH134 was obtained by inversed

PCR analysis; it was a 1,230 nucleotide long gene which presented 34 % homology with the *mdfA* gene in *E. coli* and 70 % homology with the same gene in *A. baylyi* ADP1. MdfA is an efflux pump from the Major Facilitator Superfamily (MFS) which in *E. coli* confers resistance to a diverse group of cationic or zwitterionic lipophilic compounds, including some clinically important antibiotics such as chloramphenicol, erythromycin, tetracycline and some aminoglycosides or fluoroquinolones (51). To date, there are no descriptions of the antimicrobial agents expelled by this efflux system in *Acinetobacter* spp.; therefore, we are trying to generate knock out mutants to determine the range of antibiotics covered by the MdfA efflux pump in *A. baumannii.* The prevalence of the *mdfA*-like gene was also analysed in a group of sixty-four epidemiologically unrelated isolates collected from different Spanish hospitals during the year 2000; all of them were positive for this gene, although it has not been determined whether the MdfA efflux pump was expressed in all these isolates. The constitutive expression of MdfA in *A. baumannii* could explain the high level resistance to chloramphenicol in this microorganism. Further work is necessary to analyse the real importance of this efflux pump in *A. baumannii.*

Genomic studies have deeply contributed to the current knowledge of microorganisms by determining the complete genomic sequence of several pathogens and developing sequence-based tools to study the behaviour of these genes (142); however, this genomic approach fails to take into account all the possible protein products encoded by those genes and their functional significance. On the other hand, proteomics study changes in protein expression, as well as protein-protein interactions, distribution and temporal patterns of expression (225). The bacterial membrane from Gram-negative bacteria, which acts as a permeation barrier, contains large outer membrane proteins (OMPs) called porins that allow the entrance of nutrients and possibly also the efflux of waste products (149). The presence of slow porins, such as the OmpA family (HMP-AB), could explain the high

intrinsic antibiotic resistance of *A. baumannii*; this group of porins are characterized by allowing a slower diffusion of the small solutes. Characterization of membrane proteins is necessary on account of the important role of the OMPs in resistance to antimicrobial agents, together with the transport of nutrients, cell to cell signalling, attachment to host cells and the virulence in pathogenic strains (151). The objective of **Paper 10** was to characterise the major proteins present in the *A. baumannii* cell envelope following a proteomic approach and more specifically, in the **Additional Results II** to identify new putative membrane-based mechanisms of resistance to quinolones in *A. baumannii* isogenic mutant strains.

In **Paper 10**, twenty-nine major protein spots were selected for identification; although in the polyacrylamide gel there were more proteins, only the major ones were selected and analyzed. At this time, the genomic sequence of *A. baumannii* was not available and sequence comparison was difficult; therefore, all the proteins were separated into three groups depending on the level of homology with other *Acinetobacter* spp., other bacterial species, and those unable to be identified. From the twenty-nine selected proteins, only eight were left unidentified; this lack of results could be attributed to a low level of homology with proteins of other *Acinetobacter* spp. available in the databases. Moreover, these proteins could be specific for *A. baumannii* and they may not have any significantly homologous proteins in other species. Approximately half of the analysed proteins were cytoplasmic contaminations of the cell envelope extract which highlights the difficulty of separating the cell envelope from the rest of the cellular components. Among the membrane proteins identified, there were chaperones, elongation factors and OMPs. The chaperones form part of the GroEL family and are involved in the folding of several substrates to achieve the correct tertiary structure and indirectly, also the correct quaternary structure (179,207). Membrane-bound elongation factors have been attributed a role as

adhesion factors in *Lactobacillus johnsonii*; they have also been found associated to the membrane of *Escherichia coli* and *Streptococcus oralis* (82,100,235). Finally, two OMPs were characterized: firstly, the Omp HMP, which is a monomeric porin similar to the OmpA of Enterobacteria and the outer membrane protein F (OprF) of *Pseudomonas aeruginosa*. This group of porins are characterized by having a large pore size and a slower penetration rate than the normal trimeric proteins (83). The presence in *A. baumannii* of this "slow porin" as one of the major channels could give an explanation of the higher intrinsic resistance of this microorganisms to antimicrobial agents. The second porin characterized was the 33-36 KDa outer membrane protein, which nowadays has been associated with the acquisition of imipenem resistance (215). It is important to note that Paper 10 was accepted for publication in October 2005; at this time, the genomic sequence of *A. baumannii* was not available and proteins were difficult to be correctly characterized. All this data proves the huge amount of information obtained in the past years on *A. baumannii*; nowadays, probably all the proteins would be identified.

Characterization of the major proteins present in the cell envelope of *A. baumannii* was important to understand the high intrinsic resistance to antibiotics; the Omp HMP is probably one of the most intense spots in the protein map and therefore, one of the most abundant proteins in the cell envelope of *A. baumannii*. Although the slow penetration rate of this protein can explain the high intrinsic resistance of this microorganism, it is important to study the modifications produced in the cell membrane after acquiring resistance to antimicrobial agents. In the **Additional Results II**, cell envelope proteins from two sets of isogenic quinolone-resistant mutants were analysed by 2D gel electrophoresis. As the quinolone-resistant mutants did not have any additional mutation in *gyr*A and *par*C with respect to their susceptible counterparts, the increased resistance to these antimicrobial agents would be caused by other mechanisms of resistance, probably

involving modifications in membrane permeability such as changes in porin expression or overexpression of efflux pumps. A proteomic analysis was performed on these four isolates in order to study the cell envelope modifications between the susceptible wild type isolates and the quinolone-resistant isogenic mutants.

Comparison of protein expression between the *A. baumannii* strain A15-43 and its isogenic clinafloxacin-resistant mutant showed modifications in several metabolic proteins but also on membrane-related proteins. Among the most important changes observed between these isolates, there was a shift between the chaperone Hsp60 from the wild type isolate into the chaperone Cpn60 found in the quinolone-resistant mutant. The real significance of this change is still unknown; however, as these proteins are involved in the correct folding of other proteins, this modification should be studied more in depth. With regard to the OMPs, there was a slight increment in the expression of two OMPs: a 27 KDa Omp A-like and a 24 KDa HMP-like proteins. An increased expression of these OMPs has also been observed on the second set of mutants, *A. baumannii* strain 77 and its ciprofloxacin-resistant isogenic mutant. To present, these proteins have not been identified; nevertheless, the overproduction of two porins which are probably related to the group of “slow porins” previously described, could make the cell membrane more impermeable to the entrance of antimicrobial agents. Additionally, the mutant strain 77M also presented a slight overexpression of a 35 KDa OMP and a possible ABC-transport system. Both sets of mutant also presented a modification on a biofilm-related protein (CsuA/B) which has been looked at with more detail in the **Additional Results III**.

The results obtained for both sets of mutants are slightly different; in contrast to the results obtained for the A15-43 isogenic strains which had more modification at the metabolic level, the differences observed in the *A. baumannii* 77 wt and 77M involve more OMPs than any other metabolic enzymes. Indeed, both sets of mutants do not have the

same characteristics and the mutant selection was also performed with two different antibiotics (clinafloxacin and ciprofloxacin). *A. baumannii* strain A15-43 had a double mutation in the *gyr*A and *par*C genes; being resistant to ciprofloxacin, the selection for resistant mutants was performed with clinafloxacin. On the other hand, *A. baumannii* strain 77 did not have any mutation in the *gyr*A and *par*C genes and presented a phenotype susceptible to ciprofloxacin.

Both, genomic and proteomic approaches have resulted in identification of possible membrane-related mechanisms of resistance. Nevertheless, these are still preliminary results that have to be analysed more in depth in order to obtain a real vision of the mechanisms used by *A. baumannii* to overcome the killing activity of quinolones.

- **Biofilm formation by *A. baumannii* clinical isolates (Paper 11 & Additional Results III).**

Survival in the hospital environment is one of the most challenging tasks facing the microorganisms and they have evolved several mechanisms to tolerate these adverse conditions. Nosocomial pathogens have to support the use of antimicrobial agents and disinfectants; clinical isolates also face nutrient limitation because they are trying to survive in an environment where their elimination is the priority of the medical staff. Among all these pathogenic bacteria, *A. baumannii* is probably the microorganism that has developed the best adaptation mechanisms to bear all these adverse conditions. A high intrinsic resistance to antibiotics and disinfectants prevents the rapid elimination of this microorganism from the hospital environment; in addition, the ability of *A. baumannii* to acquire new resistances has been reported in the previous papers and also in a great part of the recent literature. However, the ability to resist desiccation is a key factor that facilitates the persistence of this microorganism during long periods of time on inanimate surfaces. Biofilm formation is a possible explanation for the resistance to desiccation and it could also be responsible for an important number of the device-related nosocomial infections. Probably, in the harsh environmental conditions encountered in hospitals, biofilm structures offer better survival opportunities than existing as planktonic free-living cells.

The main objective of the following work was to study the biofilm formation in a collection of epidemiologically unrelated *A. baumannii* clinical isolates. The initial objective was to identify the clinical implication of the biofilm structure by looking at the relationship between biofilm formation and other clinically relevant associated features **(Paper 11)**. From the results obtained, an additional objective was introduced in order to analyse a possible relationship between acquisition of quinolone resistance and a reduction in biofilm formation **(Additional Results III)**.

In **Paper 11**, biofilm formation was analysed in ninety-two unrelated *A. baumannii* clinical isolates from a multicentre cohort study. Fifty-six of the clinical isolates (63 %) were able to form biofilm structures on 96-well polystyrene microtiter plates; the experiment was performed with at least two different cultures and by duplicate (two wells per culture). All clonally related isolates shared either the ability or inability to form biofilm suggesting that biofilm formation is a clone-specific feature and it does not depend on the difference in growth conditions. Complete epidemiological and clinical data were available for 78 patients; the epidemiological and clinical features of these patients which had been colonised or infected with *A. baumannii* were compared with the ability of this isolates to form biofilm. All the isolates used in this study had been previously characterized and extensive information on patients, infection and treatment was available in the database.

Biofilm-forming *A. baumannii* isolates were found to be less epidemic; therefore, these isolates would be more frequently found as pathogens with an endemic occurrence in hospitals than taking part of an epidemic outbreak. In addition, biofilm formation has been related to susceptibility to imipenem and ciprofloxacin. All these results suggest that the ability to form biofilm makes them more resistant to antibiotics, disinfection and desiccation, allowing them to persist in the hospital environment; these isolates are not as dependent as the non-biofilm-forming counterparts on antimicrobial resistance and epidemic characteristics to survive in the hospital environment. On the contrary, patients who had previously received aminoglycoside therapy were at an increased risk of being colonised or infected by biofilm-forming *A. baumannii* isolates. This suggests that the use of aminoglycosides in hospitals could exert a different selection pressure on the microorganisms, irrespectively of their own *in vitro* susceptibility to this group of antimicrobial agents. Finally, biofilm-forming *A. baumannii* isolates were more often

obtained from the blood and urine of patients than associated to respiratory tract infections; these results were not statistically significant (p=0.07 and 0.06 respectively), but it could indicate a relationship between biofilm formation and the presence of catheters. Indeed, although the results were limited by the low number of cases analysed, there is some evidence suggesting that biofilm plays a role in the pathogenesis of some device-associated *A. baumannii* infections such as those involving Foley catheters, venous catheters and cerebrospinal fluid shunts.

This study concluded that over 60 % of epidemiologically unrelated *A. baumannii* isolates from clinical samples formed biofilm and they were mainly associated with device-related infections. Multivariate analysis revealed that treatment in intensive care units, isolation from a respiratory sample and resistance to imipenem or ciprofloxacin were clearly associated with non-biofilm-forming isolates, while previous aminoglycoside use was associated with biofilm-forming isolates.

These results have shown a clear association between biofilm formation and susceptibility to ciprofloxacin (p=0.004); in order to determine the nature of this relationship, a more in depth analysis had to be performed. Two sets of quinolone mutants have previously been described in the Additional Results II; these mutants could be used to study the relationship between biofilm formation and susceptibility to quinolones. Therefore, the main objective of the **Additional Results III** was to corroborate the formerly suggested relationship between biofilm formation and susceptibility to quinolones in clinafloxacin and ciprofloxacin isogenic mutants, followed by an attempt to identify novel modifications at the protein level which could be responsible for this association.

Biofilm formation analysis determined that both sets of mutants had the same pattern; the susceptible isolates were able to form biofilm while the quinolone-resistant

mutants had this ability reduced. These results corroborate the previously established hypothesis which associated biofilm formation with quinolone susceptibility. Identification by proteomic analysis of a protein involved in type 1 pili formation (CsuA/B) could be the reason for the reduced capability to form biofilm observed in the quinolone resistant mutants; this protein was only expressed in the susceptible wild type strains. The *csuA/B* gene forms part of an operon which includes the genes *csuA* to *csuE* and it encodes for the type 1 pili subunit CsuA/B protein (214). This protein was first described in *A. baumannii* by Tomaras *et al.* (214) in a work that presented for the first time the differences in biofilm formation above, below and at the liquid-air interface. The same electron microscopy analysis was performed on the two sets of isogenic quinolone mutants to determine the differences in the biofilm structure formed by the quinolone susceptible and resistant isolates. Clear differences cannot be observed with the ciprofloxacin mutant isolates (77 wt and 77M), probably due to the different augment size of the electronic microscopy between the wild type and its respective quinolone resistant mutant. Nevertheless, although in the mutant strain there are some pili-like structures attaching the cells, above the liquid-air interface the bacterial cells are clearly in a planktonic state of growth, while the wild type strain forms biofilm in the three phases. These results are better observed when analysing the clinafloxacin isogenic mutants (A15-43 wt and A15-43M). Above the liquid-air interface, there are clear differences in the biofilm formation of the wild type strain and its isogenic mutant; the susceptible strain forms a thick biofilm structure, while the clinafloxacin resistant mutant is found in a planktonic state of growth and is missing the pili-like structures characteristic in the wild type strain.

Biofilm formation studies and electron microscopy analysis suggest a relationship between the acquisition of quinolone resistance and the inability to form a compact biofilm structure. Nevertheless, further studies are necessary to demonstrate this relationship;

expression and complementation studies of the main genes forming part of the Csu operon are currently being performed in our lab.

Thus, a relationship between biofilm formation and susceptibility to quinolones was observed *in vivo* through epidemiological analysis of clinical isolates. Afterwards, this association was corroborated with *in vitro* studies of isogenic mutants which had the same behaviour as the clinical isolates. Therefore, quinolone resistant *A. baumannii* isolates are less prone to produce biofilm than their susceptible counterparts. This association is possibly linked to a decreased expression of type 1 fimbrae, the first step in biofilm formation.

- **Mechanisms of colistin resistance in *Acinetobacter* spp. clinical isolates (Additional Results IV).**

All the previously reported mechanisms of resistance, together with an elevated number of additional mechanisms that have not been reviewed in this thesis dissertation, have left colistin as one of the last resource antimicrobial agents to treat infections caused by multidrug resistant *A. baumannii* isolates. Despite its toxicity, clinicians have been forced to reconsider colistin as a therapeutic option, and scientists are reviewing the pharmacokinetic and pharmacodynamic properties of this antimicrobial agent. Then again, how long will colistin be effective to treat infections caused by members of the *Acinetobacter* spp.? Along the years, *A. baumannii* has been developing resistance to all the antimicrobial agents used to combat these infections; aminoglycosides, tetracyclines, cephalosporins and currently the activity of carbapenems is also being jeopardized by the emergence of enzymatic and membrane-based mechanisms of resistance (160). For more than 15 years, nebulised colistimethate sodium in combination with oral ciprofloxacin has been used in Denmark and the UK to treat multidrug resistant *Pseudomonas aeruginosa* infections; during this time, resistance to colistin has not been observed. By contrast, a very high frequency of mutational resistance was observed when colistin was used alone to treat infections caused by Gram-negative microorganisms such as *P. aeruginosa, A. baumannii, Klebsiella pneumoniae,* and *E. coli* (121). Unfortunately, the increment in the use of polymyxines has resulted in the emergence of colistin resistant pathogens; the rates of resistance to these antibiotics have recently been reported to be as high as 3.2 % for MDR *A. baumannii* isolates (160). More information on the mechanisms of colistin resistance is needed in order to find solutions to maintain the antimicrobial activity of this last resort antibiotic for as long as possible. The main objective of the **Additional Results**

IV was to determine the mechanisms of resistance to colistin in clinically relevant members of the genus *Acinetobacter*.

Colistin resistant mutants were obtained from *A. baumannii, Acinetobacter* Genospecies 3 and 13TU clinical isolates, and the *A. baumannii* type strain ATCC 19606 by subjecting the isolates to increasing concentrations of colistin sulphate. In contrast to the facility encountered to obtain colistin resistant mutants from the *Acinetobacter* Genospecies 3 and 13TU, *A. baumannii* was very difficult to mutate. The initial studies were performed with the type strain ATCC 19606; fortunately, the acquisition of an *A. baumannii* clinical isolate with reduced susceptibility to colistin made possible the production of highly resistant colistin mutants on one *A. baumannii* clinical isolate. The difficulty to obtain *in vitro* mutants from *A. baumannii* clinical isolates was initially considered a positive point in favour of controlling the acquisition of resistance to colistin; nevertheless, as soon as these isolates start to tolerate this antibiotic, the acquisition of high levels of colistin resistance comes straight forward.

In all the mutants obtained, there was an inflection point in which a serial of morphologic and metabolic changes occurred; there was a change in colony morphology with an additional loss of the ability to grow on selective MacConkey agar plates. Before this point, all the isolates presented heteroresistance in front of colistin, growing in high concentrations of colistin but maintaining susceptible MIC values or presented reduced susceptibility to this antimicrobial agent. In addition, after this inflexion point, the amount of LPS increased and there were additional modifications in protein expression. The OmpW porin was downregulated on the colistin resistant mutants and a 10 KDa signal peptide was upregulated. These results are consistent with the information known to date about the mechanism of action of colistin. This antimicrobial agent penetrates into the bacterial cell due to an interaction between the positively charged antimicrobial agent and

the negatively charged LPS (242). In *P. aeruginosa*, colistin resistance has been associated to alterations of the outer membrane such as reduction in LPS, lipid alterations, reduced levels of specific outer membrane proteins and reduction in Mg^{+2} and Ca^{+2} (59). A recent work by Gooderham *et al.* (81), reports that PsrA, a regulator of the type III secretion system, regulated polymyxin and antimicrobial peptide resistance, motility, and biofilm formation. They also suggest that this PsrA regulator was able to up- or downregulate 178 genes including among others, outer membrane permeability genes (81). The results obtained so far with the colistin mutants suggest the implication of a complex regulatory network because several independent changes occur at the same inflexion point. Further work will determine whether a regulatory protein such as PsrA is involved in the acquisition of colistin resistance in the *Acinetobacter* spp.

Colistin resistant *Acinetobacter* spp. are sporadically isolated from hospitals worldwide, confirming the great ability of these microorganisms to acquire resistance to antimicrobial agents. *In vitro* analysis suggests that *Acinetobacter* spp. have an initial tolerance to colistin before they become completely resistant. Unfortunately, several publications have already reported colistin heteroresistance in the clinical environment (88,89,112,122); nevertheless, it is of special concern a report by Li *et al.* (122) demonstrating that heteroresistant *A. baumannii* isolates were susceptible to colistin on the basis of MICs (122). If the *Acinetobacter* spp. have the same behaviour *in vivo* than *in vitro*, this could be the previous step before becoming completely resistant to colistin.

VI. CONCLUSIONS

1. In a high percentage of *A. baumannii* clinical isolates from Spain carrying the IS_{Aba1}, this element is inserted into the promoter region of the bla_{ADC}-like gene. This genetic element could be acquired or lost within the hospital environment.

2. The insertion of IS_{Aba1} in the promoter region of the bla_{OXA51} gene also enhances the expression of such gene and produces a small increase in the MIC of imipenem. This mobile genetic element is becoming an important switch of resistance genes in *Acinetobacter* isolates.

3. There is a high prevalence of oxacillinases with activity against carbapenems in genetically unrelated *A. baumannii* clinical isolates from Spain; the OXA-40 is widely distributed and the OXA-58 is disseminating.

4. The OXA-58 oxacillinase is spreading over Spanish hospitals and it is increasingly identified in members of the *Acinetobacter* spp. other than *A. baumannii*. In *Acinetobacter* Genospecies 3, an additional mechanism of resistance conferred high level of resistance to imipenem.

5. Non-baumannii *Acinetobacter* clinical isolates are probably being underestimated as pathogenic agents because the resistant strains can be erroneously classified as *A. baumannii*. Although these isolates are generally more susceptible to antimicrobial agents, they are increasingly acquiring novel mechanisms of resistance.

6. Doripenem and ceftobiprole barely offer a slight improvement in front of the older carbapenems and cephalosporins. Nevertheless, doripenem has a higher activity than imipenem or meropenem in front of *A. baumannii* expressing the bla_{OXA58} gene

7. *A. baumannii* isolated in Spain are frequently resistant to tetracyclines due to the presence of TetA and TetB efflux pumps. The *tetB* gene, which affects tetracycline and minocycline, has a higher prevalence than the *tetA* gene, which affects only tetracycline.

8. A *mdfA*-like gene, which is encoding for a MDR efflux pump, has been identified for first time in an *A. baumannii* clinical isolate. The constitutive expression of MdfA in *A. baumannii* may explain the high level resistance to chloramphenicol in this microorganism.

9. Genomic and proteomic approaches have proved to be important methodologies for identification and characterisation of novel mechanisms of resistance. A reference map of proteins from *A. baumannii* was the basis for determining cell envelope molecules associated with antibiotic resistance.

10. Outer membrane protein HMP, which has a slower penetration rate than the normal trimeric porins, is one of the most abundant proteins in the *A. baumannii* cell envelope and could explain the high intrinsic resistance of this microorganism.

11. In *A. baumannii*, quinolone resistance has been associated with the overexpression of several "slow porins" still unidentified. These porins make the bacterial membrane more impermeable to the antimicrobial agents.

12. Biofilm plays a role in the pathogenesis of some device-associated *A. baumannii* infections such as those involving Foley catheters, venous catheters and cerebrospinal fluid shunts. However, treatment in an intensive care unit has been associated with non-biofilm-forming clinical isolates.

13. There is an association between biofilm formation and susceptibility to imipenem and ciprofloxacin. In addition, patients previously treated with aminoglycosides had an increasing risk of being colonised or infected by biofilm-forming *A. baumannii* isolates.

14. Biofilm-forming *A. baumannii* clinical isolates were found to be less epidemic than their planktonic counterpart. These isolates are probably less dependent on their antimicrobial resistance and epidemic characteristics to survive in the hospital environment.

15. Quinolone-resistant *A. baumannii* isolates are less prone to produce biofilm than their susceptible equivalents due to a decreased expression of type 1 fimbrae, the first step in biofilm formation. These isolates presented a down-regulation of the *csuA/B* gene which encodes for the pili subunit CsuA/B protein.

16. Colistin resistance has been associated to LPS over-production and additional modifications in protein expression which include the down-regulation of the OmpW and the up-regulation of a signal peptide.

17. *In vitro* analysis suggests that *Acinetobacter* spp. have an initial tolerance to colistin before they become completely resistant. If these microorganisms have the same behaviour *in vivo* than *in vitro*, the increasing number of colistin heteroresistant isolates could represent the previous step before becoming completely resistant to colistin.

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