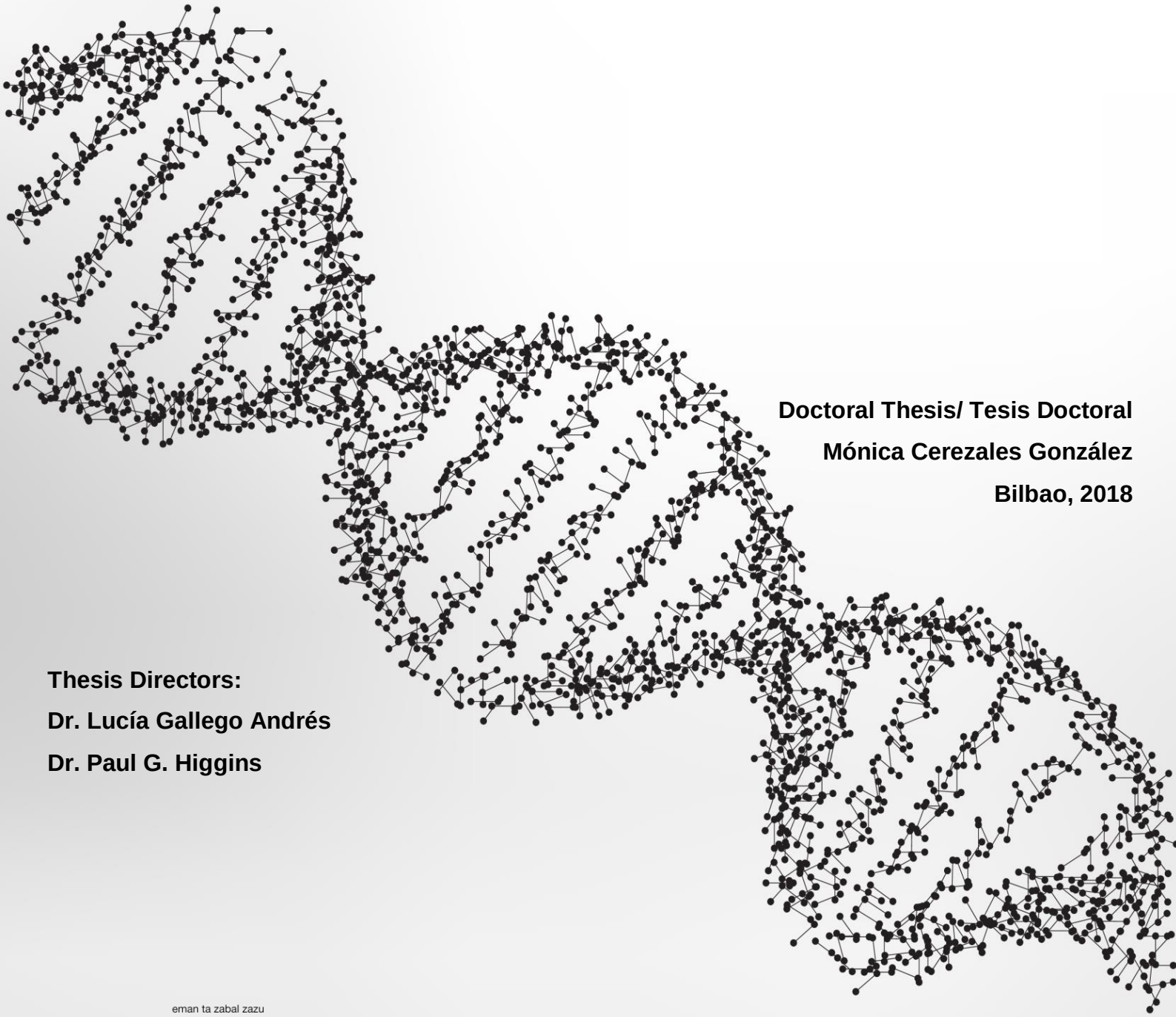
MOLECULAR CHARACTERIZATION OF *Acinetobacter* spp. AND THEIR ANTIBIOTIC RESISTANCE MOBILOME FROM CLINICAL ISOLATES COLLECTED IN BOLIVIA.

Caracterización molecular de *Acinetobacter* spp. y su mobiloma de resistencia a antibióticos en aislamientos clínicos de Bolivia.



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Where there’s a will there’s a way.

Querer es poder.

A mis padres.

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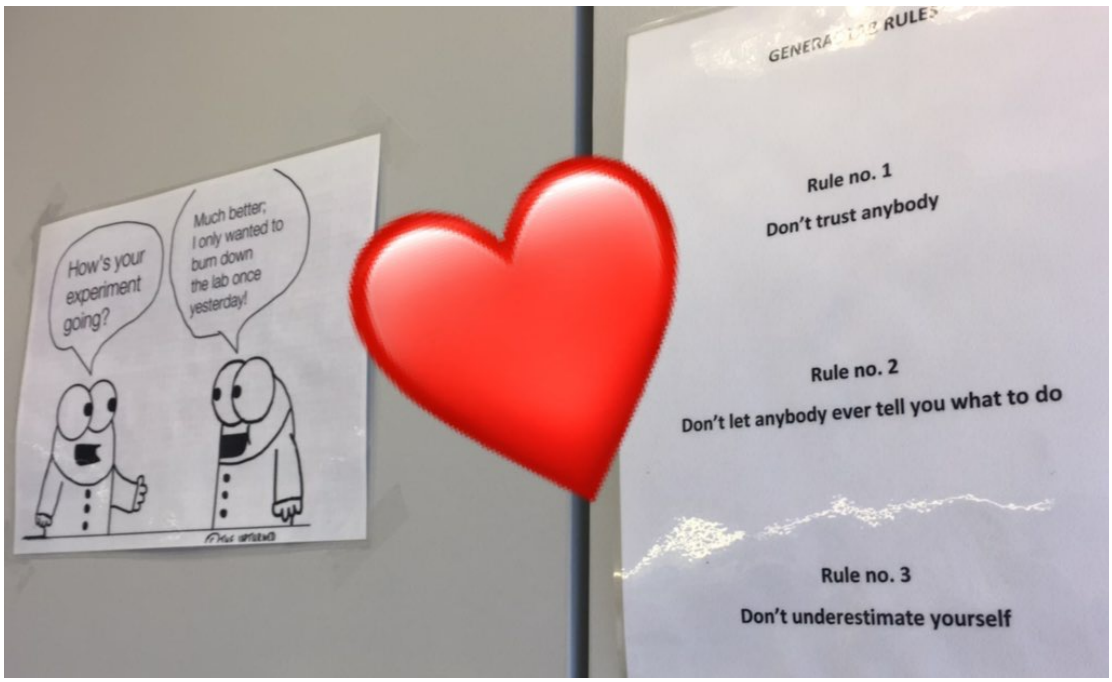

Kiki, I haven't words enough to thank you. You've been my partner in crime, my other half. Thank you for your support, for always being there for me, for being my hands in the lab when I was not there and even my brain sometimes. Thank you for being such a great colleague, and even more, an amazing person.

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Porque yo sin vosotros, no soy.

SUMMARY

Acinetobacter baumannii is a Gram-negative opportunistic pathogen causing serious hospital-acquired infections and it has been recently positioned as a priority pathogen by the World Health Organization. It has been described causing outbreaks worldwide and its antimicrobial resistance has been worryingly rising in recent times. *A. baumannii* usually causes infections such as ventilator associated pneumonia, septicaemia, urinary tract infections, wound infections or meningitis, especially in compromised patient groups such as those admitted to ICU and elderly patients, increasing the mortality rates and hospitalization costs.

Other species belonging to the *A. baumannii* group are being described more often lately as causing infections and sometimes related to antimicrobial resistance determinants.

This work is focused in the study of the *A. baumannii* isolates recovered from clinical samples in the two main hospitals of Cochabamba, Bolivia, Hospital Materno-Infantil y Hospital Viedma.

The present work is divided in four sections.

Section 1. The epidemiology and antibiotic resistance genes were studied in *A. baumannii* clinical isolates from a Children Hospital.

Section 2. We studied molecular epidemiology by cgMLST and resistome of CRAb isolates from the two main hospitals of the city of Cochabamba.

Section 3. Study of the mobile genetic elements encoding antibiotic resistance genes in *A. baumannii* isolates belonging to different ICs.

Section 4. Identification of *Acinetobacter seifertii*, an *A. baumannii* group member, isolated from both hospitals in Cochabamba.

RESUMEN

Acinetobacter baumannii es un patógeno oportunista Gram-negativo que causa infecciones nosocomiales serias, recientemente ha sido establecido como un patógeno prioritario por la Organización Mundial de la Salud. Se ha descrito como causante de brotes en todo el mundo y su resistencia a los antibióticos ha aumentado preocupantemente en los últimos tiempos. *A. baumannii* normalmente causa infecciones como neumonía, septicemia, infecciones del tracto urinario, infecciones de heridas o meningitis; especialmente en pacientes comprometidos como los que están en la UCI o los ancianos.

Otras especies pertenecientes al grupo *A. baumannii* se están describiendo como agentes causales de infecciones con más frecuencia ultimamente y en algunas ocasiones también están asociadas a determinantes de resistencia a antibióticos.

Este trabajo está enfocado en el estudio de aislamientos de *A. baumannii* de muestras clínicas de los dos hospitales principales de la ciudad de Cochambamba, Bolivia.

Este trabajo está dividido en cuatro secciones.

Sección 1. El objetivo de este studio fue el análisis y caracterización de todos los aislamientos de *A. baumannii* del Hospital Materno-Infantil de Cochabamba entre Abril de 2014 y Mayo de 2015. En un total de 36 aislamientos, se estudió la presencia de carbapenemasas de tipo D o oxacilinasas, $bla_{OXA.}$ ($bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$, $bla_{OXA\text{-}40\text{-}like}$ y $bla_{OXA\text{-}}$

$_{143\text{-like}}$), carbapenemasas tipo B o metalo-β-lactamasas (*bla*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SPM-1}$, *bla*$_{IMP}$ and *bla*$_{NDM-1}$); genes codificantes de enzimas modificadoras de aminoglicósidos (*ant(2")-Ia and aac(3)-IIa; aph(3')-Ia, aac(3)-Ia and aph(3')-VIa; aac(6')-Ih and aac(6')-Ib/cr; aac(6')-IIa*) y metilasas (*rmtB* y *rmtC*).

Además, se estudió el perfil de resistencia de los aislamientos a distintos antibióticos, se empleó el método de difusión de disco para amikacina, ampicilina-sulbactam, cefepime, cefotaxima, ceftazidime, ceftriaxona, ciprofloxacino, doxiciclina, gentamicina, imipenem, meropenem, minociclina, piperacilina-tazobactam, tetraciclina, tobramicina y trimetoprim-sulfametoxazol; se usó E-test para colistina. Se emplearon criterios establecidos para asignar los aislamientos a los grupos MDR (multidrug-resistant), XDR (extensively drug-resistant) o PDR (pandrug-resistant).

La relación clonal de estos aislamientos se estudió por restricción con la enzima *Apa*I y PFGE; los fragmentos resultants se analizaron con el software Fingerprinting II (Bio-Rad) de comparación de perfiles de bandas; el punto de corte para asumir relación clonal entre aislamientos se estableció en ≥87%. Para estudiar la epidemiología molecular también se empleó el esquema Oxford de MLST.

La localización del gen *bla*$_{OXA-23-like}$ se analizó mediante restricción con la enzima I-Ceu-I, PFGE, Southern blot e hibridación con una sonda marcada con digoxigenina.

También se estudió la producción de biofilm por parte de los aislamientos, el ensayo se llevo a cabo en pocillos en los cuales se crecieron

los cultivos, posteriormente fueron lavados y teñidos. La formación de Biofilm se cuantificó midiendo la cantidad de tinte mediante espectrofotometría.

Sección 2. Se estudió la epidemiología molecular mediante cgMLST y el resistoma de aislamientos CRAb de los dos hospitales principales de Cochabamba, Hospital Materno Infantil y Hospital Viedma. En total, 95 aislamientos se incluyeron en el estudio, que se confirmaron como A. baumannii por multiplex PCR del gen *gyrB* y presencia de $bla_{\text{OXA-51-like}}$. La presencia de β-lactamasas de clase D y B se analizó mediante multiplex PCR ($bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-143-like}}$ y $bla_{\text{OXA-235-like}}$; bla_{VIM}, bla_{KPC}, bla_{NDM} y $bla_{\text{OXA-48-like}}$; bla_{IMI}, bla_{GES}, bla_{GIM}, bla_{IMP} e IS*Aba1*- $bla_{\text{OXA-51-like}}$). Los perfiles de susceptibilidad a antibióticos se testaron mediante dilución en agar y microdilución en caldo. Todos los aislamientos resistentes a carbapenems, así como cuatro aislamientos sensibles se sometieron a secuenciación de genoma completo en la plataforma Illumina MiSeq. Los ensamblajes se realizaron con Velvet, Ridom SeqSphere+ v.3.0 y SPAdes 3.9. Los ensamblajes se emplearon para obtener el resistoma en ResFinder, los STs de los esquemas Oxford y Pasteur y el cgMLST para determiner su epidemiología molecular. Se asignaron los aislamientos a los distintos clones internacionales (IC) gracias a la variante del gen $bla_{\text{OXA-51-like}}$, el ST del esquema Pasteur y el cgMLST.

Además, también se estudió el contenido plasmídico y la localización de los genes de resistencia a antibióticos $bla_{\text{OXA-23}}$ y *strA,* mediante digestion con

la enzima S1 y separación de fragmentos con PFGE, Southern blot e hibridación con sondas marcadas con digoxigenina.

Sección 3. Estudio de elementos genéticos móviles portando genes resistencia a antibióticos en aislamientos de *A. baumannii* pertenecientes a diferentes IC. Los datos de la digestion S1-PFGE, Southern blot e hibridaciones en conjunto con los ensamblajes de genoma complete de Velvet, Ridom SeqSphere v.3.0, SPAdes y plasmidSPAdes se emplearon para hacer una predicción de los ensamblajes de los plásmidos e islas de resistencia localizadas en el cromosoma bacteriano, que se confirmaron posteriormente mediante PCR. Para confirmar estas estructuras plasmídicas se utilizó la técnica de secuenciación MinION de lecturas largas de Oxford Nanopore Technologies. Estas lecturas se ensamblaron mediante Canu. Además, las lecturas largas de MinION y las cortas, pero más precisas, de MiSeq se usaron en un ensamblaje conjunto, hybridSPAdes, en el cual las lecturas largas se utilizan como molde para ordenar las cortas de MiSeq.

Una vez obtenidas las estructuras de los plásmidos y las islas de resistencia cromosómicas, se predijeron los marcos de lectura abierta con ORFfinder con el fin de conseguir una anotación de los genes presentes. Una segunda anotación funcional se consiguió con la herramienta en línea RAST (Rapid Annotation Subsystem Technology). Con esta información se diseñaron diagramas de los plásmidos y las islas de resistencia con la herramienta SnapGene Viewer.

Sección 4. Identificación de *Acinetobacter seifertii*, un miembro del grupo *A. baumannii*, aislado en ambos hospitales de Cochabamba. Tres aislamientos identificados como *Acinetobacter* sp. por los laboratorios de diagnostico de los Hospitales Materno Infantil y Viedma, ofrecieron resultados contradictorios en la identificación mediante PCR multiplex del gen *gyrB,* las bandas obtenidas eran las específicas de *A. baumannii* y *Acinetobacter pittii*. Los equipos semiautomatizados VITEK MS y MALDI-TOF MS tampoco fueron capaces de ofrecer una identificación clara; el resultado de VITEK MS era *A. baumannii* complex para los tres aislamientos y MALDI-TOF MS daba un perfil mixto de *A. baumannii* y *A. pittii.*

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INTRODUCTION

1. GENUS *Acinetobacter*

The genus *Acinetobacter* belongs to the nonfermenting Gram-negative bacilli and it is the second most commonly found genus within this group causing bacterial infections. They have been isolated from the environment but also from clinical samples and they have became a serious problem in the intensive care units worldwide, especially *A. baumannii*. *Acinetobacter* is able to survive in unfavorable conditions and hospital reservoirs may inlclude mattresses, waterbaths and the hands of hospital staff and can cause outbreaks (1, 2). Several species within the genus have been reported to carry antimicrobial resistance determinants, which can compromise and make difficult antibiotic therapy (2–4).

Clinical *Acinetobacter* species can be easily grown in solid and liquid media at 37°C while the environmental species only grow at lower temperatures (20-30°C). All *Acinetobacter* species are oxidase negative, catalase positive, and strictly aerobic (1, 5).

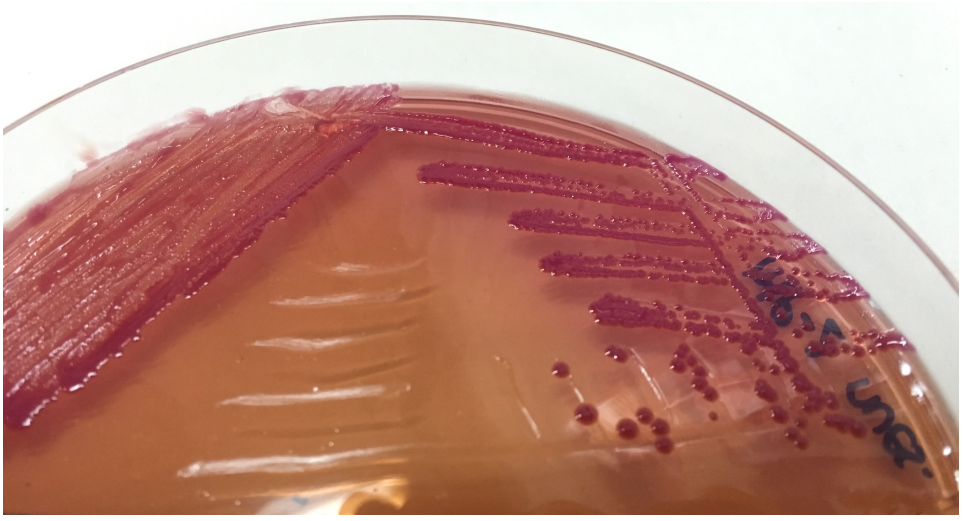

Figure 3. *Acinetobacter baumannii* in MacConkey agar.

1.1. Historical perspective

The current designation of the genus as *Acinetobacter* was first proposed by Brisou and Prévot in 1954 based on the Greek term “akinetos” which means non-motile (6), although twiching motility has been described in *Acinetobacter baumannii* (7). In 1968 this classification became widely accepted after the study of Baumann et al (8) was published. However, the first isolate of this genera was obtained from soil in 1911 and named *Micrococcus calcoaceticus* by Beijerinck (9).

The genus is still growing and species such as *Acinetobacter piscicola* and *Acinetobacter seifertii* have been validly named and published.

1.2. Current taxonomy

Currently, the genus *Acinetobacter* comprises 58 validly published species names, 6 tentative species designation and 9 effectively but not validly published species names.

Table 2. Validly published names, tentative species designation and effectively but not validly published species names (http://apps.szu.cz/anemec/Classification.pdf)

Validly published species name	Cultured from
A. albensis (10)	Soil, water
A. apis (11)	Honey bee intestine
A. baumannii (12)	Human, warm-blooded animals
A. baylyi (13)	Activated sludge, soil
A. beijerinckii (14)	Human, animals, soil, water
A. bereziniae (12, 15)	Human
A. bohemicus (16)	Soil, water
A. boissieri (17)	Floral nectar

Table 1. Continued.

A. bouvetii (13)	Activated sludge
A. brisouii (18)	Peat
A. calcoaceticus (12)	Soil, water, human
A. celticus (19)	Soil, water
A. colistiniresistens (20, 21)	Human
A. courvalinii (21, 22)	Human, animals
A. defluvii (23)	Hospital sewage
A. dijkshoorniae (=*A. lactucae*) (24, 25)	Human, water
A. dispersus (21, 22)	Soil, water, human
A. equi (26)	Horse
A. gandensis (27)	Horse, cattle, water
A. gerneri (13)	Activated sludge
A. grimontii (=A. junii) (13, 28)	Activated sludge
A. guangdongensis (=A. indicus) (29, 30)	Lead-zinc ore
A. guillouiae (12, 15)	Soil, water, human
A. gyllenbergii (14)	Human
A. haemolyticus (12)	Human
A. halotolerans (31)	Soil
A. harbinensis (32)	River water
A. indicus (33)	Soil
A. johnsonii (12)	Soil, water, human, animals
A. junii (12)	Human, animals, water, soil
A. kookii (34)	Soil, water
A. lactucae (25)	Lettuce
A. larvae (35)	Moth larval gut
A. lwoffii (12, 36)	Human, animals, soil, water
A. modestus (22, 37)	Human, water
A. nectaris (17)	Floral nectar
A. nosocomialis	Human
A. pakistanensis (=*A. bohemicus*) (38, 39)	Wastewater
A. parvus (40)	Human, animals
A. pittii (12, 41)	Human, soil, water

Table 1. Continued.

A. piscicola (42)	Fish
A. populi (43)	Populus bark
A. pragensis (44)	Soil, water
A. proteolyticus (22, 37)	Human
A. puyangensis (45)	Populus bark
A. qingfengensis (46)	Populus bark
A. radioresistens (12, 47)	Human, soil, cotton
A. rudis (48)	Raw milk, wastewater
A. schindleri (49)	Human, animals
A. seifertii (50, 51)	Human
A. soli (52)	Human, soil
A. tandoii (13)	Activated sludge, water, soil
A. tjembergiae (13)	Activated sludge
A. towneri (13)	Activated sludge, water, soil
A. ursingii (49)	Human
A. variabilis (53)	Human, animals, soil
A. venetianus (54, 55)	Salt water
A. vivianii (22, 37)	Human, soil, water
Tentative species designation	Cultured from
Genomic sp. 6 (12)	Human
Genomic sp. 15BJ (21)	Human
Genomic species 16 (21)	Human
Taxon 21 (37)	Human
Taxon 22 (37)	Human
Taxon 23 II (12, 37)	Soil, water, animals,human
Effectively but not validly published species name	Cultured from
A. antiviralis (56)	Tobacco plant roots
A. kyonggiensis (57)	Sewage treatment plant
A. marinus (58)	Sea water
A. oleivorans (59)	Soil
A. oryzae (= *A. johnsonii*) (60)	Rice

Table 1. Continued.

A. plantarum (= *A. junii*) (61)	Wheat
A. refrigeratoris (= *A. variabilis*) (62)	Refrigerator
A. seohaensis (= *A. towneri*) (58)	Sea water
A. septicus (= *A. ursingii*) (63, 64)	Human

It is important to highlight that *A. baumannii* together with *A. calcoaceticus, A. nosocomialis, A. pitti, A. seifertii* and *A. dijkshoorniae* (=*A. lactucae*) form the so called *Acinetobacter calcoaceticus- Acinetobacter baumannii* complex (ACB) (24, 50, 65). These ACB species are clinically relevant as are often isolated from clinical sample and are difficult to distiguish phenotypically. .

1.3. Habitat

Acinetobacter spp. can be isolated from a wide range of sources. Different species have been recovered from environmental samples such as soil or water (10, 12, 14–16, 19, 22, 32, 33), companion animals or cattle (12, 14, 22, 26, 27, 53), plants such as lettuce or cotton (12, 25, 47) and even from the digestive tract of insects (11, 35). The presence of these organisms in such different habitats led to the consideration that some of them could be ubiquitous in nature.

Some *Acinetobacter* species are known to be part of the normal microbiota in humans (5, 66) while the habitat of *A. baumannii*, the most relevant clinical species, remains unknown. It has been rarely found as a

colonizer among healthy individuals. However, *A. baumannii* has been isolated from several sources, human lice, vegetables and from hospital equipment such as mattresses, ventilator tubing or respirometers, and taken together with the high survival rates of *Acinetobacter baumannii,* creates a perfect source of infection that can cause outbreaks (67).

1.4. Species identification

Species identification within the genus *Acinetobacter* is difficult due to the similarities among them. Different methods have been described and used along the years, while the phenotypic methods are not easy to perform and unsuited for diagnostic purposes, while molecular techniques show a better species identification (68).

The gold-standard method for species delineation (within the *Acinetobacter* genus) is DNA-DNA hybridization, DDH, (12) while it can be used for species identification, it is very laborious and can not be used in the diagnostics' laboratories routine.

There are other techniques based on DNA fingerprinting, such as Amplified Ribosomal DNA Restriction Analysis (ARDRA), Amplified Fragment Length Polymorphism (AFLP), and ribotyping. ARDRA is based on the amplification and digestion with restriction enzymes (*Alu*I, *Cfo*I, *Mbo*I, *Rsa*I and *MSp*I) of a 16S rDNA PCR amplicon. The resultant pattern can be then compared with known ones (69); in AFLP the complete bacterial genome is digested with *Hind*III and *Taq*I, then a selective PCR-amplification using

adapters is made to obtain the DNA fingerprints and compare them (70).

With ribotyping, the complete bacterial DNA is digested, separated by electrophoresis, transferred to a membrane by Southern blot and then hybridized with a labeled probe for rDNA, the resulting profile is species specific (71).

In order to avoid time-consuming laborious techniques, more specific procedures based on sequencing have been developed. These methods rely on the amplification and sequencing of different genes and the comparison of the sequence to type strains. Different genes are used: 16S-23S rRNA spacer region (72), 16S rRNA (73), *recA* (74) and partial *rpoB* (68).

A *gyrB* multiplex PCR has been also designed to differentiate between species within the ACB complex (*A. baumannii*, *A. nosocomialis*, *A. calcoaceticus* and *A. pittii*); it is based on the interspecies heterogenicity and specific primers for each species are used (75, 76).

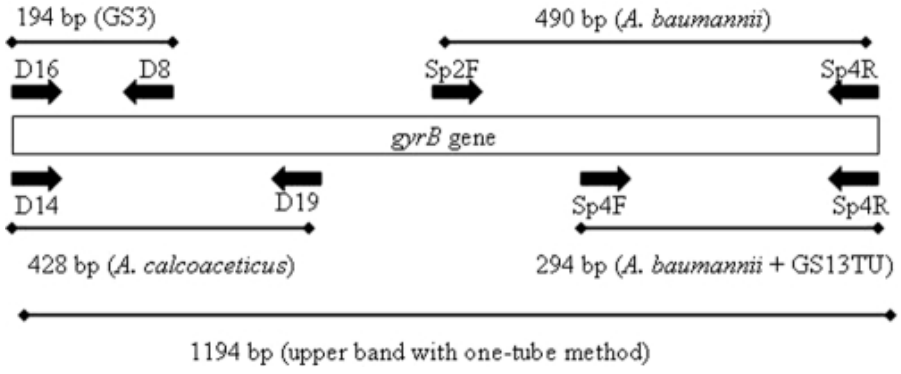

Figure 4. *gyrB* multiplex PCR, primers and PCR products (76)**.**

Another method that has been described for the identification of *A. baumannii* isolates is the detection of the intrinsic $bla_{\text{OXA-51-like}}$ carbapenemase encoding gene (77), even though further studies revealed that the detection of

bla$_{\text{OXA-51-like}}$ on its own might lead in some cases to erroneous results (78) and other species than *A. baumannii* such as *A. nosocomialis* and *A. seifertii* have been found to carry *bla*$_{\text{OXA-51-like}}$ on plasmids (79).

The most commonly used identification methods in diagnostic laboratories are semi-automated systems such as VITEK 2 (80) and MALDI-TOF MS (Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry) (81). When using MALDI-TOF MS, bacterial isolates are mixed with an organic matrix suitable for the equipment that crystalizes both matrix and sample, then the laser in a process called soft-ionization is able to separate the sample that will be analyzed and detected acording to its mass and time of flight of the different molecules, giving a spectral pattern. Every species has its own mass spectrum, which can be compared to a database to obtain the species of the unknown bacteria. As with all methods relying on databases, it is very useful for identifying already known ones, but will result in misidentification of the species that are not included in the database (81, 82). The VITEK 2 system identifies the pathogens by detecting metabolic activities such as acidification, alkalinisation, enzyme hydrolysis and growth in the presence of inhibitory substances using a fluorescence-based method, but again this system faces the same problems as MALDI-TOF MS, relying on databases of already known organisms, and in addition, both of them rely on phenotypic characteristics for the identification (80, 83).

Nowadays, with the use of next generation sequencing and the availability of a great number of complete genomes, new methods have arisen with the purpose to make identification more accurate (84, 85). As the gold

standard for species identification is still DDH, a new approach to this technique has been developed using whole genome sequencing (WGS) data and an *in silico* method, the digital DNA-DNA hybridization (dDDH) with a threshold >70% to identify the same species (86) (http://ggdc.dsmz.de). Another reliable technique using WGS data is the calculation of the average nucleotide identity (ANI) based on BLAST+ using the online tool JSpecies WS (http://jspecies.ribohost.com/jspeciesws/) (87), the identity has to be higher than 95% to be identified as the same species. In these two identification methods it is important to use type strains for comparison, as this is the official reference to which all others should be compared.

1.5. Molecular typing

After identification, typing is used in order to know the relationship among the isolates, species population. This can be achieved by different methods that are able to discriminate one strain from another, but it should be noted that these methods have different discriminatory power. The analysis of isolates' clonality within a group is important to know if the cases are unrelated or if there is an outbreak i.e. transmission of a strain, because it will allow having a deeper knowledge of the strains circulating, identifying the most virulent clones, and establishing what infection control procedures are needed, i.e. patients quarantine, identification of the source and decontamination of equipment.

Since *A. baumannii* is an opportunistic pathogen, it is important to study its epidemiology in order to prevent its spread, and establish infection control

plans as well as analyze the outbreak. Almost all the typing methods are focused in *A. baumannii* as it is the most relevant clinical species of the genus.

Different methods are available for this purpose. Pulsed Field Gel Electrophoresis (PFGE) is the Gold Standard for outbreak analysis (88). It consists of digesting the whole bacterial genome with an enzyme (*Apa*I) and then separating the resulting fragments in an agarose gel by electrophoresis switching the angle of the electric current. By this method high molecular weight DNA bands can be separated. The band pattern shows the relation among the isolates and it can be analysed by bioinformatics tools or following the Tenover criteria that classifies the isolates in four groups (indistinguishable, closely related, possibly related, and different) according to the differences among the band patterns (0, 2-3, 4-6, and ≥7, respectively (89).

Another method that has been used in the past two decades to study the clonal relatedness of the isolates have been described with two different schemes, it is called multi locus sequence typing (MLST) (90, 91). Both methods rely on the analysis of the sequences of seven housekeeping genes, each different allele for the gene has a different number and the combination of the seven numbers creates a profile for the isolate, called sequence type (ST), that can be easily compared in the online database to other STs (https://pubmlst.org/abaumannii/). The first scheme is called Oxford (90) and it uses the partial sequences of *gltA* (citrate synthase), *gyrB* (DNA gyrase subunit B), *gdhB* (glucose dehydrogenase B), *recA* (homologous recombination factor), *cpn60* (60-kDa chaperonin), *gpi* (glucose-6-phosphate isomerase) and *rpoD* (RNA polymerase sigma factor); the second scheme is Pasteur (91) and

analyses partial sequences of the following genes *cpn60* (60-kDa chaperonin), *fusA* (elongation factor EF-G), *gltA* (citrate synthase), *pyrG* (CTP synthase), *recA* (homologous recombination factor), *rplB* (50S ribosomal protein L2) and *rpoB* (RNA polymerase subunit B). Different STs that share at least 5 (double locus variant, DLV) or 6 (single locus variant, SLV) of the seven alleles, form clonal complexes (CCs). This method has a good performance for epidemiological studies but it lacks the capacity to perform a fine typing, especially the Pasteur scheme which has been already reported to have a lower discrimination capacity that the Oxford scheme (92). Another strength of this method is that can be applied to other especies within the genus.

The newest developed technology in order to deeply analyse the molecular epidemiology of *A. baumannii* is based in whole genome sequencing (93) and the comparison of a core genome, cgMLST. It is similar to MLST but the comparison is made among many more genes. The genome of *A. baumannii* ACICU strain was used as the reference genome to define the core genome, which was defined up to 2390 genes. Since this method compares over 50% of the genome, it supposes high resolution and discrimination among isolates (94).

1.6. Clinical relevant species

Not all the *Acinetobacter* species are clinically relevant, some of them have been reported to cause serious infections. The most important pathogen in the genus is *A. baumannii,* which has been isolated worldwide, causing severe

and prolonged outbreaks in hospitals, and it is often related to antimicrobial resistance (5, 95). Other species of the genus such as *A. nosocomialis* and *A. pitti* have been also described as important pathogens and they are difficult to distinguish, therefore these three species constitute the *A. baumannii* group (Ab group) (5, 96). There are two recently additions to this group, *A. seifertii* and *A. dijkshoorniae*. Both of these species have been isolated from clinical samples and can carry antimicrobial resistance genes (4, 97).

1.6.1. *Acinetobacter baumannii*

Infections caused by *Acinetobacter,* presumably *A. baumannii,* have been reported since the 1960s, in reports from Europe and North America. Most of the isolates in that time were susceptible to treatments with β-lactams or sulphonamides, in contrast to *A. baumannii* infections nowadays. By the end of 1970s, this organism was already resistant to aminoglycosides, β-lactams and sulphonamides (95).

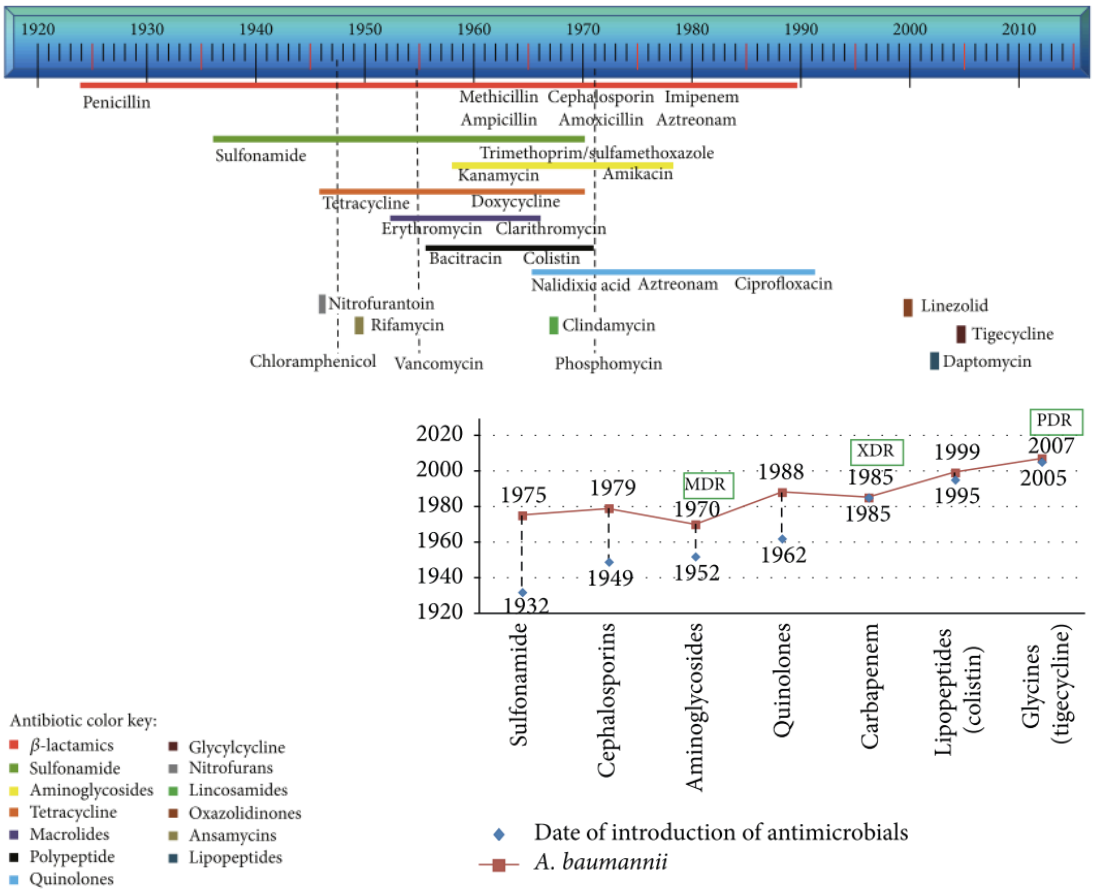

Figure 3. Top diagram shows dates of introduction of antimicrobials. The graph shows the date of introduction of antimicrobials, date of first reports of antimicrobial resistance in *A. baumannii* and the emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) (95).

Thanks to the species description in 1986 by Bouvet & Grimont (12) it was possible to more accurately identify to the species level the agent responsible for the infection, and not surprinsingly, most of the infections that were caused by *Acinetobacter* sp. were in fact caused by *Acinetobacter baumannii*.

By the end of the 1990s, multidrug-resistant *A. baumannii* causing nosocomial infections had been reported worldwide.

The most common clinical manifestations of *Acinetobacter baumannii* infections are as follows (5):

- Hospital acquired pneumonia: in almost all the studies the majority of the *A. baumannii* isolates are from the respiratory tract of the patients. It is known that the use of mechanical ventilators increase the risk of developing a ventilator-associated pneumonia caused by *A. baumannii* (5). This kind of infections are normally related to debilitated patients in ICUs (98).
- Community-acquired pneumonia: this has been described to happen in tropical areas, mostly in patients with a history of alcohol abuse. It has a mortality rate between 40-60% (5). In northern Australia, 10% of severe community-acquired pneumonia are caused by *A. baumannii* (98).
- Bloodstream infection: in a study carried between 1995 and 2002 in the USA, *A. baumannii* was responsible for 1.3% of monomicrobial bloodstream infections and the rate was higher among the ICU-acquired bloodstream infections (1.6%). The mortality was 34% to 46% in the ICU, the third highest mortality rate (5). Another study carried out in Korea analysed 180 *Acinetobacter* spp. isolated from bacteremia patients between 2003 and 2010, and *A. baumannii* comprised the 50% of the isolates, with a mortality more than double in the patients with *A. baumannii* bloodstream infection (96). The most common sources of *A. baumannii* bloodstream infections were vascular catheters and the respiratory tract; less common sources were the urinary tract and wounds (98). Some associated risk factors were mechanical ventilation, prior surgery, ICUs, prior treatment with broad-spectrum antibiotics, immunosuppression, burns, wounds, catheters and prolonged hospital stays (98).

- Skin, soft tissue and bone infection: *A. baumannii* can infect surgical or traumatic wounds, which can lead to osteomyelitis or severe tissue infection (98). These infections are normally related to prosthetic material and there has been an increase in reports of *Acinetobacter* spp. causing infections after war injuries or natural disasters such as earthquakes; *Acinetobacter* spp. were the most frequently isolated pathogens in wound infections and bloodstream infections in patients of the Vietnam war. In addition, an increase from 4% to 55% in the infections (respiratory tract, wound infections, bloodstream infections) caused by MDR *A. baumanii* in US military casualties injured in Iraq and Afghanistan was reported in a retrospective study (99).
- Urinary Tract Infection: different studies reported the presence of *A. baumannii* as the causative agent of urinary tract infection, but it is normally related to catheter-associated patients and its prevalence is always below 5% (100, 101).
- Meningitis: nosocomial meningitis caused by *A. baumannii* is related to prior neurosurgical procedures, and the mortality rate associated to this episode is 40% (91, 102).

1.6.2. *Acinetobacter seifertii*

Acinetobacter seifertii is a relatively new species within the genus *Acinetobacter*, named in 2015 by Nemec et al (50). The first two strains of this species were isolated in 1990-1991 from human clinical samples (ulcer and

blood) in Denmark (51). Since then, *A. seifertii* has been isolated from diverse clinical samples such as blood cultures or respiratory tract samples (4, 50, 103), and in some cases it carried antimicrobial encoding genes. It has also been isolated from the environment, inside (50) and outside the hospital (104).

- Bloodstream infection: in the majority of the reports *A. seifertii* causes bloodstream infections in Denmark, Japan, the United States of America and Norway (50, 51, 103).
- Ulcer: an ulcer was one of the first sources from which *A. seifertii* was recovered in Denmark (51).
- Respiratory tract infection: isolates of *A. seifertii* causing respiratory tract infections have been reported in Spain, the Czech Republic, China and Brazil (4, 50, 105, 106). The isolates found in Spain and Brazil carried the bla_{OXA-58} carbapenemase encoding gene.

2. VIRULENCE OF *Acinetobacter baumannii*

A. baumannii is an important nosocomial pathogen and this is, in part, due to its capacity to persist in the hospital environment, and the plasticity of its genome to acquire antimicrobial resistance determinants. Although its pathogenicity is not completely known, diverse virulence factors have been described.

2.1. Biofilm formation

A. baumannii is capable of forming biofilms, which are extracellular matrices that encase the bacteria within, and constitute a protection against

different hazards such as antimicrobials, macrophage attacks, desiccation, and disinfectants. Moreover, biofilms are often polymicrobial, creating the perfect environment for horizontal gene transfer, contributing to the spread of antimicrobial resistance determinants (107). It is known that biofilms are related to medical-device associated infections as biofilms are formed both in abiotic and biotic surfaces. For example, robust *A. baumannii* biofilms were found in skin and soft tissue infections as well as in health-care associated equipment such as catheter or endotracheal tubes. Factors related to biofilm formation can include: *quorum sensing* which is the capacity of the bacteria to secrete chemical signals in order to communicate to the others and measure cell density; the *csuE* gene encodes for the formation and assembly of pili, which are needed for adhesion to surfaces; the *bap* gene encoding for biofilm-associated proteins that have a role in cell-cell adhesion and maturation of the biofilm; RTX-like domain-containing protein that mediates biofilm production; PNAG (poly-β-1,6-*N*-acetylglucosamine), this extracellular polysaccharide has a role in surface and cell-to-cell adherence and it also protects bacteria against host defenses; OmpA, this outer membrane protein is involved in the bacterial attatchment to biotic surfaces; Ata, *Acinetobacter* trimeric autotransporter, contribute to adherence to extracellular matrix components (108–111).

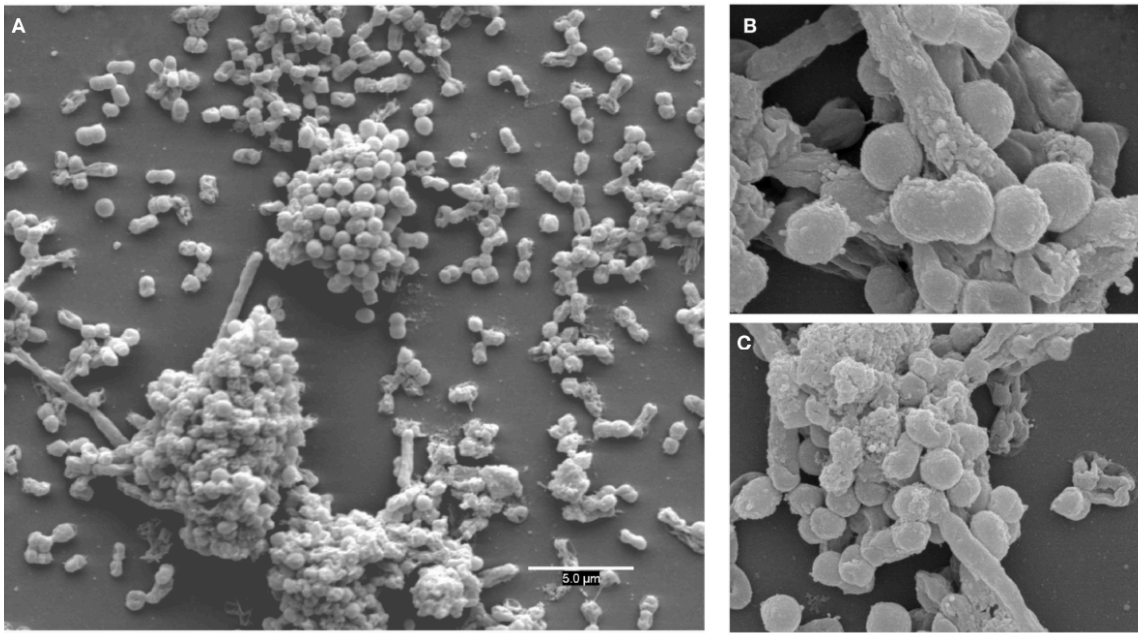

Figure 4. Scanning electron microscopy (SEM) of a biofilm-forming *A. baumannii* strain in liquid medium (108)**.**

2.2. Surface polysaccharides

The capsule in *A. baumannii* is considered to have an important role in virulence. The genes *ptk* (putative protein tyrosine kinase) and *epsA* (polysaccharide export outer membrane protein) are important for capsule polymerization and assembly, which is important for survival in human serum. PNAG, which was already described as an important component of the biofilm, also protects the bacteria against innate defenses (108).

2.3. Outer membrane proteins (OMPs)

OmpA protein from *A. baumannii* is involved in adhesion and invasion of epithelial cells and induces their apoptosis, which disrupts the mucus and helps bacteria to be internalised through the damaged epithelium. It is also related to complement resistance, increasing the capacity to cause bacteremia (108).

2.4. Outer membrane vesicles (OMVs)

OMVs have a very diverse composition (lipopolysaccharides, lipids, OMPs and DNA or RNA). Substances related to quorum sensing, transport of virulence factors or gene transfer are some of the roles OMVs play in *A. baumannii* virulence (108).

2.5. Proteolytic activity

Phospolipase C and D are important for human serum resistance, epithelial cell invasion, dissemination of bacteria, and have an important role in cytotoxicity (108).

2.6. Production of siderophores

Siderophores are structures related to iron uptake, this mechanism constitutes a virulence factor because it allows the bacteria to grow under iron-limited conditions by acquiring iron from the host, the most important siderophore in *A. baumannii* is acinetobactin (108).

2.7. Penicillin-binding proteins (PBPs)

These proteins catalyse the synthesis of peptidoglycan, the main component of the cell wall; they are also associated with cell division. The PPB-7/8 is related to cell morphology, growth and survival of *A. baumannii,* and serum resistance (108).

3. ANTIMICROBIALS FOR THE TREATMENT OF *A. baumannii* INFECTION

An antimicrobial is an agent that works be either killing the microorganisms or inhibiting their growth. An antimicrobial that can kill bacteria are called bactericidal, while those that stop their growth are called bacteriostatic. They can also be grouped according to the use they are given; disinfectants are antimicrobials that are used in abiotic surfaces such as the floor or clothes; antiseptics are used in biotic surfaces such as the skin prior to surgery, and antibiotics are used to treat patients with an infection.

There are several antimicrobial classes with different targets and sites of action, specific agents for the treatment of *A. baumannii* will be explained below in detail and a resume is shown in Figure 5.

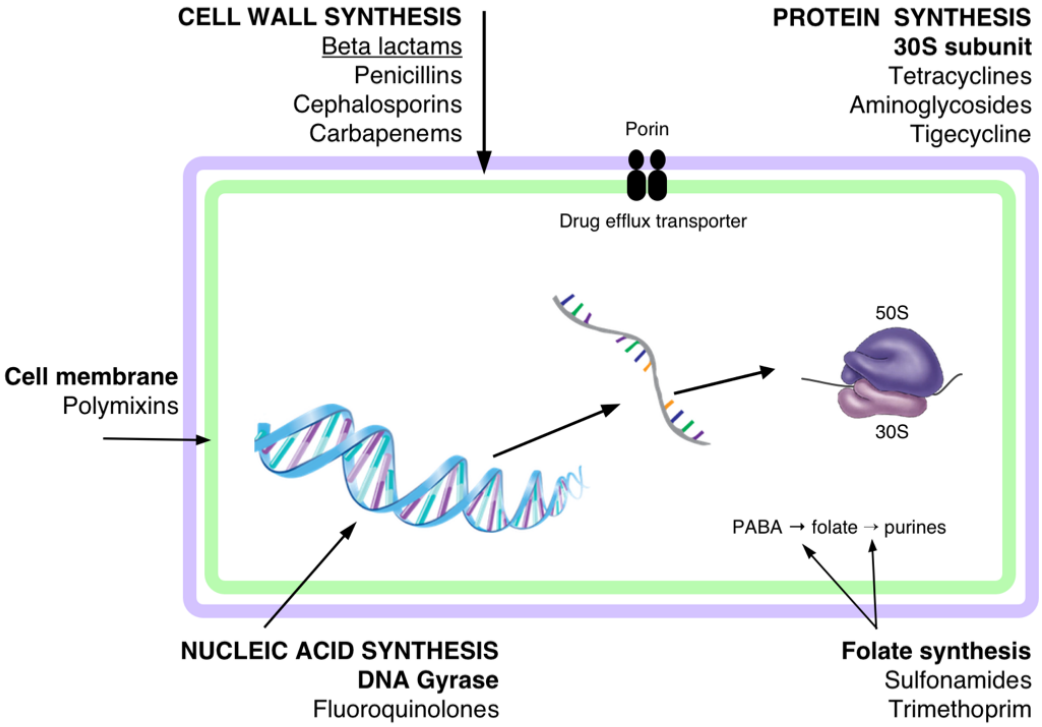

Figure 5. Mechanisms of action of different antimicrobials in Gram-negative bacteria.

3.1. Aminoglycosides

Aminoglycosided are a dose-dependent class of antibiotic that inhibits protein synthesis. They are produced by *Streptomyces* spp. and *Microsmonospora* spp. Aminoglycosides bind to the 30s ribosomal subunit, leading to the genetic code being misread, and causing an abnormal translation generating non-functional proteins. Examples of aminoglycosides that can be used to treat *A. baumannii* infections are amikacin, gentamicin, netilmicin and tobramycin. Due to their nephrotoxicity and ototoxicity, their use is restricted to severe infections such as extreme cases of sepsis (112).

3.2. β-lactams

This class of antibiotics kill bacteria by inhibiting the wall synthesis. All of them have a β-lactam ring and it targets the penicillin-binding proteins (PBPs) in the periplasm, which are involved in the wall synthesis. Penicillins (e.g. ampicillin, amoxicillin, piperacillin, ticarcillin, oxacillin), cephalosporins (e.g. cefotaxime, ceftazidime, ceftriaxone), carbapenems (e.g. imipenem and meropenem) and monobactams (e.g. aztreonam) are the four groups of antibiotics that constitute the class β-lactams. Within the group of the β-lactams, carbapenems exhibit the broadest spectrum and they, especially imipenem and meropenem, are normally used as the last resort treatment in critical patients (112, 113).

3.3. Fluoroquinolones

Fluoroquinolones are derived from quinolones, both of which are semi-synthetic compounds. Upon entering the bacterial cell, they bind to the DNA gyrase (topoisomerase II) and topoisomerase IV, which interrupts or blocks DNA replication, transcription and cell division. Some fluoroquinolones used for the treatment of *A. baumannii* infections are ciprofloxacin, levofloxacin, moxifloxacin or norfloxacin. (112).

3.4. Polymyxins

The polymyxins act upon the cell membrane, disrupting the phospholipid bilayer by a detergent-like action; this increases cell permeability and destroys the ability to act as an osmotic barrier, which leads to bacterial death. Polymixins can also affect eukaryotic cells' membranes; which is why they are nephrotoxic. Polymixin B and colistin (polymixin E) form the group and are used in infections caused by gram negatives resistant to penicillins or other broad-spectrum antibiotics (112).

3.5. Diaminopyrimidines-Sulfonamides

Trimethoprim-sulfamethoxazole (co-trimoxazole) interferes with the folic acid pathway. Sulfamethoxazole, (a sulfonamide), works at the beginning of the pathway, inhibiting the *de novo* synthesis of dihydropteroic acid; while trimethoprim works at the end of the pathway, working as a competitor of

dihydrofolate reductase, inhibiting the synthesis of tetrahydrofolic acid in the bacteria. The effect of these combination is the blocking of the production of folic acid by the bacteria that is needed for the biosynthesis of many cellular components.

3.6. Tetracyclines

Tetracyclines bind to the 30s ribosomal subunit preventing the attatchment of the tRNA. Thus the addition of amino acids to the peptide chain is stopped, preventing protein synthesis. Examples of tetracyclines are doxycycline, minocycline, tetracycline and tigecycline (112).

3.7. First line agents and alternative agents for resistant organisms

Antibiotic-susceptible *A. baumannii* infections have a broad range of therapeutic options such as broad-spectrum cephalosporin (ceftazidime or cefepime), β-lactam combined with a β-lactamase inhibitor (ampicillin-sulbactam) or a carbapenem (imipenem, meropenem or doripenem). Sometimes these agents are used in combination with antipseudomonal fluoroquinolones (e.g. imipenem + ciprofloxacin) or aminoglycosides (e.g. imipenem + amikacin) (114).

When *A. baumannii* isolates are resistant to already mentioned antimicrobials, the options for treatment are limited. Polymixins are the first choice when treating carbapenem-resistant *A. baumannii,* but these agents are

nephrotoxic and neurotoxic. Minocycline is another agent of use but few data about its outcome is available. Combination therapy is also used to treat difficult infections but there is not enough data to support or reject this approach, i.e. a polymixin plus a carbapenem (114).

4. ANTIMICROBIAL SUSCEPTIBILITY TESTING

There are different methods to test the antimicrobial susceptibility of clinical isolates.

4.1. Disk diffusion method

The oldest and most widely used is the Kirby-Bauer disk diffusion method. The EUCAST (European Committee on Antimicrobial Susceptibility Testing) disk diffusion is a standarized method based on studies and experience of some expert groups (115). Discs containing known concentrations of antibiotics are placed on Mueller-Hinton plates inoculated with a swab with a 0.5 McFarland ($1\text{-}1.5 \cdot 10^8$ CFU/mL) of the organism in saline solution. The antibiotic diffuses through the medium and inhibits bacterial growth, this inhibition can be measured and establish whether the isolate is susceptible or resistant to the tested antimicrobial. The procedure as well as the breakpoints are available for interpretation (115, 116).

This method is useful to know if the isolate is susceptible or resistant to certain antimicrobial but it is not able to get the minimal inhibitory concentration

of the antimicrobials, which is the lowest concentration that is able to inhibit bacterial growth.

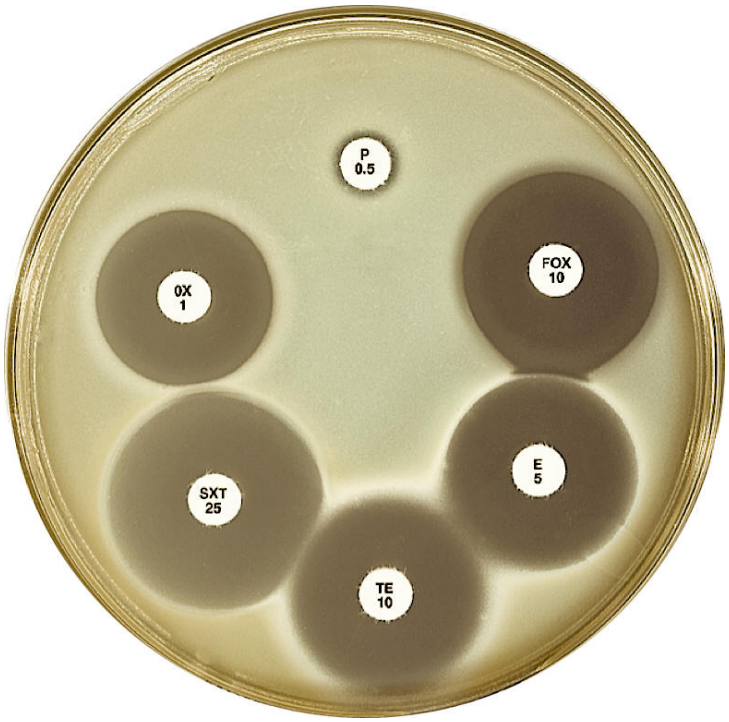

Figure 6. Disk diffusion method with inhibition areas (197)**.**

4.2. Agar dilution method

This method is used in order to determine the minimum inhibitory concentration (MIC) of an antimicrobial agent against the bacterium, the results obtained by this technique are more accurate than those obtained by disk diffusion. Plates of Mueller-Hinton agar with increasing concentration of antibiotic are prepared and several isolates are inoculated on the same plate. The MIC is the lowest concentration of the antibiotic that inhibits the growth of the bacteria.

The breakpoints for the controls as well as the isolates can be checked in the EUCAST breakpoints table (116).

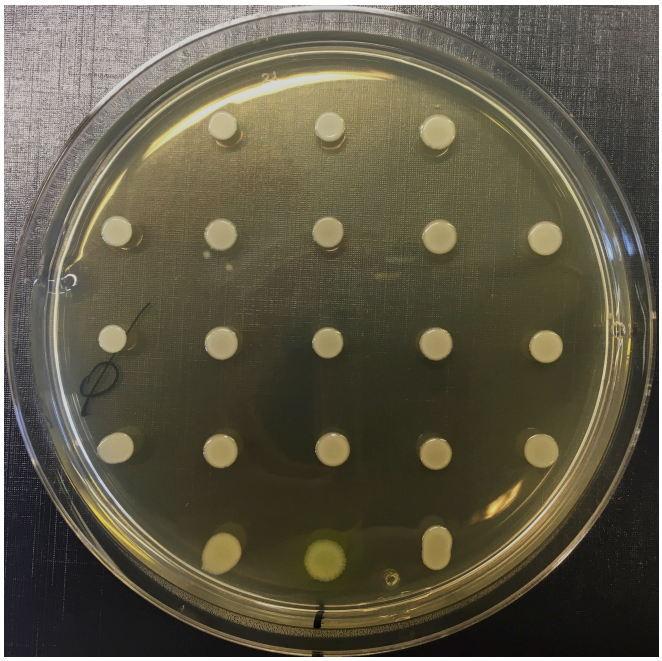

Figure 7. Agar dilution method testing 18 isolates and three controls.

4.3. Broth microdilution method

This method has been set as the only valid one in order to test MICs of antimicrobials such as colistin (polymyxin E), because colistin molecule is large and it is no able to diffuse in the agar plates. The principle of the technique is the same as in agar dilution, but using Mueller-Hinton broth instead of agar. Quality control has to be performed with *E. coli* ATCC 25922 or *P. aeruginosa* ATCC 27853 and with the colistin resistant *E. coli* NCTC 13846 (116, 117).

4.4. Multidrug-resistant, Extensively drug-resistant and Pandrug-resistant

In the last years, antimicrobial resistance rates have risen among the most common nosocomial pathogens. Because different terminology was being used to define the antimicrobial resistance patterns among the isolates, the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) gathered an expert committee to create a standardized international definition. This criterion was based on the Clinical Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the United States Food and Drug Administration (FDA). The antimicrobial categories, agents and criteria used to define MDR, XDR and PDR *Acinetobacter* spp. can be seen in Table 2.

Table 2. Antimicrobial categories and antimicrobial agents used to define MDR, XDR and PDR in *Acinetobacter* spp.

Antimicrobial category	Antimicrobial agent
Aminoglycoside	Gentamicin Tobramycin Amikacin Netilmicin
Antipseudomonal carbapenems	Imipenem Meropenem Doripenem
Antipseudomonal fluoroquinolones	Ciprofloxacin Levofloxacin
Antipseudomonal penicillins + β-lactamase inhibitors	Piperacillin-tazobactam Ticarcillin-clavulanic acid
Extended-spectrum cephalosporins	Cefotaxime Ceftriaxone Ceftazidime Cefepime
Folate pathway inhibitors	Trimethoprim-sulphamethoxazole
Penicillins + β-lactamase inhibitors	Ampicillin-sulbactam
Polymyxins	Colistin Polymyxin B
Tetracyclines	Tetracycline Doxycicline Minocycline

Criteria for defining MDR, XDR and PDR in *Acinetobacter* spp.
MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories
XDR: non-suscpetible to ≥1 agent in all but ≤2 categories
PDR: non-susceptible to all antimicrobial listed

5. MECHANISMS OF ANTIMICROBIAL RESISTANCE

Resistance to antimicrobials can be innate (intrinsic to a species), or acquired. For example, *A. baumannii* can carry genes conferring resistance to antimicrobials in both the chromosome and plasmids and this is in part due to its ability to acquire antimicrobial resistance determinants (118). For example oxacillinases, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-143-like}}$ or $bla_{\text{OXA-235-like}}$, have been reported worldwide. Some point mutations have been also described to be involved in increased antimicrobial resistance (5). Another mechanism of

resistance is the modification of the target site, which impairs its binding and activity (5).

5.1. β-lactamases

Inactivation of β-lactams is mainly produced by β-lactamase enzymes (bla). According to sequence homology, β-lactamases are grouped into four classes, A, B, C and D. Class A, C and D have serine at the active site while class B have zinc.

Class A β-lactamases hydrolyze penicillins and cephalosporins and are inhibited by clavulanate. Some of them are narrow-spectrum while others are extended-spectrum β-lactamases (ESBLs). ESBLs are enzymes that confer resistance to β-lactam antibiotics such as penicillins and cephalosporins. Diverse class A β-lactamases have been described in *A. baumannii* isolates: TEM-1, TEM-92, GES-1, PER-1, PER-2, CTX-M-2, SCO-1, VEB-1 (118). The $bla_{\text{VEB-1}}$ has been described to be associated with an upstream IS (IS26) that could be responsible for its spread (5).

Class B β-lactamases are also called metallo-β-lactamases (MBLs) and their activity is dependant on metals such as zinc. As their substrate spectrum is broad, they can hydrolyze almost all β-lactams. MBLs described in *A. baumannii* $bla_{\text{IMP-like}}$, $bla_{\text{VIM-like}}$, $bla_{\text{NDM-1}}$, $bla_{\text{SIM-1}}$, $bla_{\text{SPM-1}}$ and $bla_{\text{GIM-1}}$ (118).

A. baumannii carries an intrinsic chromosomally encoded class C β-lactamase, AmpC cephalosporinase (ADC, *Acinetobacter* derived cephalosporinase), which is sometimes found with an upstream IS (IS*Aba1*) which provides a strong promoter and leads to its overexpression (5). These β-

lactamases complicate the therapeutic treatment as they confer resistance to cephamycins, penicillins and cephalosporins, in addition they are not inhibited by β-lactamase inhibitors (118).

Class D β-lactamases are the most frequently detected carbapenem-hydrolyzing enzymes within *A. baumannii* isolates. They are called OXAs (oxacillinases) and there are six major subgroups associated with *A. baumannii*; the intrinsic *bla*$_{\text{OXA-51-like}}$, and the acquired *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{OXA-143-like}}$ and *bla*$_{\text{OXA-235-like}}$. Most of these enzymes have been, since they were first detected, reported worldwide i.e. *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$ and *bla*$_{\text{OXA-58-like}}$. Some of these enzymes are associated with upstream insertion elements such as IS*Aba1*, IS*Aba2,* IS*Aba3,* leading to their overexpression (5, 118).

5.2. Genome plasticity

A. baumannii has been shown to have a great genome plasticity, which is the capacity to acquire and disseminate genes, especially those related to antimicrobial resistance. These processes are achieved thanks to mobile elements combined with resistance genes such as insertion sequences (IS), transposons, integrons, plasmids and resistance islands (RI).

5.2.1. Insertion sequences and transposons

IS are very small mobile DNA elements (< 2.5 Kb), encoding only the information needed for their mobilization. The mobilization is mediated by a

transposase and this coding region is flanked by two inverted repeats (IR) and two direct repeats (DR) generated after transposition (119).

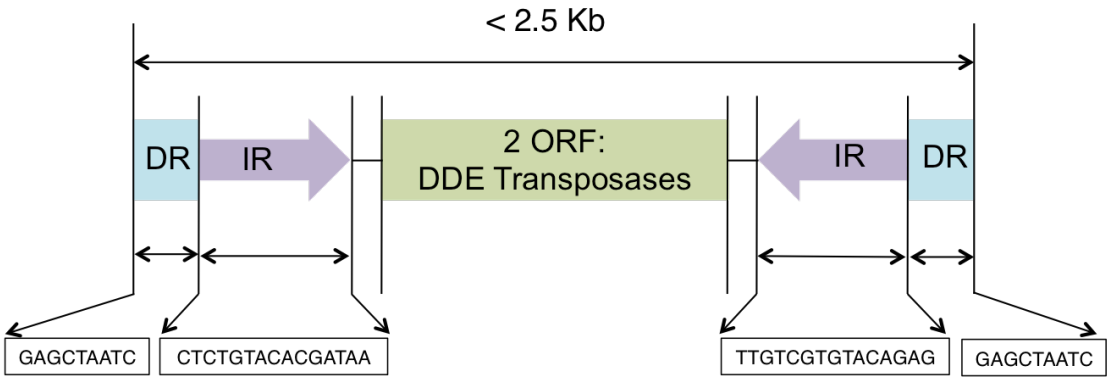

Figure 8. IS*Aba1* sequence.

IS are involved in antimicrobial resistance by three mechanisms:

- Insertion of the IS upstream the gene, which can lead to its overexpression. For example IS*Aba1* upstream the *bla*$_{\text{OXA-51-like}}$ gene is known to increase resistance to carbapenems because it promotes the overexpression of the gene (120). Other IS such as IS*Aba2,* IS*Aba3,* IS*Aba4,* IS*Aba10,* IS*Aba16,* IS*Aba18,* IS*Aba125* and IS*Aba825* have been found upstream *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58}}$ (108).

- IS elements can also insert into a coding sequence and disrupt the gene. This is the case, for example, of ISAba1 inserted into the transcriptional regulator *adeS* which controls the expression of the AdeABC efflux pump, causing its overexpression (121).

- The main mechanism of antimicrobial resistance mediated by IS is the mobilization of genes within the chromosome, between the chromosome and

plasmids, between different strains and even among bacteria from different genus.

There are different mechanisms to mobilize gene cassettes, the simplest one is when the cassette is flanked by two IS, these IS act together and move the complete DNA sequence from one IS to another, these are called compound transposons. For example the *bla*$_{OXA-23}$ gene flanked by two IS*Aba1* in inverse or direct orientation, called Tn*2006* and Tn*2009* respectively. When there is just one IS, a mechanism called one-ended transposition can move the genes by using one of the ends of the IS working together with a sequence of certain similarity in the surroundings, this is a simple transposon, the *bla*$_{OXA-23}$ gene with an inverted IS*Aba1* upstream form Tn*2008*. Another way of mobilization is the one mediated by IS from the IS*91* family (IS*CR1* and IS*CR2*) by a rolling-circle replication mechanism (108).

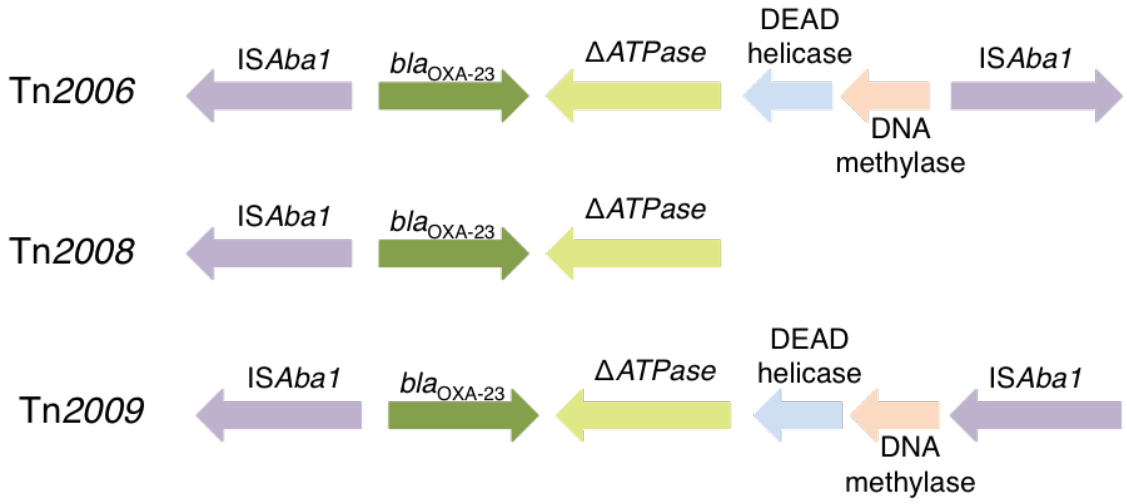

Figure 9. Genetic structure of simple and compound transposons carrying *bla*$_{OXA-23}$ in *A. baumannii.*

The genes *tet(A)* and *tetR*, encoding for a tetracycline efflux pump and its regulator, respectively, have also been found in Tn*1721-like* transposons that can be present in multiple copies in the bacterial chromosome (108).

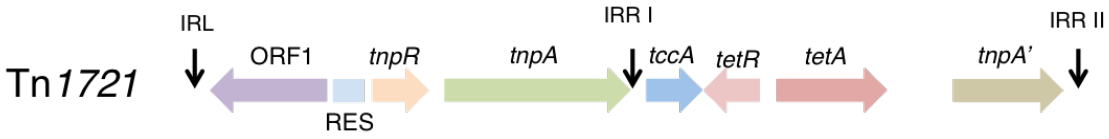

Figure 10. Genetic structure of Tn*1721*.

5.2.2. Integrons

Integrons are genetic structures that coordinate the site-specific integration of gene cassettes and regulate their expression. Integrons have a conserved region with three genes, *attI*, the specific recombination site; *intI*, integrase encoding gene and a promoter region, P1-P2; and a variable region where the resistance genes are located. Integrons are not mobile but together with IS elements or by homologous recombination they can spread to chromosomal locations, plasmids or to other strains.

Integrons in *A. baumannii* are classified by the homology of their integrases; class 1 integrons normally encode aminoglycoside and sulphonamide resistance genes and genes conferring resistance to antiseptics, as well as β-lactams. Class 2 integrons are related to Tn7 and the diversity of the genes they can carry is smaller than class 1. Some of the genes confer resistance to aminoglycosides, chloramphenicol, or trimethoprim (108).

5.2.3. Resistance islands

Large clusters of antimicrobial resistance genes together with genes conferring resistance to heavy metals have been found in the *A. baumannii* chromosome, and they are called resistance islands (RI). Mobile structures such as IS, transposons and integrons are part of RI. Usually these resistance islands are integrated in a specific site, disrupting the *comM* gene but other RI were found in other locations of the chromosome (108). Several RIs have been characterized up to now and they have been found to be related to the international clone (IC) of the strain, which provides further evidence of the independent evolution of the *A. baumannii* clonal lineages The first and largest RI in *A. baumannii* was found in the resistant strain AYE, it was called AbaR1 and carried 45 antibiotic and heavy metals resistance genes in a 86 Kb structure (108, 122). Although, several RI with very diverse structures have been reported since then.

5.2.4. Plasmids in *Acinetobacter baumannii*

Plasmids are extrachromosomal genetic material within the cell. Some of them exhibit a self-inducible transfer to new bacterial cells; this is called conjugation and it is part of the horizontal gene transfer (HGT) that is the movement of genetic material between different cells, which differs from the parent-daughter vertical transfer. Plasmids are capable of self-replication and they ensure their own survival in the bacterial cell with systems such as post-segregational killing or partitioning systems, which can cause cellular death

(123).

Conjugation is a process that takes place between two bacteria, the donor and the recipient, the donor carries plasmid encoding genes for multiplication and transfer proteins, and by the establishment of a channel connecting both cells, the replicated plasmid is able to transfer. Plasmids can also be non-transmissible or mobilizable, which means that they can only be transferred by cell division or by conjugative elements' help. Plasmids also encode stability genes, including partitioning regulation systems (type I: *parA*, *parB, parC*) that contribute to the equal distribution of plasmids between daughter cells, or toxin-antitoxin systems, that are involved in vertical stability, which means that if the daughter cell does not contain the plasmid, the toxin kills the cell. The activity of this toxin-antitoxin system relies on the stability of both components, if the daughter cell does not inherit the plasmid, the antitoxin that is higly unstable is degraded while the toxin protein is very stable and kills the cell (123).

Plasmids carry beneficial genes for the bacteria, for example virulence genes or acquired antimicrobial resistance determinants that are generally integrated in resistance cassettes related to translocation elements, that lead to an accumulation of resistance to multiple drugs. Multi-drug resistance encoding plasmids are often larger that 50 Kb and have a strict control for their replication, controlling their copy number and the stability when with another plasmid in the cell; plasmids with the same Rep (replication initiator proteins) cannot be in the same cell, this is known as plasmid incompatibility.

Plasmids in *Acinetobacter* spp. have been described to be unique and

not related to those of other genera. Different groups of plasmids within *A. baumannii* have been described according to the nucleotide identity of their replicase genes; this method is called replicon-typing and the groups are represented by AbGR (124, 125). The plasmid replicase gene or replicon is the origin of replication of the DNA. Nineteen groups comprising twenty-seven replicase genes have been stablished (AbGR1-AbGR19) in order to classify *A. baumannii* plasmids, although some other replicons that cannot be grouped according to these groups have since then been found (126, 127).

In *A. baumannii* very diverse plasmids can be found, ranging in size from 2 Kb to more than 150 Kb. The larger plasmids normally carry more than one resistance gene, but up to now little is known about these plasmids (125, 128).

Diverse plasmids have been described in the different *A. baumannii* ICs. For example, four different plasmids were found in two IC2 *A. baumannii* isolates from Malaysia; the smallest one was an 8.7 Kb plasmid with two replicons, a TonB-dependent receptor, involved in transmiting signals from the outside of the cell leading to transcriptional activation of target genes; a septicolysin encoding gene that is a cytolytic enzyme toward eukariotc cells and is involved in pathogenesis; and a Toxin-Antitoxin system. Other plasmids that were found in these two isolates were two very similar conjugative 70 Kb plasmids, encoding a *tra* locus, responsible for mating pair formation spans and plasmid mobilization, as well as genes for the T4SS, this secretion system is homologous to conjugation mechanism and is able to secrete or take up both proteins and DNA. The uptake of naked DNA from the environment is known as natural competence. The main difference among these two plasmids is that one

of them carries the *bla*$_{OXA-23}$ and the isolate carrying this plasmid has another copy of the *bla*$_{OXA-23}$ gene in the chromosome (129). An *A. baumannii* isolate belonging to IC1 contained three plasmids, a 8.7 Kb one carrying *aadB* conferring resistance to gentamicin, kanamycin and tobramycin; this 8.7 Kb plasmid was also found in IC2 along with a larger plasmid (200 Kb) carrying *sul2*, sulphonamide resistance gene, *strAB*, streptomycin resistance gene and a mercuric resistance operon (128). Different studies have reported large plasmids within IC7 *A. baumannii* isolates, all of them carrying the resistance genes *sul2, strA* and *strB* and the efflux pump encoding gene *tetB* (130, 131).

The biggest group of small plasmids in *A. baumannii* comprises the Rep-3 superfamily (*repB*). This replicon is sometimes accompanied by a conserved domain called *repA*, although it has no similarities to any known replicase; *repB* can also be on its own. This Rep-3 superfamily group of plasmids encode for Type II toxin-antitoxin systems, TonB-dependent receptors, septicolysin and Sel1-repeat protein, and some of them also encode MobL or MobA mobilization proteins, making them mobilizable by other self-transmissible plasmids.

There is a group of very small enigmatic plasmids grouped in the Rep-1 superfamily, they encode a rep gene and from two to five ORF of unknown function (132).

Another plasmid group derived from the same ancestor plasmid has been described, these plasmids carry the *aadB* gene conferring resistance to aminoglycosides such as gentamicin, kanamycin and tobramycin; as well as mobilization proteins (MobA) and other hypothetical proteins (132).

5.3. Efflux pumps

Efflux pumps play an important role in *A. baumannii* antimicrobial resistance as they can confer resistance to many different classes of antibiotics such as β-lactams, chloramphenicol, tetracycline, aminoglycosides and tigecycline, as well conferring resistance to antiseptics and disinfectants. Five super-families of chromosomally encoded efflux pumps have been associated with antimicrobial resistance in *A. baumannii,* ATP-Binding Cassette transporters (ABC), multi-drug and toxic compound extrusion (MATE), resistance-nodulation-cell division (RND), major facilitator superfamily (MFS) and small multi-drug resistance families (SMR) (133). The ABC family, MATE family, MFS family and SMR family are located on the inner membrane and all of them have a narrow substrate specificity, being able of transporting drugs from the cytosol into the periplasm, while the RND family are transmembrane proteins with a broad substrate specifity and they pump drugs from the periplasm out of the cell. These efflux pumps may be specific for one substrate or able to transport a wide range of substrates.

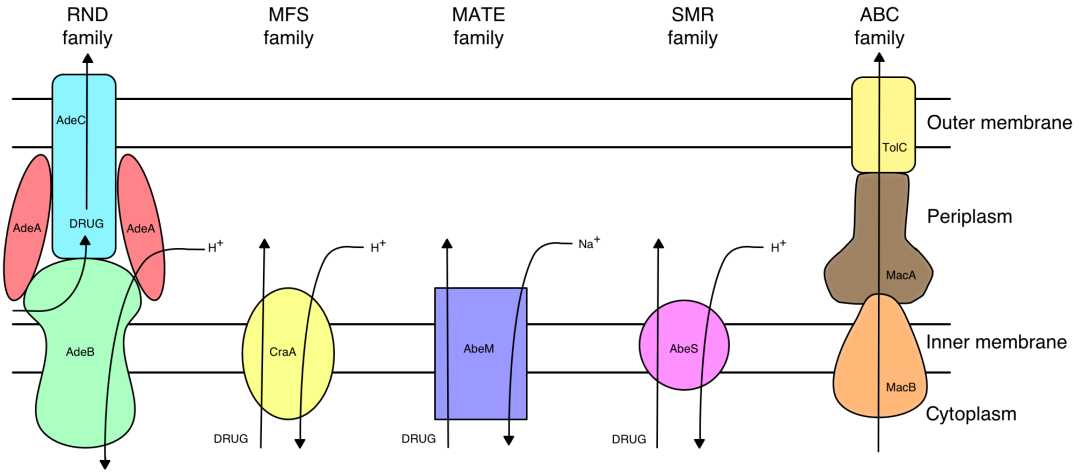

Figure 11. Efflux pumps involved in antimicrobial resistance in *A. baumannii* (134)**.**

5.3.1. RND efflux systems

AdeABC: it was the first RND system characterized in *A. baumannii*. It is encoded in an operon and when it is overexpressed, either by mutations in the *adeRS* genes, or by the insertion of IS*Aba1* in *adeS,* it confers resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, tigecycline, macrolides, chloramphenicol and trimethoprim. In a study, this *adeABC* operon was found in 80% of *A. baumannii* isolates (133).

AdeIJK: it was the second RND efflux system described in *A. baumannii.* This RND pump confers intrinsic resistance to β-lactams, fluoroquinolones, tetracycline, tigecycline, lincosamides, rifampin, chloramphenicol, cotrimoxazole, novobiocin and fusidic acid. This pump works in a synergistic way with AdeABC to eliminate some compounds (118).

AdeFGH: it is encoded in an *adeFGH* operon, regulated by the upstream *adeL* and its overexpression confers high-level resistance to

fluoroquinolones, chloramphenicol, trimethoprim and clindamycin and some low level resistance to tetracyclines, tigecycline and sulfamethoxazole (133).

AdeABC and AdeIJK are able, in addition to extrude other compounds such as detergents, biocides, antiseptics and dyes.

5.3.2. MFS family

CraA: it confers resistance to chloramphenicol but it is still not known if it is constitutively or only after overexpression. The gene that encodes the pump structure is overexpressed in presence of NaCl (133).

5.3.3. MATE

AbeM: the role of this pump is still hypothetical although it is known that it extrudes aminoglycosides, fluoroquinoloes, chloramphenicol, trimethoprim, ethidium bromide and dyes (133).

5.3.4. SMR

AbeS: this pump confers low-level resistance to chloramphenicol, fluoroquinolones, erythromycin and novobiocin, as well as, dyes and detergents (133).

5.3.5. ABC transporters

MacB: this inner membrane transporter has been described in *A. baumannii* forming a transmembrane pump together with MacA, a membrane fusion protein, and TolC, an outer membrane protein that pumps out of the cell. Macrolides are one of its substrates (135).

5.3.6. Acquired efflux systems

Several studies have reported the acquisition of efflux pumps by *A. baumannii* as being part of mobile structures such as plasmids, transposons or resistance islands. For example, the genes encoding for efflux pumps TetA (conferring resistance to tetracycline) and TetB (conferring resistance to tetracycline and minocycline) from the MFS have been found in plasmids in *A. baumannii*. Structural genes for MFS, CmlA and FloR, that confer resistance to chloramphenicol have been described within the resistance island AbaR1. Additionally, the gene *qacE* has been found in the same resistance island, it encodes a SMR efflux pump, which can confer resistance to quaternary ammonium compounds (133).

5.4. Outer membrane proteins

Porins are pores in the outer membrane that allow the passive diffusion of various compounds into the periplasm, some of which are involved in virulence, also play a role in antimicrobial resistance. Reduced expression of the porins, CarO, Omp22-33, Omp33-36, Omp37, Omp43, Omp44 and Omp47

are associated with carbapenem resistance. Reduced expression of OmpA is also involved in aztreonam, chloramphenicol and nalidixic acid resistance (118).

5.5. Aminoglycoside modifying enzymes

There are many genes encoding for aminoglycoside modifying enzymes (AMEs), which are the major aminoglycoside resistance mechanism; they are grouped into acetyltransferases (AACs), adenyltransferases (ANTs) and phosphotransferases (APHs). Some examples are AACs: AAC3, AAC(6'); ANTs: ANT(2") or *aadB*, ANT(3") or *aadA1*; and APHs: APH(3') or *aphA1* and APH(3"). The specificity of these enzymes normally confers resistance to only one or two aminoglycosides; they catalyse the modification of the aminoglycoside, inhibiting its capability to bind to the 30s ribosomal subunit and confering high-level resistance to gentamicin, tobramycin and amikacin (5, 118, 136).

5.6. Target modification

The modification of the target site of an antimicrobial can lead to antimicrobial resistance as its action is impaired. For example, ribosomal modification by 16s rRNA methyltransferases (16s RMTases) which modifies the binding site of the antibiotic, is another mechanism of aminoglycoside resistance. Some of the 16s RMTases that have been described in *A. baumannii* are ArmA and RmtB (136). Mutations in the genes *gyrA* and *parC* confer quinolone resistance, as these mutations change the antibiotic binding

site. By the action of TetM binding to the ribosome, tetracycline resistance is also increased as TetM protects the ribosome and the drug is released from its binding site; loss of LPS, or addition of phospoethanolamine to LPS by the PmrABC system have also been described to decrease the susceptibility to many antibiotics such as polymixins (5, 118)

6. EPIDEMIOLOGY OF *Acinetobacter* spp. IN LATIN AMERICA

6.1. *A. baumannii*

A. baumannii has been defined as clonal in nature and eight major groups to classify the isolates have been identified and were termed International Clones (ICs) due to the fact that they are spread worldwide (IC1, IC2, IC3, IC4, IC5, IC6, IC7 and IC8). Although some of these clones have been reported in many parts of the world, published data suggests that the clones have originated in different locations and then they have spread to other places (3). *A. baumannii* isolates can persist in the hospital settings for many years while acquiring multiple antimicrobial resistance determinants and then they can spread again and cause an outbreak (137). The presence of different international clones (ICs), such as IC4, IC5, and IC7 have been isolated in several Latin American countries, and this differs from the epidemiological situation found in Europe or North America, where the majority of the isolates belong to IC1, IC2 or IC3; with IC2 the most prevalent and widespread worldwide (3, 138). IC2 has been reported related to $bla_{\text{OXA-23-like}}$ carbapenemase gene, but this clone can acquire other OXAs, and OXA-23 is

foundin other ICs (139). But even though the situation is different, the incidence of carbapenem-resistant *A. baumannii* (CRAb) is also increasing in Latin America (3, 137, 140–142). The nonsusceptibility rates to ceftazidime (76%), ciprofloxacin (65%), gentamicin (72%), imipenem (53%) and meropenem (58%) of this pathogen in Latin America appear to be higher than in other parts of the world according to the PAHO report from 2014, however the results in this study might be skewed because of inaproppiate species identification which can lead to the inclusion of non-baumannii isolates that are normally less resistant to antimicrobials (143).

The dissemination of these ICs is often associated with antibiotic resistance, especially resistance to carbapenems. The spread of these ICs mirrors the increase of circulating carbapenemase-encoding genes such as $bla_{\text{OXA-23}}$ which has now been widely reported worldwide (137, 141, 144). In previous studies carried in Latin American countries the predominant carbapenem resistance determinant was $bla_{\text{OXA-58}}$ but it has recently been replaced by $bla_{\text{OXA-23}}$ (137, 145, 146).

For example, IC4 ($CC15^{P}$) has been described in a study comprising 69.4% of the isolates between 2009-2011 in a Brazilian hospital, all these isolates carried the carbapenem-resistance gene $bla_{\text{OXA-23}}$ (142). This IC has been also found in other Latin American countries such as Argentina and Chile, normally representing unrelated or sporadic cases (3). In the previously mentioned study, IC5 ($CC79^{P}$) comprised just 10% of the isolates, it appears to be the most prevalent among other studies carried out in Latin American countries (147, 148). IC5 has been mainly isolated in North, Central and South

America and it received the name Pan-American clone because of this (3). On the other hand, IC7 ($CC25^{P}$) has been reported worldwide and associated with diverse antimicrobial resistance determinants such as bla_{NDM-2}, bla_{OXA-72}, bla_{OXA-58} and bla_{OXA-23} (3, 139, 149). In Latin America, this clone has been reported in hospitals in Brazil, Paraguay, Bolivia, Argentina, Colombia, Mexico and Venezuela (3, 141, 142). Two sporadic *A. baumannii* isolates belonging to IC5 and IC7, respectively, have recently been isolated from neonates in a hospital in Brazil (150) while in Colombia most of the isolates recovered in a 2017 study belonged to $CC636^{Ox}$ (IC5) followed by $CC110^{Ox}$ (IC7) (151).

Furthermore, there has been an increase of CRAb isolates from 27% in 2006 to 76% in 2009 along with the high prevalence of this pathogen in Latin America, however Bolivia had the lowest resistance rates of 19% and 7% to imipenem and meropenem, respectively, but as the identification method is not always clear, the same problem of misidentification as previously mentioned can be faced (137). The increasing incidence of CRAb was confirmed in studies carried out in 2012 and 2015 in the hospitals of the city of Cochabamba, where carbapenem resistance was found to be 35% and 90%, respectively (145, 149).

6.2. *A. seifertii*

Acinetobacter seifertii is one of the more recently named members of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (50). The first two strains of this species were isolated from human clinical specimens (ulcer and blood) in Denmark in 1990-1991 and they were named *Acinetobacter* gen. sp. ‘close to 13TU’ (50, 51). *A. seifertii* has since been found in diverse clinical

samples mainly in countries in Southeast Asia and South America, and it has been also isolated from environmental samples both inside and outside the hospital (4, 50, 103, 104, 106). Although the clinical relevance of *A. seifertii* seems to be lower than that of other members of the complex, reports of its incidence causing infections have been published recently. In addition, some isolates were shown to have acquired antimicrobial resistance genes such as the plasmid encoded carbapenemase *bla*$_{\text{OXA-58}}$, and it has even been reported to have acquired the *A. baumannii* intrinsic *bla*$_{\text{OXA-51}}$ and can therefore make anti-infective therapy difficult (4, 79).

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OBJECTIVES

The objectives of this thesis were to:

1. Characterize the clinical *Acinetobacter* spp. isolates from the main hospitals of Cochabamba, Bolivia, Hospital Materno-Infantil and Hospital Viedma, and investigate their molecular epidemiology.

2. Determine their resistance to antibiotics and the associated mechanisms conferring resistance to different groups of antibiotics.

3. Investigate the structure of mobile genetic elements such as transposons, plasmids, or resistance islands that are responsible for the spread of the antimicrobial resistance determinants among isolates.

4. Correctly identify *Acinetobacter* spp. isolates from Hospital Materno-Infantil and Hospital Viedma.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Bacterial isolates

Two independent groups of isolates were studied. A total of thirty-six *A. baumannii* isolates were collected between March 2014 and May 2015 at Hospital Materno-Infantil in Cochabamba, Bolivia. This 208-beds children hospital is a tertiary-care hospital with 14 beds in the intensive care unit. The isolates were first identified at the hospitals' laboratories as *Acinetobacter* spp by phenotypic methods such as Gram-negative by Gram staining; non-fermenter (K/K) by triple sugar iron agar; negative motility in brain heart infusion broth; negative oxidase, negative gelatin liquefaction, and negative hemolysis.

Table 3. *A. baumannii* Isolates recovered between March 2014 and May 2015 in Hospital Materno Infantil.

Isolate	Hosp. code	Date	Sex	Age	Sample	Diagnostic
MI1	287	03/14	F	5 d	Blood Culture	Sepsis
MI2	247	03/14	-	3 d	Respiratory Tract	Preterm neonate
MI3	243	04/14	M	1 m	Respiratory Tract	Neumonía neonatal
MI4	296	04/14	M	10 d	Blood Culture	Preterm neonate Congenital syphilis
MI5	275	04/14	M	11 d	Catheter	Preterm neonate Congenital syphilis
MI6	275	04/14	M	4 d	Blood Culture	Gastroschisis
MI8	200-2	04/14	F	4 d	Blood Culture	Sepsis
MI9	295	04/14		4 d	Blood Culture	Sepsis
MI11	287	06/14	F	7 d	Blood Culture	Preterm neonate
MI12	286	06/14	F	13 d	Blood Culture	Pneumonia
MI14	260	10/14	M	2 d	Catheter	Preterm neonate
MI17	262	10/14	M	20 d	Surgical wound	Sepsis
MI18	423	11/14	F	3 m	Blood Culture	Sepsis
MI19	269	11/14	M	8 d	Blood Culture	Sepsis
MI22	434	11/14	F	2 y	Surgical wound	Cerebral tumor
MI23	7	11/14			Environmental	Anesthesiology machine
MI24	432	11/14	F	2 y	Cerebrospinal fluid	Cerebral tumor
MI25	16	11/14			Environmental	Stretcher
MI26	268	11/14	M	6 d	Cerebrospinal fluid	Sepsis
MI28	44	12/14	F	14 y	Aspirate	Pneumonia
MI30	13	12/14	F	14 y	Aspirate	Pneumonia
MI31	410	12/14	F	11 m	Aspirate	Uremic hemolytic syndrome
MI33	424	12/14	F	11 m	Peritoneal fluid	Uremic hemolytic syndrome
MI38	413	01/15	F	1 d	Blood Culture	Sepsis
MI39	407	02/15	F	1 y	Cerebrospinal fluid	Meningitis
MI41		02/15	M	2 y	Abscess	Abscess
MI42		03/15	M	8 y	Respiratory Tract	Pneumonia
MI44	406	04/15	M	1 m	Respiratory Tract	Pneumonia
MI45	421	04/15	M	1 m	Respiratory Tract	Pneumonia
MI47	423	04/15	M	2 y	Respiratory Tract	Acute respiratory infection
MI50	412	04/15	-	-	Respiratory Tract	Pneumonia
MI51	46	05/15			Environmental	Operating room tripod
MI52	15	05/15	M	8 y	Exudate	Tissue infection
MI53	33	05/15	F	10 y	Catheter	Sepsis
MI54	3	05/15			Environmental	Student's hands
MI59	8	-	M	10 y	Respiratory Tract	Pneumonia

A total of ninety-eight isolates recovered between September 2015 and December 2016 from two geographically close hospitals, Hospital Materno Infantil and Hospital Viedma in the city of Cochabamba, Bolivia, were included in the study. These two hospitals have a combined total of 408 beds and 21 ICU beds. All isolates were previously identified by biochemical methods at the hospital clinical laboratories as previously stated.

Table 4. *A. baumannii* isolates recovered between September 2015 and July 2016 in Hospital Materno Infantil.

Isolate	Hosp. code	Date	Sex	Age	Sample	Diagnostic
MC1	421-269	09/15	M	9 m	Catheter	Sepsis
MC2	26-268	10/15	M	9 y	Endotracheal tube	Myeloid leukemia
MC3	14-386	10/15	F	28 y	Tracheal Secretion	Organophosphate poisoning
MC4	200-24-501	10/15	F	33 y	Retroculture	Hysterectomy
MC5	417-517	10/15	F	1 m	Tracheal aspirate	Bronchiolitis
MC6	421-237	11/15	M	9 m	Endotracheal tube	Pericardial effusion
MC7	35-481	11/15	F	35 y	Tracheal Secretion	Pulmonary fibrosis
MC8	43-735	11/15	F	8 y	Tracheal Secretion	Tracheal infection
MC9	363-830	01/16	M	7 m	Abscesse Secretion	Parapharyngeal abscess
MC10	47-942	01/16	M	12 y	Catheter	Ileostomy
MC12	25-299	02/16	M	13 y	Pleural Tube	Pleural effusion
MC13	30-324	03/16	F	59 y	Abscesse Secretion	Abdominal abscess
MC14	200-26-501	03/16	F	5 d	Blood Culture	Sepsis
MC15	201-825	04/16	M	3 d	Blood Culture	Sepsis
MC16	421-133	04/16	F	2 y	Catheter	Sepsis
MC17	405-156	04/16	F	5 d	Blood Culture	Sepsis
MC18	412-201	04/16	F	2 y	Foley Catheter	Sepsis
MC19	413-202	04/16	F	2 y	Blood Culture	Sepsis
MC21	5-849	05/16	M	8 y	Tracheal Secretion	Pneumonia
MC22	236-21	07/16	F	43 y	Bronchial Secretion	Puerperium

Table 5. *A. baumannii* isolates recovered between January 2016 and December 2016 in Hospital Viedma.

Isolate	Hosp. code	Date	Sex	Age	Sample	Diagnostic
MC23	226	01/16	M	71 y	Urine Culture	-
MC24	271	01/16	M	28 y	Surgical Wound	Necrotizing fasciitis
MC25	300	01/16	F	17 y	Abdominal Secretion	Septicemia
MC27	758	02/16	F	76 y	Pressure ulcer	Traumatic brain injury
MC29	866	02/16	F	51 y	Urine Culture	-
MC30	863	02/16	M	37 y	Knee-joint exudate	Fracture
MC31	928	02/16	M	19 y	Catheter	-
MC32	1054	02/16	F	53 y	Ulcer	Infected ulcer
MC33	1229	02/16	M	74 y	Tracheal secretion	Sepsis
MC34	1330	02/16	F	30 y	Tracheal secretion	Burn
MC35	1369	02/16	M	33 y	Knee-joint exudate	Pressure ulcer
MC37	1371	02/16	M	33 y	Knee-joint exudate	Pressure ulcer
MC38	1417	02/16	M	75 y	Urine Culture	-
MC39	1614	03/16	M	75 y	Urine Culture	-
MC40	1657	03/16	F	33 y	Tracheal secretion	Epidural hematoma
MC41	1718	03/16	M	63 y	Urine Culture	-
MC42	1735	03/16	M	76 y	Tracheal secretion	Traumatic brain injury
MC43	1738	03/16	M	76 y	Blood culture	-
MC44	1987	03/16	F	53 y	Wound secretion	Burn
MC45	2053	03/16	M	65 y	Bronchial aspirate	Septic shock
MC47	2071	03/16	M	40 y	Tracheal secretion	Hypovolemic shock
MC48	2074	03/16	M	65 y	Secretion	Prostate cancer
MC49	2079	03/16	M	37 y	Tracheal secretion	Craniotomy
MC50	2082	03/16	M	65 y	Tracheal secretion	Sepsis
MC51	2090	03/16	M	65 y	Lumbosacral ulcer	-
MC52	2104	03/16	M	28 y	Secretion	Necrotizing fascitis
MC53	79	04/16	M	20 y	Wound secretion	Wound
MC54	155	04/16	F	61 y	Ulcer	Infected ulcer
MC55	185	04/16	M	38 y	Tracheal secretion	Craniotomy
MC56	235	04/16	M	38 y	Dental Abscess	Dental abscess
MC57	542	04/16	M	62 y	Tracheal secretion	Pneumonia
MC58	842	05/16	M	37 y	Wound secretion	Bone surgery
MC59	1110	05/16	M	34 y	Catheter	-
MC60	1131	05/16	F	75 y	Catheter	-
MC61	1133	05/16	F	75 y	Tracheal secretion	Sepsis
MC62	1154	05/16	F	34 y	Tracheal secretion	Sepsis
MC63	1237	05/16	F	86 y	Fluid (Bone tissue)	Bone tissue infection
MC64	1245	05/16	M	36 y	Urine Cult	-
MC65	1319	05/16	F	17 y	Tracheal secretion	Tube exchange catheter
MC66	1350	05/16	F	61 y	Tracheal secretion	Cerebellum tumor
MC67	1783	06/16	M	76 y	Urine Culture	-
MC68	1797	06/16	M	27 y	Tracheal secretion	Tube exchange catheter
MC69	1826	06/16	F	86 y	Wound secretion	Tissue infection
MC70	1955	06/16	F	41 y	Fuid	-

Table 5. Continued.

MC71	87	07/16	M	66 y	Exudate (leg)	Osteomyelitis
MC72	316	07/16	F	61 y	Tracheal secretion	Septic shock
MC90	939	08/16	M	90 y	Blood culture	-
MC79	1409	09/16	M	20 y	Tracheal secretion	Tube exchange catheter
MC80	1642	09/16	F	90 y	Exudate (leg)	Infected ulcer
MC81	1670	09/16	M	76 y	Wound secretion	Infected wound
MC82	1798	09/16	M	75 y	Secretion	Septic arthritis
MC83	1861	09/16	M	24 y	Secretion	Fasciitis
MC84	1864	09/16	F	56 y	Ulcer	Pressure ulcer
MC85	1886	09/16	F	82 y	Secretion (tumor)	Surgical wound
MC86	1892	09/16	F	67 y	Tracheal secretion	-
MC87	1901	09/16	M	83 y	-	Pneumonia
MC88	1951	09/16	M	72 y	Cervical abscess	-
MC89	1978	10/16	F	56 y	Ulcer	Infected ulcer
MC73	138	10/16	F	56 y	Ulcer	Infected ulcer
MC74	265	10/16	F	45 y	Wound secretion	Abscesse
MC75	289	10/16	M	84 y	Ulcer	Septicemia
MC76	383	10/16	M	52 y	Tracheal secretion	Tube exchange catheter
MC78	485	10/16	M	63 y	-	Gastric cancer
MC77	386	10/16	F	30 y	Tracheal secretion	Acute respiratory infection
MC91	679	11/16	M	71 y	Urine Cult	Chronic kidney disease
MC93	739	11/16	F	66 y	Wound secretion	Burn
MC94	740	11/16	F	66 y	Wound secretion	Burn
MC95	863	11/16	F	75 y	Tracheal secretion	Traumatic brain injury
MC96	1000	11/16	F	41 y	Tracheal secretion	Meningoencephalitis
MC98	1200	11/16	M	66 y	Wound secretion	Chronic kidney disease
MC99	1307	11/16	F	70 y	Tracheal secretion	Traumatic brain injury
MC100	1318	11/16	F	68 y	Tracheal secretion	Tube exchange catheter
MC101	1349	11/16	M	71 y	Blood culture	Chronic kidney disease
MC102	1428	11/16	M	63 y	Ulcer	Renal tumor
MC103	1429	11/16	M	29 y	Pleural Fluid	-
MC104	1481	12/16	F	52 y	Wound secretion	Cellulitis
MC105	1620	12/16	M	66 y	Diabetic Foot	Diabetic Foot

2. Species identification

All PCR based methods were performed using the Taq PCR Master Mix Kit (Qiagen) or the KAPA2G Fast HotStart ReadyMix PCR kit (KapaBiosystems).

2.1. *gyrB* multiplex PCR

The isolates were confirmed to be *A. baumannii* by *gyrB* multiplex PCR. *gyrB* is a conserved gene within *Acinetobacter* spp. but using the differences in its sequence among the different species, a multiplex PCR was designed in order to differentiate the following species: *A. baumannii, A. pitti, A. nosocomialis* and *A. calcoaceticus* (75, 76).

2.2. Presence of *bla*$_{\text{OXA-51-like}}$

The presence of the *bla*$_{\text{OXA-51-like}}$ has been described to be an indicator of the isolates belonging to *A. baumannii* as this gene is intrinsic to this species (77).

2.3. Semi-automated systems

Semi-automated systems such as VITEK®2 7.01 with the GN ID card (bioMérieux) and MALDI-TOF MS, Bruker MALDI Biotyper®, Compass IVD software (Bruker Daltonik GmbH) with HCCA portioned matrix were used for species identification.

2.4. Whole genome sequencing

Total DNA extraction was performed from an overnight culture of a single colony incubated in 10 mL Luria-Bertani broth at 37°C, using the MagAttract HMW DNA Kit (Qiagen) following the manufacturer's instructions. Quality of the DNA was determined by UV spectrophotometry using a NanoDrop Spectrophotometer (Thermo Scientific) considering a ~1.8 A260/280 ratio as good; DNA concentration was determined using a Qubit dsDNA HS assay system (Thermo Fisher Scientific).

DNA concentration was diluted to 0.2 ng/μL in nuclease-free water (SIGMA) and sequencing libraries were prepared using the Nextera XT library prep kit (Illumina GmbH). Tagmentation of the samples was performed using the Nextera XT transposome, which fragments the DNA and adds adapter sequences to its ends in a program at 55°C for 5 min and then decreasing the temperature to 10°C. Once the samples were tagmented, a limited-cycle PCR (Table 10) was performed in order to add the indexes/barcodes to the sequences; a different combination of an i7 and an i5 index/barcode for each sample. The addition of different indexes/barcodes to each sample allows for multiplexing and the consequent identification of the sequences belonging to each sample.

Table 6. Limited-cycle PCR for indexing

Time	Temperature	
3 minutes	72°C	
30 seconds	95°C	
10 seconds	95°C	
30 seconds	55°C	12 cycles
30 seconds	72°C	
5 minutes	72°C	
Hold	10°C	

AMPure XP beads were used to purify the PCR products, a size selection is carried out in the library DNA, removing the very short DNA fragments in the library with two ethanol washings as well as primers, DNA templates and all the components that are no further required. After the cleanup, a normalization step was performed in order to ensure all libraries are equally represented. As the final step, prior to starting the sequencing, equal volumes of the normalized libraries were combined into a pooled sample, diluted in hybridization buffer and denaturated by heating. The pooled library was loaded into the reagent cartridge together with a PhiX control and MiSeq sequencer for a 250bp paired-end sequencing run on an Illumina MiSeq sequencer.

Sequencing quality had to fulfill the manufacturer's minimum specifications. The resulting FASTQ files containing paired reads were *de novo* assembled with the Velvet 1.1.04 assembler using the Ridom SeqSphere+ v.3.0 software (152) and SPAdes 3.9 (https://cge.cbs.dtu.dk/services/SPAdes/) (153).

Further analysis was performed in some isolates.

- The CGE web-tools SpeciesFinder 1.2 (154), based on the complete 16s rRNA gene for species identification, and KmerFinder 2.0 (154, 155), that evaluates the number of co-occurring k-mers (strings of k nucleotides in DNA sequence data) were used (https://cge.cbs.dtu.dk/services/).

- Multi locus sequence analysis (MLSA) using CLUSTAL 2.1 was performed; the concatenated sequences of the seven housekeeping genes used for the Pasteur MLST scheme were compared to those of forty-three reference strains (seven *A. calcoaceticus*, eighteen *A. baumannii,* six *A. nosocomialis,* ten *A. pittii*, two *Acinetobacter* genomic species "between 1 and 3"*)* and fifteen *A. seifertii* strains used by Nemec *et al* to describe the species (50).

- The digital DNA–DNA hybridization (dDDH) parameter was calculated between the three studied strains and the type strains, *A. baumannii* CIP 70.34 ^{T}*, A. calcoaceticus* CIP 81.8^{T}*, A. dijkshoorniae* JVAP01^{T}*, A. nosocomialis* NIPH 2119^{T}*, A. pittii* CIP 70.29^{T} and *A. seifertii* NIPH 973^{T} using the GGDC 2.1 (http://ggdc.dsmz.de) program, with the recommended parameters and threshold value for species, 70%.

- In addition, JSpeciesWS webserver (http://jspecies.ribohost.com/jspeciesws/) was used to determine the average nucleotide identity (ANI) based on BLAST+ (ANIb) for species identification using the complete genomes and comparing them to the type strains *A. baumannii* CIP 70.34^{T}*, A. calcoaceticus* CIP 81.8^{T}*, A. dijkshoorniae* JVAP01^{T}*, A. nosocomialis* NIPH 2119^{T}*, A. pittii* CIP 70.29^{T} and *A. seifertii* NIPH 973^{T}. A cut-off at 95% was used.

3. Identification of antibiotic resistance genes

3.1. Molecular determination of carbapenemases

A multiplex PCR was performed to detect the presence of genes encoding *bla*$_{OXA}$ carbapenemases (*bla*$_{OXA-51-like}$, *bla*$_{OXA-23-like}$, *bla*$_{OXA-58-like}$, *bla*$_{OXA-40-like}$, *bla*$_{OXA-143-like}$ and *bla*$_{OXA-235-like}$) (156–158).

The presence of metallo-β-lactamases (class B) was determined either by single PCR for the following genes: *bla*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SPM-1}$ and *bla*$_{IMP}$ following previously established protocols (156, 157, 159) or together with some *bla*$_{OXA}$ carbapenemases by using two in-house multiplex PCRs. The first multiplex PCR included the following genes: VIM, KPC, *bla*$_{OXA-40-like}$, NDM, *bla*$_{OXA-48-like}$ and *bla*$_{OXA-23}$, while IMI, *bla*$_{OXA-58-like}$, GES, GIM, IMP and IS*Aba1*-*bla*$_{OXA-51-like}$ were screened by a second multiplex PCR.

3.2. Detection of IS*Aba1* upstream *bla*$_{OXA-23-like}$

The presence of the *ISAba1* insertion upstream of *bla*$_{OXA-23}$ was determined by PCR mapping and sequencing of PCR products as previously described (120).

The PCR products were purified using GeneJet™ PCR Purification Kit (ThermoFisher Scientific) according to the manufacturer's recommendations and then sequenced through an external resource (Macrogen Inc).

3.3. Phenotypic determination of class D and B β-lactamases

The Modified Hodge Test for detection of class D-carbapenemase-producing strains and the Hodge Test Imipenem-EDTA Double-Disk Synergy Test for the detection of metallo-β-lactamases were used (160).

3.4. Molecular determination of aminoglycoside resistance

Detection of genes encoding aminoglycoside-modifying enzymes *ant(2")-Ia, aac(3)-IIa, aph(3')-Ia, aac(3)-Ia, aph(3')-VIa, aac(6')-Ih, aac(6')-Ib/cr* and *aac(6')-IIa*, and the 16 rRNA methylases *armA, rmtB and rmtC* was performed by PCR as previously described (161–166).

4. Resistome by WGS

The assembled genomes of all sequenced isolates were used to identify the acquired resistome by using ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/), a web-based tool from CGE (Centre for Genomic Epidemiology). It identifies the acquired antibiotic-resistance genes in the genome by using BLAST (167). Same results were obtained by using The Comprehensive Antibiotic Resistance Database (CARD) (168).

5. Antimicrobial susceptibility

5.1. Method 1. Disk diffusion

Antibiotic susceptibility testing against 15 antibiotics was performed by disk diffusion on Mueller-Hinton agar: amikacin (30 μg), ampicillin-sulbactam (20 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), doxycycline (30 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), piperacillin-tazobactam (110 μg), tetracycline (30 μg), tobramicin (10 μg) and trimetoprim-sulphametoxazole (25 μg). Etests® (bioMérieux) were used to test colistin susceptibility (Etest 0.016-256 μg). This method is performed inoculating a Mueller-Hinton agar plate with a 0.5 McFarland inoculum of the isolate in NaCl by using a sterile cotton swab. Then, the antimicrobial disks were applied to the surface of the inoculated plate and the incubation is performed at 35±1ºC in air for 16-20 h. After incubation the inhibition zone can be measured and checked in the breakpoint tables (116). Quality control strains were used to check the test performance, for example *Escherichia coli* ATCC® 25922™, *Pseudomonas aeruginosa* ATCC® 27853™ or *Staphylococcus aureus* ATCC® 29213™. The results were interpreted according to the clinical breakpoints recommended by EUCAST (116).

5.2. Method 2. Agar dilution

Antimicrobial susceptibility testing for some of the isolates was performed by agar dilution method according to the EUCAST guidelines and breakpoints (116, 169). Minimum inhibitory concentrations (MICs) were evaluated for

ciprofloxacin, colistin, gentamicin, imipenem, meropenem, and tigecycline. Stock solutions of the powder of tested antimicrobials are daily prepared accordingly to the following instructions.

$$\text{Volume of Solvent (mL)} = \frac{\text{Weight of powder (mg) x Potency of powder (mg/g)}}{\text{Concentration (mg/L)}}$$

For most of the antimicrobials water is the solvent. Alternative solvents include phosphate buffer 0.01 M pH 7.2 for imipenem. Mueller-Hinton agar plates with different antimicrobial concentration (512-0.04 mg/L) were inoculated with a 0.5 McFarland standard from each isolate. The plates were incubated at 35-37ºC in air for 18 h. The MICs were determined as the lowest concentration plate in which there is no growth of the isolate. The EUCAST tigecycline MIC breakpoint for Enterobacteriaceae was used as no MIC breakpoints are available for *A. baumannii*. The reference strains *Escherichia coli* ATCC® 25922™, *Pseudomonas aeruginosa* ATCC® 27853™ and *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC® 29213™ were used as control strains (169). The experiments were repeated three times for all the isolates.

5.3. Method 3. Broth microdilution

Broth microdilution was used in order to analyze colistin MICs, according to EUCAST recommendations. MICRONAUT-S microplates (MERLIN Diagnostika GmbH) were used for this purpose. This microplates have lyophylized antibiotic and the principle is based on rehydratation with a

standarized bacteria suspension. A bacterial suspension 0.5 McFarland was prepared in NaCl 0.9% and 50 μL of this suspension were inoculated in 11 mL Mueller-Hinton broth. Inoculation of the plates was done with 100 μL of the bacterial suspension in Mueller-Hinton broth. The plates were covered and incubated at 35-37ºC in air for 24 h. Colistin MICs were interpreted according to EUCAST breakpoints. The reference strains *Escherichia coli* ATCC® 25922™, and *Pseudomonas aeruginosa* ATCC® 27853™ were used as control strains.

6. Biofilm formation experiments

Biofilm formation was assessed as described by O'Toole (170) with some modifications. Biofilms were developed in 24 well plates (Nunc, Thermo Fisher Scientific). Overnight cultures of bacteria were adjusted to an optical density of 0.2 at 600 nm; one hundred microliters were placed in each well containing 900 μL of M63 medium (minimal salts medium) supplemented with casamino acids (0.5% w/v) and incubated 24 h at 37°C. Planktonic cells were removed by taking out the medium (using a pipette) and the wells containing biofilms were rinsed three times with distilled water and air dried for approximately 20 min. The remaining adherent bacteria were stained with 1 mL/well of 0.7% crystal violet (wt/vol) solution (Sigma-Aldrich) for 12 min. Excess stain was removed by three washes with distilled water. Crystal violet-stained biofilm was solubilized in 1 mL of 33% acetic acid (vol/vol), and the plates were incubated at RT in an orbital shaker for 1 min at 400 rpm and the amount of dye (proportional to the density of adherent cells) was determined at

620 nm using a microplate reader (Infinite® 200 PRO, Tecan). Results were corrected for background staining by subtracting the value for crystal violet bound to uninoculated control wells. The biofilm assay was performed independently four times.

7. Molecular typing and analysis of the molecular epidemiology

7.1. Method 1. Pulsed Field Gel Electrophoresis (PFGE)

Plugs of genomic DNA were prepared from an overnight Luria-Bertani broth culture. The cultures were washed with saline solution and adjusted to a 0.8-1 absorbance at 600 nm. 500 μL of the solution were mixed with 500 μL 2% agarose for plugs in TE.

Once the plugs were solid, cell lysis was performed (Apendix 2).

The plugs were incubated for 5 hours at 37°C. Then the plugs were washed with TE. This was followed by a proteinase K digestion (Apendix 2) at 56°C overnight.

After proteinase K digestion, the plugs were washed ten times with TE at 50°C and digested with *Apa*I (30 U/μL) overnight at 37°C. The restricted fragments were separated on 1% Pulsed Field Certified™ Agarose (Bio-Rad) gels in 0.5x TBE (Tris-borate-EDTA) buffer using a CHEF-DR II system (Bio-Rad) for 18.5 h at 14°C with 5-20 s of linear ramping at 6 V/cm. DNA fingerprintings were analyzed using the Fingerprinting II software package (Bio-Rad) using the band-based Dice coefficient. The band tolerance and

optimization were both set at 1.5%. The threshold level for similarity was set as ≥87% to assume clonal relatedness.

7.2. Method 2. Whole Genome Sequencing, WGS

7.2.1. cgMLST

A core-genome multi locus sequence typing (cgMLST) scheme was defined for *A. baumannii* using the Ridom SeqSphere+ v.3.0 software using *A. baumannii* ACICU as reference genome. The resulting core-genome of 2390 alleles was used to investigate their molecular epidemiology (94). A minimum spanning tree based on the core-genome of 2390 alleles was generated using Ridom SeqSphere+ ignoring the missing values. Results of the cgMLST were also used to cluster the isolates into the different ICs using in-house reference strains (94).

7.2.2. *bla*$_{\text{OXA-51-like}}$

The *bla*$_{\text{OXA-51}}$ variant has been described to be related to the ICs (140), and the *bla*$_{\text{OXA-51}}$ variants in this study were used, as well, as another evidence to classify the isolates into the ICs.

7.3. Method 3. Multi-locus sequence typing (MLST)

7.3.1. MLST by Sanger sequencing

MLST was performed on selected isolates representing all the pulsotypes

as according to the Oxford scheme with the primers described in Apendix 1 (90, 171). The PCR products of the amplified genes were purified by using GeneJet™ PCR Purification Kit (ThermoFisher Scientific) according to the manufacturer's recommendations and sequenced thorugh an external resource (Macrogen Inc.).

Use of the PubMLST database allowed to identify the isolates and assign the new alleles and STs (https://pubmlst.org/abaumannii/).

7.3.2. MLST by WGS

The assembled genomes of the sequenced isolates were used to determine the traditional seven loci MLST from both the Oxford and Pasteur Schemes (90, 91). The online database PubMLST was used as explained above (90, 91).

7.3.3. Molecular epidemiology by MLST

eBURST software (http://eburst.mlst.net/) was used to analyze the sequence types (STs) and the relationship among them (171).

The Pasteur Scheme STs helped to elucidate the International Clone that the isolates belonged to (92).

8. Determination of chromosome or plasmid encoded antimicrobial resistance

8.1. Plasmid characterisation

Plasmid DNA was extracted using a commercial kit (Plasmid Midi Kit, Qiagen) following the manufacturers instructions. Plasmid content was analyzed by electrophoresis on 0.7% agarose gels in 0.5x TBE buffer and plasmid size was determined by comparison to plasmid DNA extracted from the type strains *E. coli* NCTC 50193 (CECT678) and NCTC 59192 (CECT679) carrying plasmids ranging in size from 2 Kb to 154 Kb.

8.2. S1-PFGE

S1 nuclease-pulse field gel electrophoresis (S1-PFGE) and Southern blot hybridization were perfomed to determine the plasmid size and the plasmid/chromosomal location of bla_{OXA-23} and *strA*.

Overnight cultures in 10 mL LB broth were used to prepare a bacterial suspension in cell suspension buffer (CSB).

300 μL of the bacterial suspension were mixed with 15 μL Proteinase K and incubated 10 min at 56°C. After that, bacterial suspensions were mixed with 1% SeaKem Gold Agarose prepared in TE buffer with 1% SDS (sodium dodecyl sulfate) and plugs were made.

Once the plugs were prepared, they were incubated in Cell lysis buffer (CLB) with 25 μL Proteinase K in the waterbath during 3 h at 56°C.

Two water washings (10 min with 500 μL sterile water at 56°C) and three TE washings (15 min with 500 μL sterile TE at 56°C) were carried out. Total bacterial DNA embebded in agarose plugs were digested with 50U of S1 nuclease (Thermo Fisher Scientific), that linearizes plasmids, and incubated at 37°C for 45 minutes. The restriction digestion was stopped with 2 μL 0.5 M EDTA pH 8.0 at 70°C for 10 min and the resulting fragments were subsequently separated in a 1% PFGE agarose gel using a CHEF-DR II system (Bio-Rad). The PFGE conditions were 17 hours at 6 V/cm and 14°C, inital and final pulses were conducted at 4 and 16 s, respectively. The gel was stained with a 3 mg/mL ethidium bromide solution.

8.3. S1-PFGE and I-CeuI-PFGE

Total DNA plugs were prepared following the protocol explained in 6.1.1., and digested with 10U of S1 enzyme (Thermo Fisher Scientific) at 37ºC for 45 minutes. The resulting fragments were visualized on 1% Pulsed Field Certified™ Agarose (Bio-Rad) gels by PFGE using ramping of 5-20 s at 14ºC for 20 hours.

To determine plasmid location of *bla*$_{OXA-23}$, total DNA plugs were digested with I-CeuI endonuclease at 37°C O/N and the resulting fragments were separated by PFGE on 1% Pulsed Field Certified™ Agarose (Bio-Rad) gels in 0.5x TBE buffer under the following conditions: temperature, 14°C; voltage, 6 V/cm; and switch angle, 120°, with one linear switch ramp of 5 to 125 s for 22 h.

8.4. Southern Blot and hybridization

DNA from the PFGE gels was transferred to a Hybond-N membrane (Sigma-Aldrich) by capillary transfer followed by hybridization with digoxigenin (DIG)-labeled specific probes (Roche) for *bla*$_{\text{OXA-23-like}}$ and *strA*. Chromosomal location was shown by colocalization with the *bla*$_{\text{OXA-51-like}}$ probe.

The gel was subjected to a depurination step with 0.25 M HCl for 10-20 min and then rinsed with sterile nanopure water. Then the DNA was twice denaturized in a denaturation solution for 15 min and then rinsed with sterile nanopure water.

Consequently, it was submerged in neutralization solution for 15 min, and then the solution was changed for another 15 min.

The gel was equilibrated for 10 min in 20x SSC. And a construction of Whatman® 3MM paper (Merck) soaking into 20x SSC, gel, Hybond-N membrane (Sigma-Aldrich), Whatman® 3MM paper (Merck), 9 cm of absorbent paper with a metalic lid and a weight on top was built. The construction was left overnight in order to obtain the migration of the DNA from the gel to the Hybond-N membrane. DNA was crosslinked to the membrane using a UV transilluminator and then the membrane was dried for at least 2 h.

When the membrane is dry, proceed with the prehibridization. Roll the membrane into a net and introduce it in a glass tube with 10 mL/100 cm^2 hybridization buffer and place it in the hybridization oven at 42°C for 3.5 h.

For the hybridization, a hybridization solution was prepared. Prewarm the hybridization buffer to 42°C. Take 45 μL of hybridization buffer and add the

probe, boil at 80°C for 5 min. Add the probe to the prewarmed hybridization buffer (5 μL probe/30 mL buffer). Add the hybridization solution to the tube containing the membrane and incubate overnight at 42°C in the hybridization oven.

Two washings (15 min) with 200 mL of low stringency buffer and two washings (15 min) with 200 mL prewarmed (68°C) high stringency buffer were performed.

Signal detection was performed according to manufacturer's instructions using CDP-Star® ready-to-use (Roche) chemiluminescent substrate by autoradiography on a X-ray film (GE Healthcare).

8.5. Plasmid analysis and scaffolding

8.5.1. PCR-based gap closure

By using the resistome, S1-PFGE and Southern blot data, plasmid analysis was performed in three isolates belonging to different international clones; IC4, IC5 and IC7.

S1-PFGE allows to assess the plasmids size and by hybridization, the plasmidic or chromosomal location of the resistance genes. Chromosomal location can be demonstrated by co-localization with chromosomal gene markers.

Contigs of the WGS carrying resistance genes were examined together with other contigs of the *de novo* assembled genome. Plasmid assemblies and

predicted gaps were further confirmed using PCR-based gap closure. The contigs of interest were checked out for overlaps with others, this analysis allowed for a preliminary organization of the contigs which was further confirmed by designing specific primer pairs where overlaps were found and analyzing the obtained amplicons.

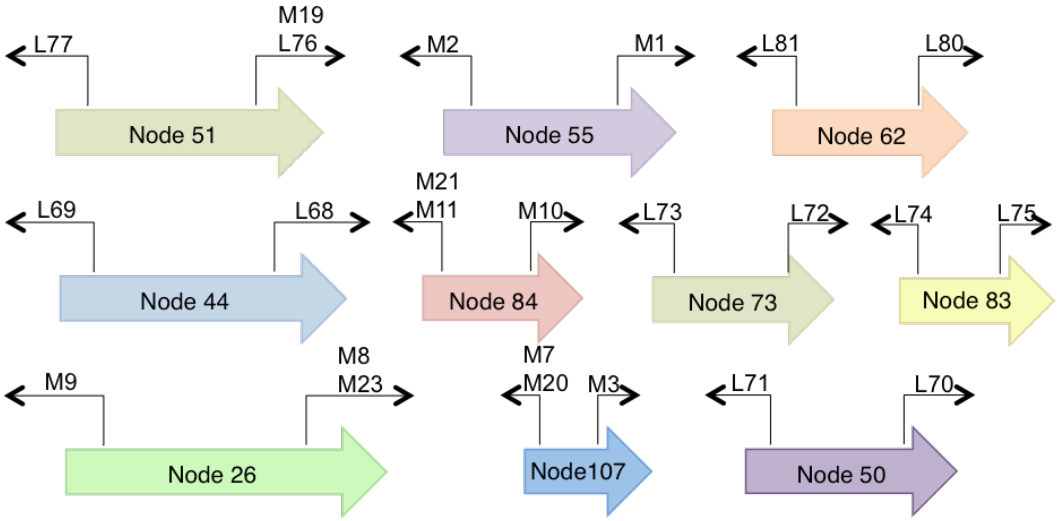

Figure 12. Some nodes of MC1 assembly and some primers designed for the hypothetical nodes of a plasmid.

As a further confirmation of the plasmid scaffold, MinION long-read sequencing was performed.

8.5.2. MinION

Oxford Nanopore Technologies (ONT) MinION long-read sequencer was used in order to obtain longer reads and confirm the plasmid structure.

DNA extraction was performed using the Genomic-tip 100/G kit (Qiagen) from 2.5 mL overnight cultures in Luria-Bertani according to manufacturer's specifications. The DNA was eluted in 300 µL TE buffer at 55°C for 1.30 h.

DNA concentration was measured with NanoDrop™ 2000/2000c and Qubit® 2.0 Fluorometer.

Table 7. DNA concentration using Qubit® 2.0 Fluorometer for MinION sequencing.

Isolate	DNA concentration
MC1	180.8 ng/μL
MC23	64.4 ng/μL
MC75	58.8 ng/μL

Library preparation was carried out according to manufacturer's indications using a combination of Native Barcoding Kit 1D and Ligation Sequencing Kit 1D; EXP-NBD103 and SQK-LSK108 respectively.

1-1.5 μg DNA were repaired using NEBNext® FFPE DNA Repair Mix (New England BioLabs) in order to improve the read lenght. End-repair and dA-tailing of fragmented DNA was performed by NEBNext End repair/dA-tailing Module (New England BioLabs), to achieve an aim of 700 ng repaired DNA. Subsequently, ligation of the barcodes (Native Barcoding Kit 1D) was perfomed using Blunt/TA Ligase Master Mix (New England BioLabs) using 500 ng of each sample DNA. After barcoding, equimolar amounts of each sample were pooled to obtain in total 700 ng DNA in 50 μL nuclease-free water. The pooled DNA went through adapter ligation with the Barcode Adapter Mix and then it was loaded in a R9.4 flowcell (FLOMIN 106). MinION sequencer was run for approximately 19 hours.

The tool Albacore was used for demultiplexing the reads which were later used to perform the Canu assembly. Another assembly was made with hybridSpades, that combines both long and short reads from MinION and MiSeq, respectively.

8.5.3. Plasmid annotation and visualization

First, ORFfinder (NCBI) (https://www.ncbi.nlm.nih.gov/orffinder/) was used in order to predict the open reading frames (ORF) in the plasmid sequences, it uses an algorithm which looks for a start codon (ATG and alternative initiation codons), followed by a protein encoding sequence (longer than 75 nt) and a stop codon. Consequently, putative gene function assignments were assigned with amino acid sequences using BLASTp (NCBI) and the non-redundant protein sequences database (NCBI).

A second functional annotation of the genomes was performed using the online tool Rapid Annotation Subsystem Technology (RAST) (172); this server uses two different approaches to generate gene function: subsystem-based inferences rely on the recognition of functional variants of subsystems, which are defined as a set of functional roles and protein families; while other approaches are used for the nonsubsystem-based assertions, for example identification of tRNA and rRNA encoding genes using tRNAscan-SE and search_for_rnas, respectively.

Consequently, the tool SnapGene Viewer (GSL Biotech) was used in order to obtain a circular diagram of the plasmids. The resulting diagrams were manually curated.

RESULTS AND DISCUSSION

1. High Prevalence of Extensively Drug-Resistant *Acinetobacter baumannii* at a Children Hospital in Bolivia.

RESULTS

1. Bacterial isolates

Thirty-six isolates recovered between March 2014 and May 2015 in Hospital Materno Infantil were confirmed as *A. baumannii* by the presence of $bla_{\text{OXA-51-like}}$.

2. Analysis of the cases

Of the 36 *A. baumannii* isolates, fifteen (41.7%) were isolated from male patients and fourteen (38.9%) from female patients; four of them (11.1%) were from hospital environmental samples and for three of them (8.3%) the sex of the patient was not given.

The patients (n=32) were grouped according to age in five groups, ≤1 month [n=17, 53%]; >1 month ≤ 1 year [n=4, 12.4%]; >1 year ≤5 years [n=4, 12.4%] and >5 years [n=6, 18.7%]. For one of the patients the age could not be precisely determined.

The isolates were recovered from different sources: blood cultures (n=10, 31.3%), respiratory samples (n=11, 34.4%), catheter (n=3, 9.4%), cerebrospinal fluid (n=3, 9.4%), surgical wound (n=2, 6.3%), abscess (n=1 3.1%), exudate (n=1, 3.1%) and peritoneal fluid (n=1, 3.1%).

The most prevalent diagnostics were pneumonia (n=10, 31.3%) and septicaemia (n=9, 28.1%). There were other infections such as meningitis (n=1), skin and soft tissue infections (n=2), hemolytic uremic syndrome (n=2) or congenital syphilis (n=2).

3. Susceptibility of *A. baumannii* isolates to antibiotics and mechanisms of resistance

Antimicrobial susceptibility testing was performed by disk diffusion method and Etest® for colistin.

The susceptibility and resistance rates to the different antibiotics can be seen in Figure 13. The majority of the isolates (n=30, 80.6%) were extensively-drug resistant (XDR) and 8.3% (n=3) were multidrug-resistant (MDR) following the criteria of Magiorakos et al (173).

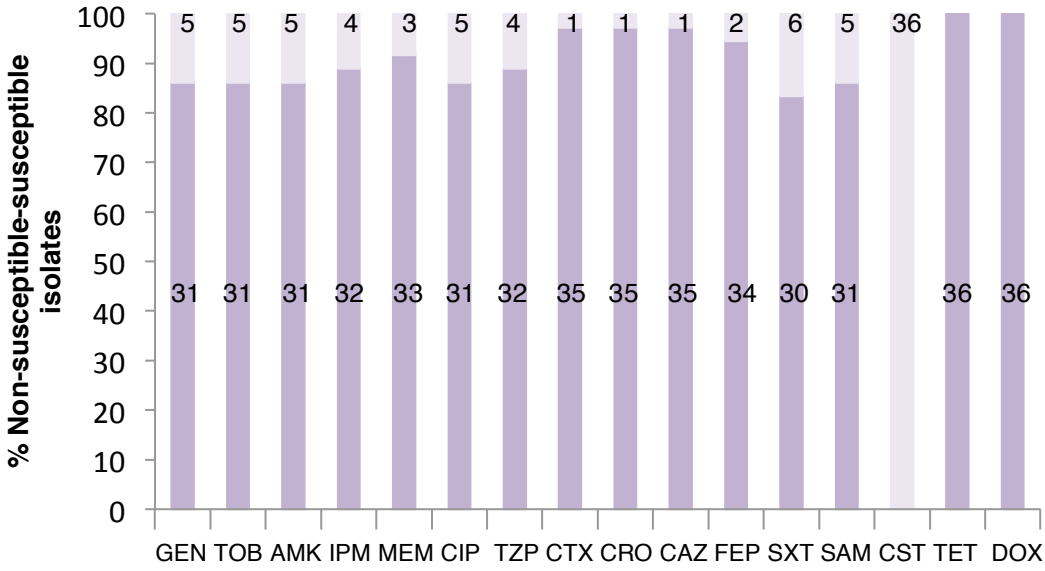

Figure 23. *A. baumannii* susceptibility-resistance rates. The dark bars indicate the percentage of resistant isolates and the light bars the susceptible isolates. The numbers within the bars represent the number of isolates in each category.

All the carbapenem-resistant isolates carried the *bla*$_{\text{OXA-23-like}}$ gene as determined by *bla*$_{\text{OXA}}$ multiplex PCR, while in those isolates susceptible to carbapenems it was not present. Only the carbapenem resistant isolates gave a positive result for the Hodge Test. The *aac(3)-IIa* gene was present in all the aminoglycoside-resistant *A. baumannii* isolates (86.1%) but not in the aminoglycoside-susceptible ones. No other acquired class D or B β-lactamase genes, or aminoglycoside resistance encoding genes were detected by PCR.

4. Molecular typing methods

Molecular epidemiology of the isolates was evaluated by *ApaI*-PFGE (Figure 14, Figure 15). The majority of the isolates were grouped into two predominant pulsotypes named E (n=10) and F (n=15). These pulsotypes had subclonal variants that differed by up to six bands, two within pulsotype E (E1-E2) and six within pulsotype F (F1-F6). Nine isolates were considered sporadic strains because they were singletons and were isolated one or two times during all the study period. Two environmental isolates were non-typeable (NT) by using PFGE despite the experiment being repeated three times, no bands were obtained, *Apa*I was not able to cut the isolates DNA.

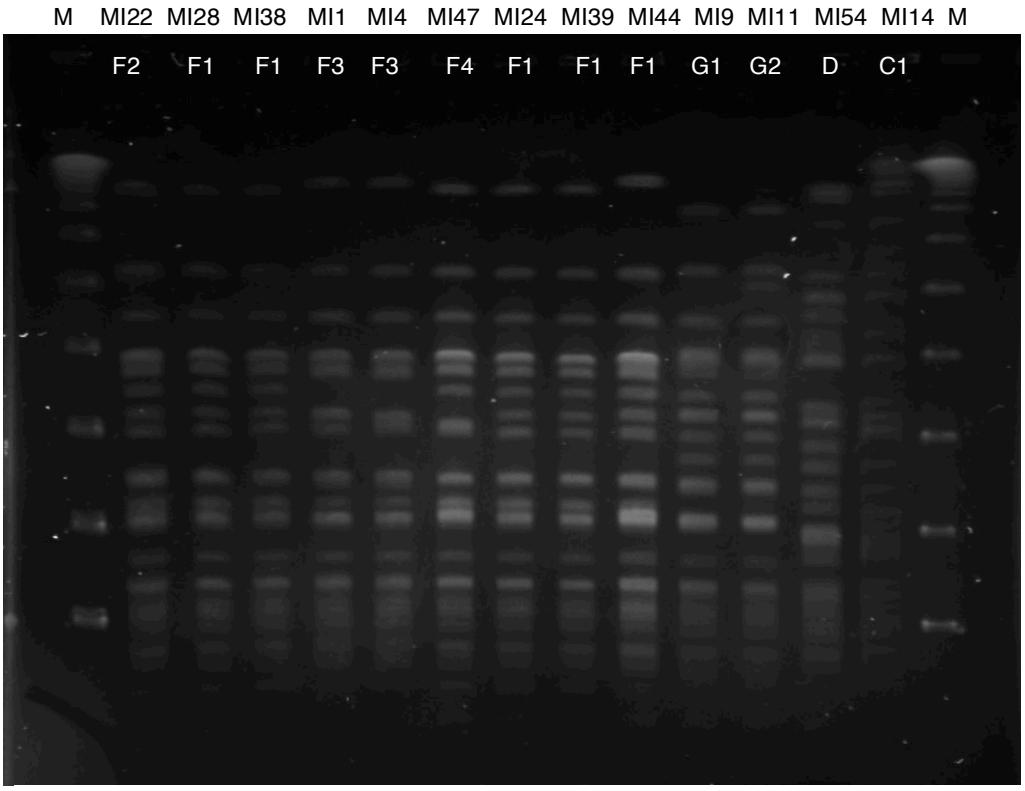

Figure 14. *ApaI* PFGE of thirteen *A. baumannii* isolates. M, marker. Isolate and pulsotype.

MLST according to the Oxford scheme was performed by Sanger secuencing, and eBURST was used in order to analyze the clonal relatedness. The isolates of the two predominant clones were single locus variants (SLV) of the clonal complex CC110 (Table 8) which belongs to the international clone 7 (IC7), and differ only in the *gpi* allele with the exception of the double locus variant (DLV) ST1521. Five of the unrelated sporadic strains as determined by PFGE were also SLV of CC110 (STs 1489, 1518 and 1522). The rest of the isolates were assigned to ST1482, an SLV of CC741, and ST1531 and ST1532 that are DLV of the same CC.

Table 8. STs and CC of the isolates according to the Oxford scheme.

Clone (n of isolates)	ST	CC	*gltA*	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*
A (3)	ST1489	CC110 (IC7)	1	15	2	28	1	283	32
B (1)	ST1532	CC741	21	35	2	28	107	302	4
C (2)	ST1482 ST1532	CC741	21 21	35 35	2 2	28 28	1 107	175 302	4 4
D (1)	ST1531	CC741	21	35	2	28	107	25	4
E (10)	ST1518 ST1521	CC110 (IC7)	1 1	15 15	2 2	28 43	1 1	299 102	32 32
F (15)	ST1489 ST490 ST1518	CC110 (IC7)	1 1	15 15	2 2	28 28	1 1	283 102	32 32
G (2)	ST1522 ST1518	CC110 (IC7)	1 1	15 15	21 2	28 28	1 2	102 299	32 32

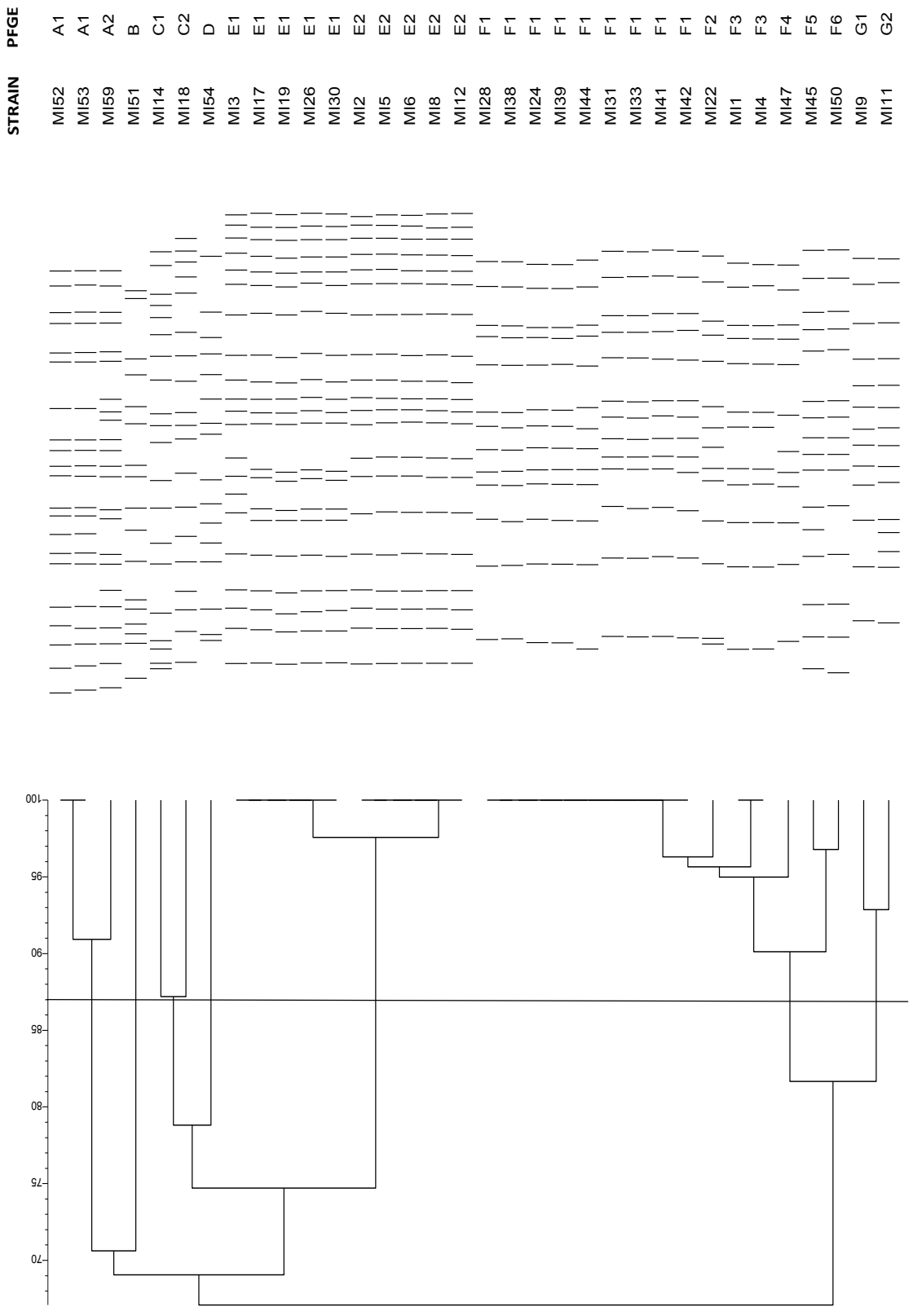

Figure 15. Dendogram obtained from the analysis of *ApaI* PFGE patterns

5. Plasmid characterization

In order to analyze plasmids, S1-PFGE was used; this analysis showed an ~180 Kb plasmid in 30 isolates, while one isolate also had an additional plasmid of ~39 Kb, two isolates carried two plasmids of ~100 Kb and ~80 Kb, and no plasmids were found in four isolates.

6. Genetic context of *bla*$_{\text{OXA-23-like}}$ gene

PCR-mapping of the *bla*$_{\text{OXA-23}}$ genetic environment was performed in the 32 strains harboring this gene. In 29 strains a highly similar structure (99%) to a previously described Tn*2008* (LN877214.1) was revealed.

In order to determine whether the *bla*$_{\text{OXA-23-like}}$ gene was located on the chromosome or a plasmid, digestion with I-CeuI and hybridization with a *bla*$_{\text{OXA-23}}$ probe were carried out. By Southern blotting, a positive signal was detected for the *I-Ceu*I digested DNA hybridized with a specific *bla*$_{\text{OXA-23}}$ probe, indicating a chromosomal location of the *bla*$_{\text{OXA-23}}$ encoding gene.

7. Biofilm formation

Varying degrees of biofilm production were demonstrated among all the strains. It was observed that stronger biofilm-producers were isolated from the hospital environment (MI51, MI54, MI25 and MI23); catheter (MI14), blood (MI18) and CSF (MI24), and all of them, with the exception of MI24, were part of the sporadic strains (Figure 16). The isolates that clustered in the two

predominant pulsotypes E and F did not produce high levels of biofilm. The lowest levels of biofilm production were found in isolates belonging to pulsotype G, which were isolated from blood cultures.

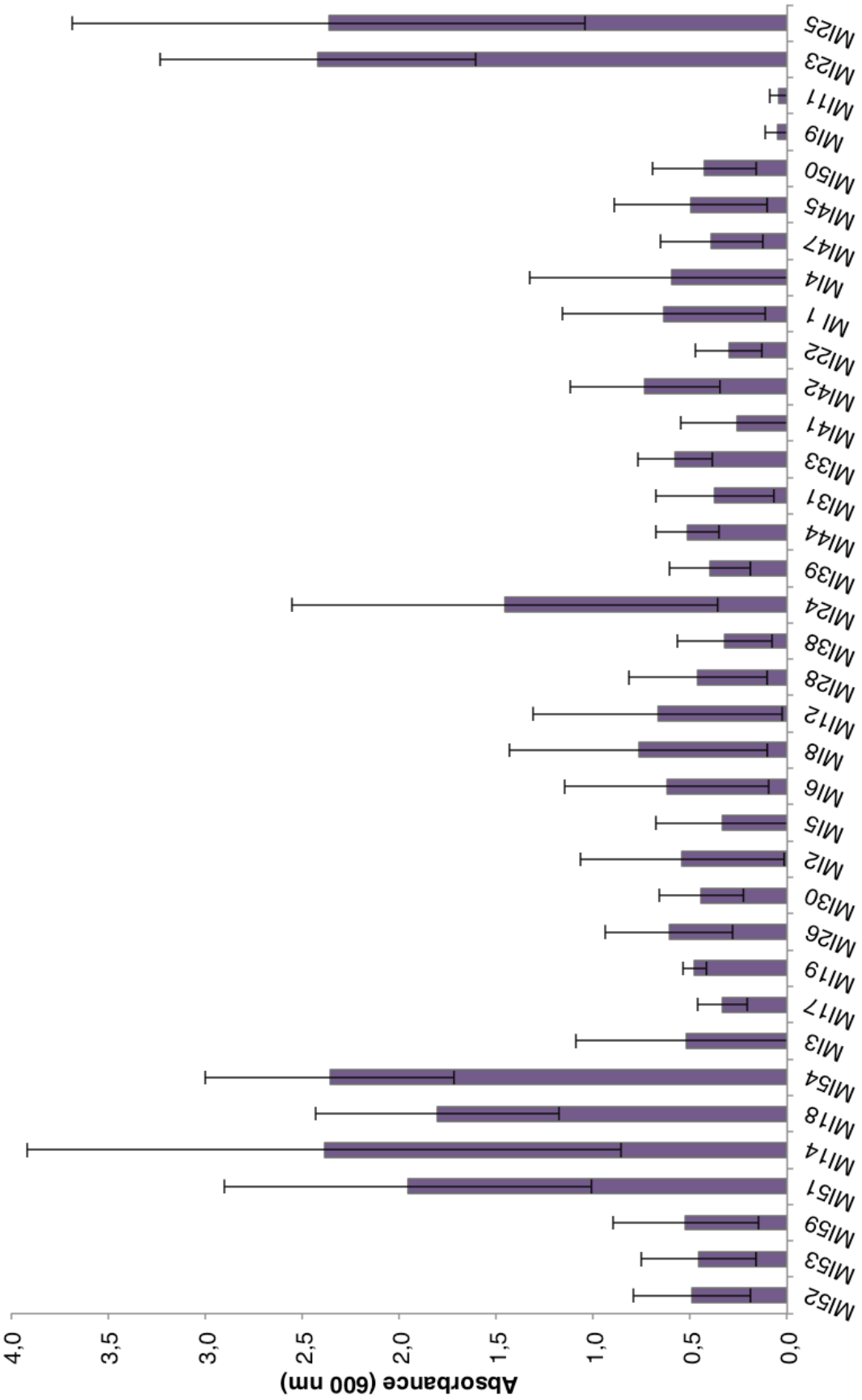

Figure 16. Biofilm formation, mean ± standard deviation

DISCUSSION

In this study the clonal relatedness and antmicrobial resistance profiles of *A. baumannii* from a Bolivian children hospital have been analyzed. The microbiology laboratory service in this hospital was established in 2014, and this is the first survey they have investigated in depth. The majority of the isolates were clustered in two pulsotypes, both of which are SLVs of CC110 that belongs to IC7 (92), which is one of the endemic ICs in Latin America (174). It is difficult to establish a connection between pulsotypes and sequence types because the same ST can be present in different pulsotypes. This can be due to the fact that MLST lacks the resolution of PFGE, in addition genomic rearrangements that will not necessarily change the ST will have an impact in the band pattern of the PFGE (92, 175). The presence of $bla_{\text{OXA-23-like}}$ in 88.9% of the isolates (in related and unrelated clones) matches the situation in other Latin American countries in which the presence of this class D carbapenemase ranges between 75-100% (144). The similarity of the context of the $bla_{\text{OXA-23-like}}$ in these isolates with the previously reported Tn*2008* in an isolate from another city in Bolivia (176) could be due to the mobilization and spread of this transposable unit among *A. baumannii* isolates (177). The gene encoding for the aminoglycoside modifying enzyme, *aac(3)-II*a was also present in the majority of the isolates. The combination of these two antimicrobial resistance genes, makes colistin the last resort treatment (113), increasing the risk for adverse events (29%) and mortality in these patients (16%). Furthermore, the dose of colistin in children is still not accurately established (178, 179).

To our knowledge, this is the first report of XDR *A. baumannii* isolates in infants in South America. The high rates of resistance to antimicrobials, 83.3% of the isolates were XDR, together with the complicated diagnostics (septicaemia and pneumonia the most prevalent) combined with the age of the patients (more than the half of them were newborns), increase the complications of these infections. XDR *A. baumannii* infections in infants are associated with high morbidity and mortality (28.2%) (180). We also detected that two of the isolates obtained from the hospital environment were XDR and MDR, respectively, therefore this environmental presence can lead to a rapid spread of the MDR and XDR isolates within the hospital and suggests that stricter infection control measures should be introduced. Moreover it was seen that the results matched the study of other authors (181) as the hospital environmental isolates and those related to medical devices were the ones that presented higher rates of biofilm formation, in contrast to what Bardbari et al reported (182). Biofilm is a good persistence mechanism that can help these strains to survive in the hospital environment for long periods of time and contribute to the colonization and spread of these XDR *A. baumannii* isolates.

Despite the relatively new creation of the microbiology laboratory service in this hospital, an infection control plan has been created because of this work, in order to prevent and reduce the number of infections among patients. Further studies are needed to track the evolution of the XDR *A. baumannii* infections.

The present study demonstrated the predominance and spread of closely related XDR *A. baumannii* isolates in a Children Hospital in Bolivia. The location

of the *bla*$_{\text{OXA-23-like}}$ gene in a transposon-like structure could be responsible for the dissemination of this carbapenemase-encoding gene among other isolates. The high prevalence of XDR *A. baumannii* clones confers increasing risk to children and is of major concern due to the kind of infections and the lack of therapeutic alternatives to treat them.

2. *Acinetobacter baumannii* analysis by core genome MLST in two hospitals in Bolivia: endemicity of international clone 7 isolates.

RESULTS

1. Bacterial isolates

A total of ninety-five isolates recovered between September 2015 and December 2016 in Hospital Materno Infantil and Hospital Viedma, were confirmed to be A. baumannii and included in this study.

Figure 17. Location of both hospitals, Hospital Viedma and Hospital Materno-Infantil.

The most common source of the isolates was the respiratory tract, n=34 (35.8%) followed by wound secretions, n=17 (17.9%); ulcers, n=9 (9.5%) and urine culture, n=8 (8.4%). The remaining isolates were recovered from diverse sources such as blood culture, catheter, abscesses and exudates.

2. Antimicrobial susceptibility and PCR experiments

All the isolates were confirmed as *A. baumannii* by *gyrB* multiplex PCR and the presence of the intrinsic $bla_{\text{OXA-51-like}}$ carbapenemase gene.

MICs were tested by agar dilution. Resistance to ciprofloxacin, gentamicin, imipenem, meropenem, and tigecycline was as follows 90.6% (n=86); 86.4% (n=82); 53.7% (n=51); 53.7% (n=51) and 61.1% (n=58) respectively (Table 9, Figure 18). Colistin MICs were tested by broth microdilution (BMD) for all the CRAb isolates which colistin MIC was ≤2 by agar dilution. All the isolates but one, were susceptible to colistin.

By using multiplex PCR, $bla_{\text{OXA-23-like}}$ gene was detected in the 51 CRAb isolates. No other acquired class D β-lactamases was detected in any of the isolates.

Table 9. Minimum inhibitory concentration of colistin (COL), imipenem (IPM), meropenem (MEM), ciprofloxacin (CIP), tigecycline (TG) and gentamicin (GEN). Breakpoint interpretations were in accordance to EUCAST (116)

	COL	IPM	MEM	CIP	TGC	GEN
MC1	0.5 S*	32 R	64 R	>128 R	16 R	32 R
MC2	1 S	32 R	32 R	32 R	1 S	>128 R
MC3	1 S	32 R	32 R	128 R	4 R	>128 R
MC4	1 S	1 S	1 S	64 R	4 R	>128 R
MC5	1 S	32 R	32 R	128 R	4 R	>128 R
MC6	1 S	32 R	32 R	64 R	1 S	>128 R
MC7	2 S	1 S	2 S	64 R	4 R	>128 R
MC8	1 S	32 R	64 R	32 R	1 S	>128 R
MC9	2 S	1 S	1 S	64 R	4 R	>128 R
MC10	2 S	0.5 S	1 S	>128 R	4 R	>128 R
MC12	1 S*	32 R	32 R	>128 R	8 R	32 R
MC14	1 S	32 R	32 R	>128 R	8 R	>128 R
MC15	2 S	1 S	1 S	>128 R	4 R	>128 R
MC17	1 S*	1 S	2 S	>128 R	4 R	>128 R
MC18	0.5 S*	32 R	64 R	128 R	8 R	>128 R
MC19	1 S	32 R	32 R	64 R	1 S	>128 R
MC21	0.5 S*	32 R	32 R	64 R	0,5 S	>128 R
MC22	1 S	32 R	32 R	64 R	1 S	1 S
MC23	1 S*	1 S	2 S	>128 R	4 R	>128 R
MC24	4 R	1 S	2 S	>128 R	4R	>128 R
MC25	1 S	0.5 S	1 S	>128 R	4 R	>128 R
MC27	1 S	32 R	32 R	32 R	0.5	>128 R
MC29	0.5 S*	32 R	64 R	>128 R	8 R	>128 R
MC30	2 S	2 S	4 S	64 R	1 S	>128 R
MC31	1 S*	32 R	32 R	128 R	8 R	>128 R
MC32	1 S*	32 R	32 R	128 R	8 R	>128 R
MC33	0.5 S*	32 R	32 R	32 R	1 S	>128 R
MC34	0.5 S*	32 R	64 R	128 R	8 R	>128 R
MC35	1 S*	32 R	64 R	32 R	1 S	>128 R
MC38	1 S*	0.5 S	1 S	>128 R	2 S	>128 R
MC39	1 S*	32 R	64 R	>128 R	8 R	4 S
MC40	2 S	1 S	2 S	>128 R	4 R	>128 R
MC41	2 S	0.5 S	4 S	>128 R	2 S	>128 R
MC42	1 S	0.5 S	4 S	>128 R	4 R	>128 R
MC43	1 S	0.5 S	4 S	>128 R	1 S	>128 R
MC44	0.25 S*	32 R	64 R	>128 R	4 R	>128 R
MC45	4 R	0.5 S	2 S	>128 R	2 S	>128 R
MC47	1 S*	0.5 S	2 S	128 R	0.5 S	>128 R
MC48	1 S	32 R	64 R	128 R	8 R	>128 R
MC49	2 S	0.5 S	2 S	>128 R	4 R	>128 R
MC50	0.25 S*	32 R	64 R	32 R	1 S	>128 R
MC51	0.5 S*	32 R	32 R	128 R	8 R	>128 R
MC52	1 S	2 S	4 S	>128 R	4 R	>128 R
MC53	1 S	32 R	32 R	32 R	0.5 S	>128 R

Table 9. Continued.

	COL	IPM	MEM	CIP	TGC	GEN
MC54	2 S	0.5 S	2 S	>128 R	4 R	>128 R
MC55	1 S	0.5 S	2 S	0.5 S	0.25 S	2 S
MC56	1 S	0.125 S	2 S	1 S	0.25 S	2 S
MC57	0.5 S*	32 R	32 R	128 R	4 R	>128 R
MC58	1 S	0.5 S	0.5 S	0.5 S	0.25 S	1 S
MC59	0.25 S*	32 R	32 R	128 R	8 R	>128 R
MC60	1 S	32 R	32 R	64 R	1 S	>128 R
MC61	2 S	1 S	1 S	>128 R	4 R	>128 R
MC62	0.25 S*	32 R	32 R	32 R	0.5 S	>128 R
MC63	0.25 S*	32 R	32 R	>128 R	8 R	>128 R
MC64	1 S	32 R	32 R	128 R	4 R	>128 R
MC65	2 S	1 S	2 S	>128 R	4 R	>128 R
MC66	2 S	0.25 S	0.125 S	0.25 S	0.25 S	2 S
MC67	1 S	1 S	2 S	>128 R	4 R	>128 R
MC68	2 S	0.5 S	2 S	>128 R	4 R	>128 R
MC69	1 S	32 R	32 R	>128 R	4 R	>128 R
MC70	1 S	1 S	1 S	>128 R	4 R	>128 R
MC71	1 S	32 R	64 R	64 R	0,5	>128 R
MC72	2 S	1 S	1 S	>128 R	4 R	>128 R
MC73	2 S	1 S	0.5 S	>128 R	4 R	>128 R
MC74	1 S	1 S	2 S	>128 R	2 S	>128 R
MC75	1 S*	32 R	64 R	128 R	2 S	>128 R
MC76	2 S	1 S	2 S	128 R	0.5 S	32 R
MC77	1 S*	32 R	32 R	632 R	0.5 S	>128 R
MC78	1 S	32 R	32 R	128 R	8 R	>128 R
MC79	1 S	0.25 S	2 S	0.5 S	0.25 S	2 S
MC80	2 S	0.5 S	2 S	128 R	2 S	64 R
MC81	0.5	0.25 S	1 S	0.5 S	0.5 S	2 S
MC82	0.5	0.25 S	1 S	0.5 S	0.5 S	2 S
MC83	1 S	0.5 S	2 S	64 R	0.5 S	16 R
MC84	0.5	0.5 S	2 S	>128 R	4 R	>128 R
MC85	1 S	0.5 S	1 S	>128 R	4 R	>128 R
MC86	0.5 S	1 S	1 S	>128 R	4 R	64 R
MC87	1 S*	32 R	64 R	128 R	8 R	>128 R
MC88	1 S	0.25 S	0.25 S	0.125 S	0.5 S	2 S
MC89	1 S*	32 R	64 R	64 R	8 R	>128 R
MC90	1 S*	32 R	64 R	64 R	4 R	>128 R
MC91	1 S*	32 R	64 R	128 R	8 R	>128 R
MC93	1 S*	32 R	64 R	64 R	8 R	>128 R
MC94	1 S*	32 R	64 R	64 R	8 R	>128 R
MC95	1 S*	32 R	64 R	128 R	8 R	>128 R
MC96	8 R*	32 R	64 R	128 R	8 R	>128 R
MC98	1 S*	32 R	32 R	64 R	8 R	>128 R
MC100	1 S*	32 R	32 R	64 R	8 R	>128 R
MC101	1 S*	32 R	32 R	64 R	8 R	>128 R

Table 9. Continued.

	COL	IPM	MEM	CIP	TIG	GENTA
MC102	2 S*	32 R	32 R	64 R	8 R	>128 R
MC103	1 S*	32 R	64 R	64 R	8 R	>128 R
MC104	1 S*	32 R	32 R	128 R	4 R	>128 R
MC105	1 S*	32 R	32 R	64 R	1 S	>128 R

*Colistin MICs for these isolates was tested by BMD.

1

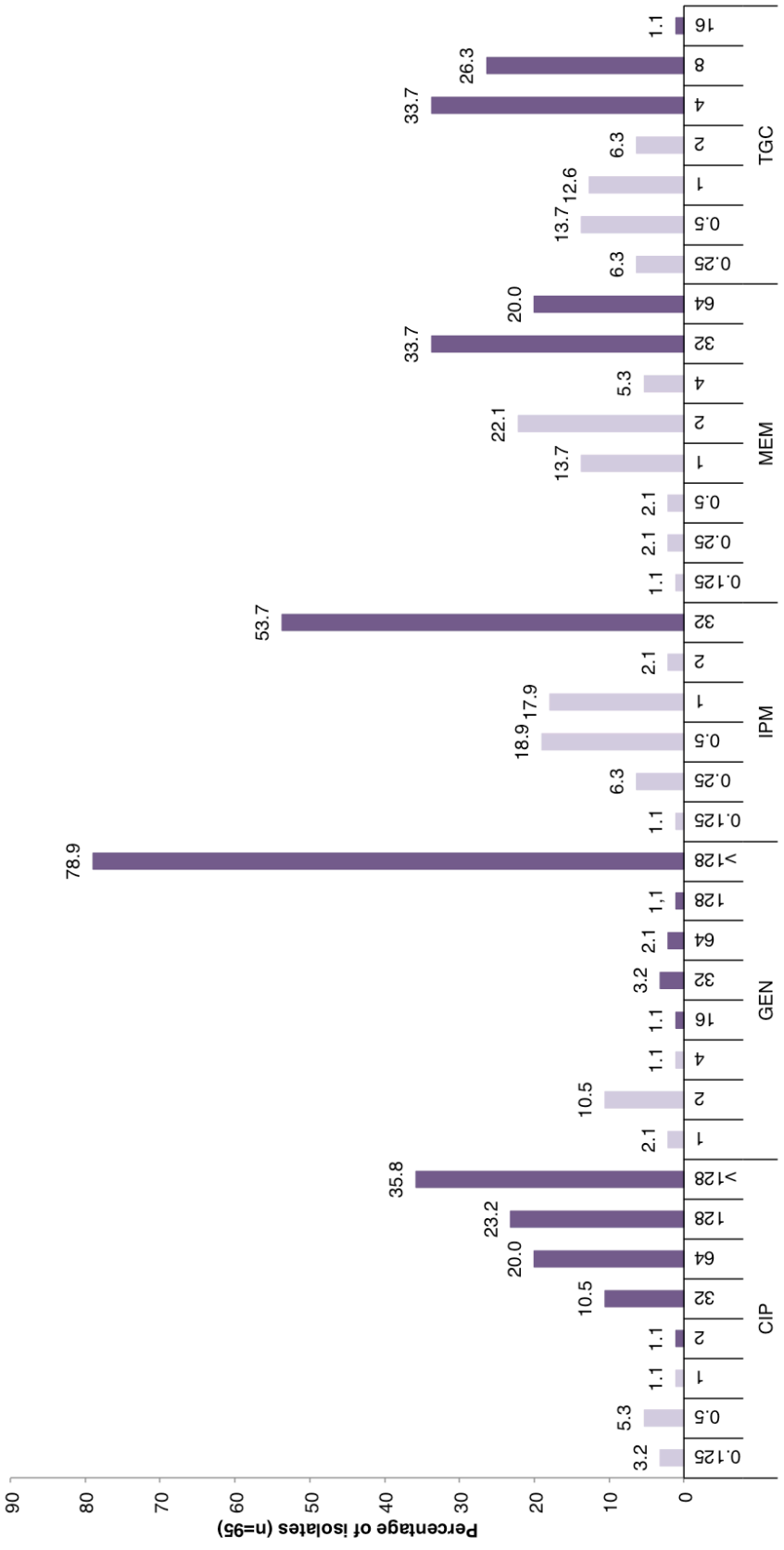

Figure 18. Minimum inhibitory concentrations as determined by agar dilution. CIP, ciprofloxacin; GEN, gentamicin; IPM, imipenem; MEM, meropenem and TGC, tigecycline. Dark bars represent the resistant MICs and light bars represent the susceptible MICs.

3. DNA extraction for WGS

The DNA concentration in ng/μL was as follows: MC1, 0.86; MC2, 0.81; MC3, 0.67; MC5, 0.97; MC6, 0.89; MC8, 1.09; MC12, 1.12; MC13, 1.74; MC17, 1.95; MC18, 2.30; MC19, 1.39; MC21, 0.97; MC22, 1.08; MC27, 1.06; MC29, 1.04; MC31, 2.16; MC32, 1.21; MC33, 1.52; MC34, 1.39; MC35, 0.66; MC37, 1.12; MC38, 1.95; MC39, 0.84; MC44, 0.69; MC48, 0.99; MC50, 1.22; MC51, 0.86; MC53, 1.39; MC57, 0.74; MC59, 1.87; MC60, 1.13; MC62, 0.99; MC63, 1.23; MC64, 1.31; MC69, 0.63; MC71, 0.85; MC75, 2.26; MC77, 2.18; MC78, 1.55; MC87, 1.59; MC89, 0.90; MC90, 0.89; MC91, 0.96; MC93, 1.06; MC94, 1.76; MC95, 1.33; MC96, 1.16; MC98, 1.84; MC100, 1.17; MC101, 0.83; MC102, 1.97; MC103, 1.09; MC104, 2.04 and MC105, 1.40.

4. Molecular epidemiology and WGS analysis

All the CRAb isolates (n=51) and subset of carbapenem susceptible isolates recovered along the study period (n=4) were subjected to whole genome sequencing in order to investigate their molecular epidemiology as well as their antimicrobial resistance mechanisms. A minimum spanning tree generated using the cgMLST scheme is shown in Figure 18. Most of the isolates, 90.9% (n=50) belonged to ST25 and its SLV ST991 by Pasteur scheme (clonal complex 25, CC25) which is associated with IC7, and also had *bla*$_{\text{OXA-64}}$, the characteristic *bla*$_{\text{OXA-51}}$ variant from this lineage. Furthermore these isolates clustered with IC7 control strains using cgMLST. These three pieces of evidence showed that IC7 was predominant among these *A. baumannii* isolates. Following the Oxford scheme these fifty isolates were further

delineated into five different groups belonging to CC110 (single locus variant, SLV, ST1489, ST1519, ST1529 and ST1518 and double locus variant, DLV, ST1528). Furthermore, cgMLST showed that most isolates were not considered as transmissions (i.e. the same clone). However, there were five potential transmission clusters using a cutoff of 0-10 allelic differences. The other CC25 isolates differred by up to 110 alleles.

Five unrelated isolates were also present, differing in ≥ 1950 alleles from the IC7 cluster. Three isolates carried the *bla*$_{\text{OXA-65}}$, were ST79 (IC5) by Pasteur scheme and ST233 and DLV ST1520 according to Oxford, and clustered with the IC5 control by cgMLST. One isolate clustered with IC4 controls, carried *bla*$_{\text{OXA-51}}$ and was ST15 by Pasteur scheme and ST236 by Oxford. One isolate was a singleton, carrying the *bla*$_{\text{OXA-180}}$ and was ST267/ST942 (Pasteur/Oxford).

turbosiesta

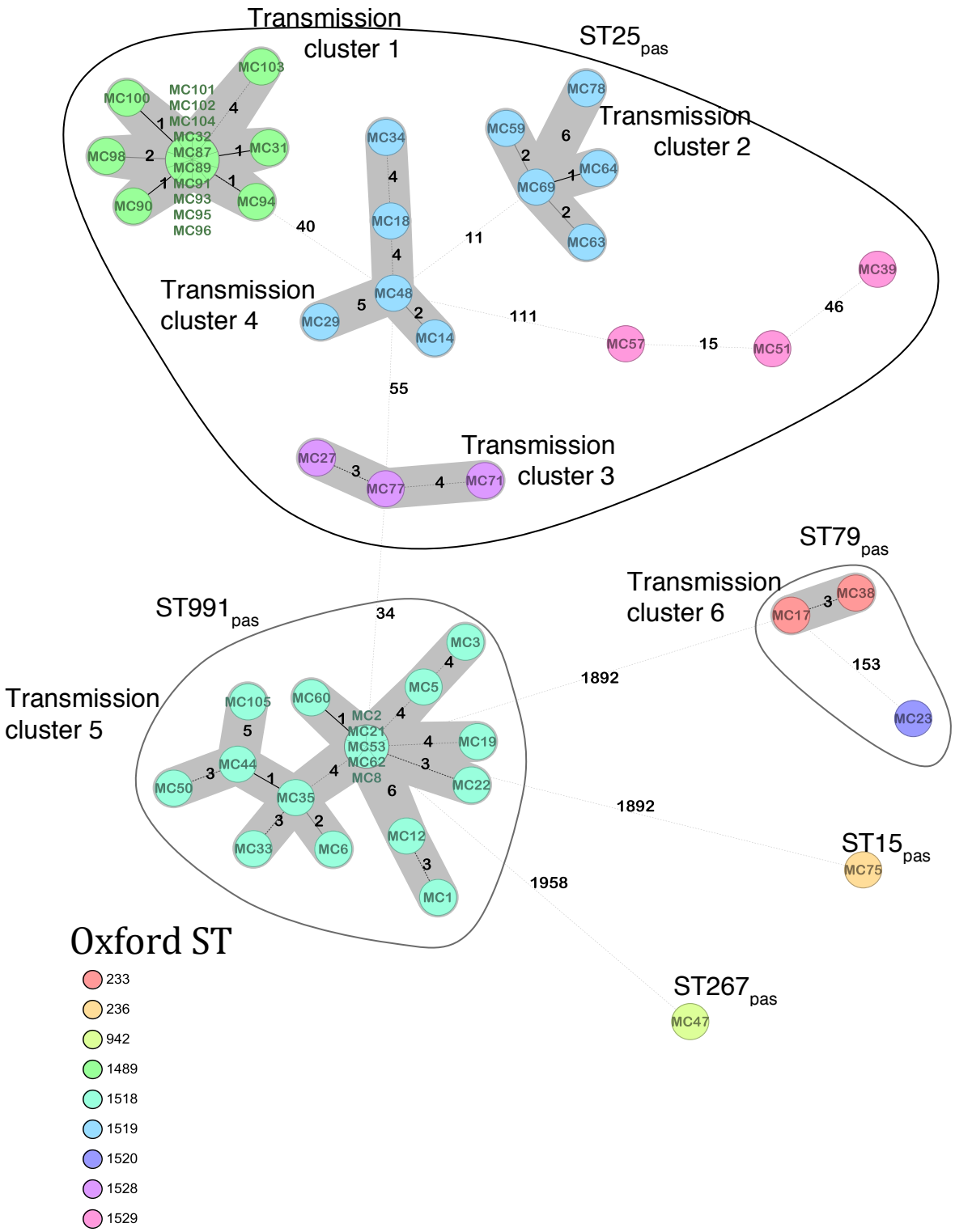

Figure 19. Minimum spanning tree generated using Ridom SeqSphere+ for 55 samples ignoring missing values.

- **Distance based on columns from *A. baumannii* cgMLST 2390 targets ACICU.**
- **Numbers between the nodes indicate the number of allelic differences.**
- **Shaded isolates represent transmission clusters.**

WGS analysis revealed that in total 51 isolates (88%) carried bla_{OXA-23} on Tn*2008*. Other genes conferring resistance to antibiotics such as aminoglycosides (*strA*, n=49; *strB*, n=49; *aac(3')-IIa*, n=49; *aac(3')-VIa*, n=5; *aadA1*, n=3; *aadB*, n=3); β-lactams (bla_{ADC-25}, n=55; bla_{TEM-1A}, n=4; bla_{TEM-1B}, n=2); sulphonamides (*sul2*, n=52); tetracyclines (*tet(B)*, n=48); trimethoprim (*dfrA*, n=3) and phenicols (*floR*, n=3) were also present. The gene *aac(3')-VIa* was located on a transposon, Tn*aphA6*, in five of the isolates. Results on the detected resistance genes of the isolates are summarised in Table 10.

Table 10. Resistome of the fifty-five sequenced *A. baumannii* isolates and location of the antimicrobial resistance genes as discovered by S1-PFGE.

		Isolate	Hospital	S1-PFGE+Southern blot	Resistome
CRAb	IC7	MC1	HMI	bla_{OXA-23} chrom*/ *strA* ~180 Kb p#	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC2	HMI	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC3	HMI	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC5	HMI	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC6	HMI	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC8	HMI	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC12	HMI	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC19	HMI	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC21	HMI	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC22	HMI	-	bla_{OXA-64} bla_{OXA-23} bla_{ADC-25}
		MC33	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC35	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC44	HV	-	bla_{OXA-64} bla_{OXA-23} bla_{ADC-25}
		MC50	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC53	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC60	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC62	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC105	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
	IC7	MC31	HV	bla_{OXA-23} chrom*/ *strA* ~180 Kb p#	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC32	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC87	HV	bla_{OXA-23} chrom*/ *strA* ~180 Kb p#	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC89	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC90	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC91	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC93	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC94	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC95	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC96	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC98	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC100	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC101	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC102	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC103	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*
		MC104	HV	-	*strA strB aac(3)-IIa* bla_{OXA-64} bla_{OXA-23} bla_{ADC-25} *sul2 tet(B)*

Table 10. Continued.

		Isolate	Hospital	S1-PFGE+Southern blot	Resistome
CRAb	IC7	MC14	HMI	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC18	HMI	*bla*$_{OXA-23}$ chrom*/ *strA* ~180 Kb p#	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC29	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC34	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC48	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC59	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC63	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC64	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC69	HV	*bla*$_{OXA-23}$ chrom*/ *strA* ~180 Kb p#	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC78	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
	IC7	MC39	HV	-	*bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$
		MC51	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC57	HV	*bla*$_{OXA-23}$ chrom*/ *strA* ~180 Kb p#	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
	IC7	MC27	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC71	HV	*bla*$_{OXA-23}$ chrom*/ *strA* ~180 Kb p#	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
		MC77	HV	-	*strA strB aac(3)-IIa bla*$_{OXA-64}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *sul2 tet(B)*
	IC4	MC75	HV	*bla*$_{OXA-23}$ chrom*/ *strA* ~150 Kb p#	*strA strB aac(3)-IIa aph(3')-Via bla*$_{OXA-51}$ *bla*$_{OXA-23}$ *bla*$_{ADC-25}$ *bla*$_{TEM-1B}$ *sul2*
Non-CRAb	IC5	MC23	HV	*strA* chrom*	*strA strB aadA1 aadB aph(3')-Via bla*$_{OXA-65}$ *bla*$_{ADC-25}$ *bla*$_{TEM-1A}$ *sul2 floR dfrA1*
		MC17	HMI	-	*strA strB aadA1 aadB aph(3')-Via bla*$_{OXA-65}$ *bla*$_{ADC-25}$ *bla*$_{TEM-1A}$ *sul2 floR dfrA1*
		MC38	HV	-	*strA strB aadA1 aadB aph(3')-Via bla*$_{OXA-65}$ *bla*$_{ADC-25}$ *bla*$_{TEM-1A}$ *sul2 floR dfrA1*
	Sg.$^{\beta}$	MC47	HV	*strA* ~180 Kb p#	*strA strB aac(3)-IIa aph(3')-Via bla*$_{OXA-180}$ *bla*$_{ADC-25}$ *bla*$_{TEM-1B}$ *sul2 tet(B)*

5. Location of resistance genes

S1-nuclease pulsed field gel electrophoresis (S1-PFGE) and Southern blot hybridization were perfomed to determine the plasmid size and the plasmid/chromosomal location of *bla*$_{OXA-23}$ and *strA* in a selection of isolates representing all the present ICs and unique Oxford STs (n=10) (Figure 19). Southern blot revealed that *bla*$_{OXA-23}$ was located on the chromosome in all tested isolates (n=8). However, the *strA* gene was encoded on a 184 Kb plasmid in all the IC7 isolates as well as in the singleton; the IC4 isolate carried the *strA* gene on a ~150 Kb plasmid. *strA* was always linked to *strB, sul2, aac(3)-IIa* and *tet(B)* in the IC7 isolates, as identified by contig overlap and confirmed using PCR-based gap closure. No *tet(B)* gene was detected in the IC4 isolate. In contrast, Southern blot revealed that *strA* was located on the chromosome in the IC5 isolates and was associated with *strB, sul2* and *floR*. Additionally, the IC5 isolates harbored *aadA1* and *aadB* on a 6.2 Kb plasmid (Table 10, Table 11).

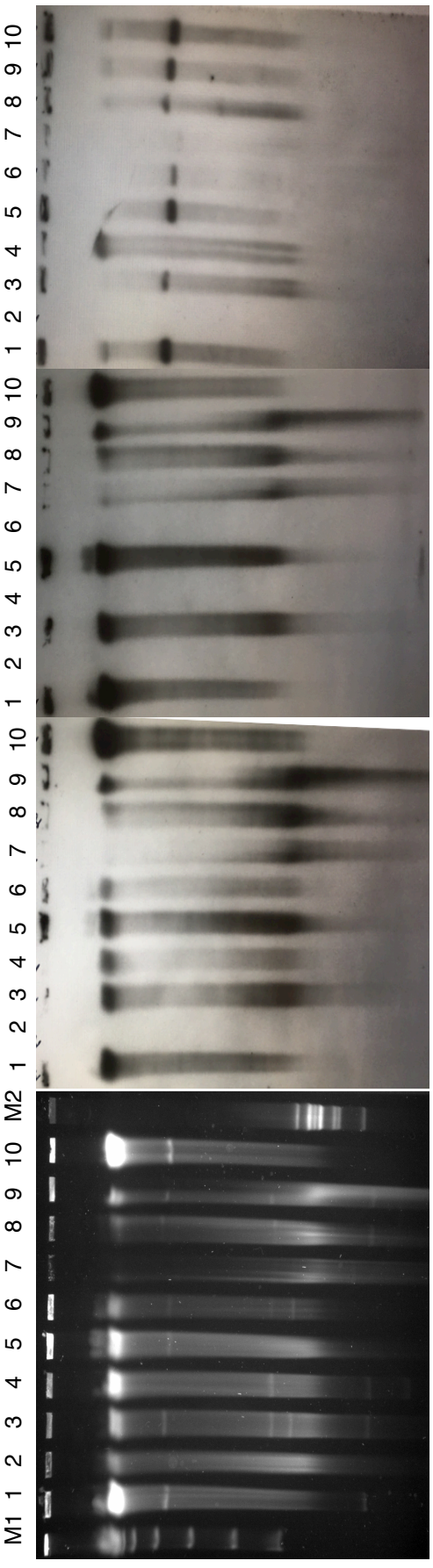

Figure 20. S1-PFGE and Southern blot with DIG-labelled bla_{OXA-51}, bla_{OXA-23} and *strA* probes. Isolates MC1, MC16, MC18, MC23, MC31, MC47, MC57, MC69, MC71 and MC87. M1, PFG marker; 1, MC1; 2, MC16; 3, MC18; 4, MC23; 5, MC31; 6, MC47; 7, MC57; 8, MC69; 9, MC71; 10, MC87; M2, λ monocut.

Table 11. Summary of the molecular epidemiology and resistance genes.

IC	ST Pasteur	ST Oxford	No. of isolates	*bla*$_{OXA-51-like}$	*bla*$_{OXA-23}$ Tn*2008*	*bla*$_{ADC-25}$	*bla*$_{TEM-1A}$	*bla*$_{TEM-1B}$	*strA+strB+ sul2+tetB*	*aac(3)-IIa*	*strA+strB+ sul2+floR*	*aadB+aadA 1*	*aph(3')-VIa* Tn*aphA6*
IC7	25	1489	16	*bla*$_{OXA-64}$	16	16	0	0	16	16	0	0	0
		1519	10		10	10	1	0	10	10	0	0	0
		1529	3		3	3	0	0	2	2	0	0	0
		1528	3		3	3	0	0	3	3	0	0	0
	991	1518	18		18	18	0	0	16	16	0	0	0
IC5	79	233	2	*bla*$_{OXA-65}$	0	2	2	0	0	0	2	2	2
		1520	1		0	1	1	0	0	0	1	1	1
IC4	15	236	1	*bla*$_{OXA-51}$	1	1	0	1	*strA+strB+sul2*	1	0	0	1
Singleton	267	942	1	*bla*$_{OXA-180}$	0	1	0	1	1	1	0	0	1
Total			**55**		51	55	4	2	48(+1)*	49	3	3	5

*48 isolates carried *strA+strB+sul2+tetB* and 1 isolate *strA+strB+sul2*

DISCUSSION

Formerly IC7 isolates have been described in some Latin American countries such as Paraguay or Argentina, but normally they were sporadic and not associated with outbreaks (142). However, IC7 was the most prevalent group in studies performed in Bolivia or Uruguay (141), which is in concordance with our findings. We found only one carbapenem-resistant IC4 isolate and three carbapenem-susceptible IC5 isolates, although, IC5 is the prevalent lineage in Latin America, the so called Pan-American clone, followed by IC4 (141, 144, 174, 183). The prevalence of IC7 isolates suggests a change in the epidemiology of carbapenem-resistant *A. baumannii* isolates in Bolivia and in particular the city of Cochabamba, when comparing these results to previous studies (183).

According to the Pan American Health Organization (PAHO) annual study, in Bolivia in 2010 19% of *Acinetobacter* spp. were resistant to imipenem and 7% to meropenem; in 2014, 51% of *A. baumannii* isolates were resistant to imipenem and 57% to meropenem (184, 185). In our study, similar results to those obtained by the PAHO in 2014 were found, and the resistance rates were similar to the ones in Colombia, where the presence of ST229 (Oxford) isolates (with $bla_{\text{OXA-64}}$), that belong to IC7, have also been reported (186, 187). High rates of carbapenem resistance in Hospital Materno Infantil have been already described in a previous study (149). In addition, the CRAb isolates present higher resistance rates to other antimicrobials such as ciprofloxacin and gentamicin, in comparison to the non-CRAb isolates, thus complicating

antimicrobial treatment options. When analyzing the population within both hospitals, it can be seen that different clusters are associated with each of them; almost all the isolates from Hospital Materno Infantil were ST991Pas while ST25^{Pas} isolates were mainly found in Hospital Viedma, just two ST25Pas isolates belonged to Hospital Materno Infantil. Some ST991^{Pas} isolates were also isolated in Hospital Viedma (n=8), which can suggest that there is cross-transmission from Hospital Materno Infantil to Hospital Viedma.

Diverse *bla*$_{OXA-51}$ variants such as *bla*$_{OXA-65}$, *bla*$_{OXA-64}$, *bla*$_{OXA-51}$ or *bla*$_{OXA-66}$ have been reported in Latin America but until now no *bla*$_{OXA-180}$ carbapenemase had been found (144). Moreover, Sennati et al described the presence of Tn*2008* in a ST25 (Pasteur) *A. baumannii* isolate from Bolivia that also carried *bla*$_{OXA-64}$ (176). In this transposon, IS*Aba1* is not only serving as a promoter for the carbapenemase encoding gene, but is also involved in the mobilization of the gene (177). This carbapenemase-encoding vehicle has spread worldwide (177), and similar to what Sennati et al found, in our isolates from the current study Tn*2008* is present in all the isolates belonging to ST25 (IC7) as well as in the ST15 isolate (IC4). Thus the mobilization of Tn*2008* has led to its prevalence among diverse ICs, increasing the resistance rates to carbapenems (177). Furthermore, our group of isolates carried resistance genes encoded on different structures such as transposons or plasmids that can also spread and confer antimicrobial resistance to other groups of drugs such as aminoglycosides, which in combination with carbapenem resistance, drastically reduces the therapeutic options (114).

In conclusion, IC7 was endemic in these two Bolivian hospitals, clustering together 50 isolates with different resistomes. In the present study we report the dissemination of several clones of CC25 (IC7) carrying the carbapenem-resistance determinant Tn*2008*. Although some studies are being carried out in South America, the situation in Bolivia is not very well known although epidemiological information is essential to implement infection control strategies in the hospital settings. To the best of our knowledge, this is the first study carried in Bolivia in which the molecular epidemiology of all the CRAb isolates has been analysed and the obtained data will be useful in order to know the *A. baumannii* population dynamics within these two hospitals.

3. Plasmid content in three Bolivian *Acinetobacter baumanni* isolates belonging to different International Clones.

RESULTS AND DISCUSSION

Three isolates representing all ICs from the previous study were selected in order to deeper study their plasmid content; MC1, an IC7 carbapenem-resistant isolate, isolated from Hospital Materno-Infantil; and two isolates from Hospital Viedma; MC23, a carbapenem susceptible isolate belonging to IC5, and MC75, a carbapenem-resistant IC4 isolate (Figure 20, Table 12). MC1 was isolated from a catheter in a patient with a bloodstream infection; MC23 from an urine sample and MC75 from a ulcer.

Table 12. Plasmid content according to S1-PFGE

Isolate	Plasmids*		
MC1	~180 Kb	~8 Kb	
MC23	~60 Kb	~8 Kb	~6 Kb
MC75	~150 Kb	~14 Kb	

*Size determined by PFGE.

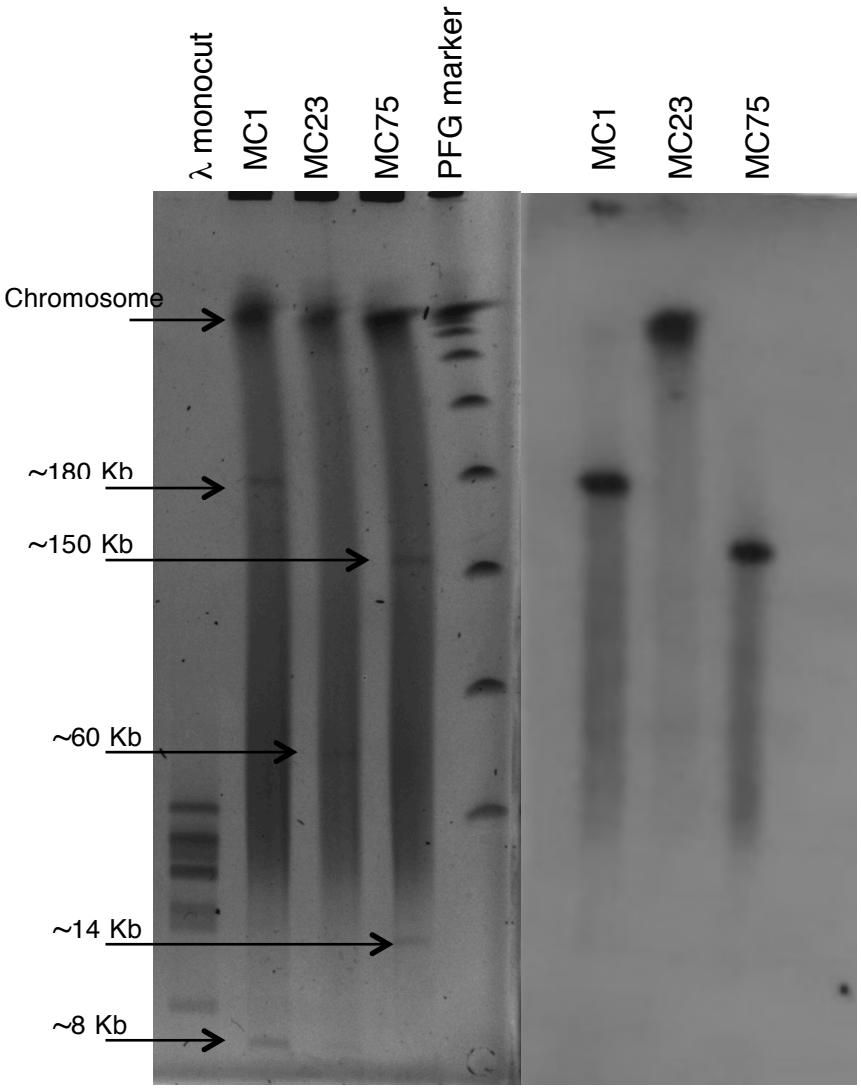

Figure 21. S1-PFGE and Southern blot with DIG-labelled *strA* probe. Left hand side shows S1-PFGE of isolates MC1, MC23 and MC75. All the plasmids but the 6 Kb can be seen. Right hand side correspond to the Southern blot-hybidization of the same isolates with *strA,* which is located on the chromosome in MC23 and in plasmids in MC1 and MC75..

Primers were designed for confirming the plasmid structure in MC1 and MC23 isolates. PCR mapping confirmed the contigs' overlaps (Table 13).

Table 13. PCR mapping using the primers designed for closing the ~180 Kb plasmid in isolate MC1. Green boxes indicate the contigs that belong together according to PCR results.

PRIMER	L68	L69	L70	L71	L72	L73	L74	L75	L76	L77	L80	L81	M1	M2	M3	M7	M8	M9	M10	M11
L68	-																			
L69	-	-																		
L70	1,5 Kb	-	-																	
L71	-	-	-	-																
L72	-	-	-	-	-															
L73	-	-	-	-	-	-														
L74	-	-	-	1,5 Kb	1,5 Kb	-	-													
L75	-	750 bp	-	-	-	-	-	-												
L76	-	-	-	-	-	-	-	-	-											
L77	-	-	-	-	750bp	-	-	-	400 bp	-										
L80	-	-	-	1,5 Kb	-	1,2 Kb	750 bp	-	-	-	-									
L81	-	-	-	-	-	-	-	-	-	-	-	3 bands								
M1	-	-	-	750bp	-	1,1 Kb	3 bands	-	-	5,8 Kb	-	-	-							
M2	-	-	-	-	-	-	600 bp	700 bp	-	-	-	6 Kb	-	800 bp						
M3	-	-	-	2Kb	1,9 Kb	2 Kb	1,2 Kb	-	-	-	-	-	-	-	-					
M7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
M8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
M9	-	-	-	-	-	3 Kb	2,5 Kb	-	-	-	-	-	-	-	-	3,5 Kb	-	4 Kb		
M10	-	-	-	-	-	1,5 Kb	-	3 Kb	-	-	-	-	-	-	-	-	-	4 Kb	-	
M11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4 Kb	-	-

For the ~8 Kb plasmid in MC1 two primers were designed L64 and L65. The plasmid structure was confirmed by PCR. The same results were obtained for MC23, showing that both isolates carried the same plasmid.

Another two pairs of primers were designed to confirm plasmid assemblies in MC23. M5-M6 primers to confirm the ~6 Kb plasmid and M17-M18 to confirm the ~60 Kb plasmid. The structure of each plasmid was confirmed by PCR products confirmed these two plasmids as well.

Long-read sequencing allowed us to confirm the plasmids' scaffolds and assemblies using hybridSpades, that uses both short and long reads, confirmed both the scaffold and the genes sequences (Table 14).

Table 14. Parameters of the different sequencing techniques and assemblies.

		MiSeq sequencing SPADES assembly		minION sequencing Canu assembly[#]			hybridSpades
Isolate	Plasmid	Contig (size)	Coverage	Contig	Coverage	Reads	
MC1	184 Kb	26 (61280 bp)	40.93	211335 bp	221.2	368	212554 bp
		44 (29233 bp)	22.09				
		50 (23758 bp)	18.89				
		51 (23406 bp)	26.35				
		55 (18260 bp)	27.09				
		62 (12764 bp)	21.54				
		73 (4171 bp)	68.85				
		83 (1817 bp)	36.49				
		84 (1810 bp)	34.64				
		87 (1309 bp)	698.65				
		92 (1101 bp)	53.38				
		95 (909 bp)	253.21				
		96 (906 bp)	673.91				
		97 (820 bp)	161.49				
		107 (418 bp)	28.68				
	8.7 Kb	65 (8858 bp)	729.06	8679 bp	4.91	9	8765 bp

Table 14. Continued.

		MiSeq sequencing SPADES assembly		minION sequencing Canu assembly[#]			hybridSpades
Isolate	Plasmid	Contigs (size)	Coverage	Contig	Coverage	Reads	
MC23	6 Kb	61 (6205 bp)	260.49	8575 bp	3.26	16	6205 bp
	8.7 Kb	53 (8858 bp)	582.06	8896 bp	16.26	33	8850 bp
	68 Kb	1 (62207 bp)* 10 (423 bp)* 5 (4769 bp)*	133.54 277.57 120.23	-	-	-	62207 bp 4769 bp 423 bp
MC75	13.9 Kb	77 (10281 bp) 104 (2361 bp) 130 (821 bp)	51.40 82.02 181.23	35615 bp	1.14	24	10281 bp 2361 bp 821 bp
	150 Kb	52 (25967 bp) 57 (19895 bp) 58 (19866 bp) 68 (15622 bp) 73 (12618 bp) 76 (11480 bp)	20.46 28.31 16.74 16.09 18.56 35.14	187286 bp	108.84	91	1841620 bp 34987 bp 53911 bp

Table 14. Continued.

		MiSeq sequencing SPADES assembly		minION sequencing Canu assembly[#]			hybridSpades
Isolate	Plasmid	Contigs (size)	Coverage	Contig	Coverage	Reads	
MC75	150 Kb	78 (10110 bp)	15.21	187286 bp	108.84	91	
		85 (8115 bp)	25.05				
		89 (5794 bp)	25.49				
		95 (3981 bp)	7.47				
		109 (2037 bp)	4.40				
		110 (1911 bp)	34.54				
		112 (1786 bp)	21.78				
		114 (1772 bp)	13.63				
		116 (1710 bp)	66.31				
		118 (1320 bp)	1047.67				
		125 (1087 bp)	446.14				
		127 (906 bp)	414.16				
		129 (821 bp)	61.18				
		132 (670 bp)	31.71				
		134 (580 bp)	73.14				

*These contigs correspond to a plasmidSpades assembly. [#] Contigs of the Canu assembly include the overlapping DNA.

Annotation of pMC1.1 (39% GC content) revealed many different IS such as IS*1006*, IS*1007*, IS*1008*, IS*Acsp1* (from *Acinetobacter* spp.), IS91 family, IS*Aha2 (*from *Acinetobacter haemolyticus)*, IS*Aba11*, IS*Aba12* and IS*17*. This plasmid carried a mercuric resistance operon, similar to an already described mercuric transposon in an IC1 *A. baumannii* 200 Kb plasmid (pA297-3, this isolate A297/RUH875 was recovered in The Netherlands in 1984) but lacking the *merP* open reading frame (128), that encodes for a protein that specifically binds to a mercuric ion in the periplasm and passes it to *merA,* a cytoplasmic reductase, via the transport protein *merT* (188). Different antimicrobial resistance determinants such as *strA, strB*, *aac(3)-IIa* and *aac(6')-Ian,* conferring resistance to aminoglycosides, *sul2* conferring resistance to sulphonamides and *tet(B)* conferring resistance to tetracycline were also present in two regions. The region of the plasmid carrying *strAB and sul2* shared a great homology (99%) with Tn*6172,* located in pA297-3 as well. However, *arsR, tetR* and *tet(B)* genes were also located within the transposon together with an IS*CR2* transposable element (IS91 family). The IS*CR2* element has been described related to different antimicrobial resistance genes, especially to *sul2*, contributing to their mobilization thanks to a rolling circle transposition mechanism (189). This structure was similar to formerly described ones such as those in Argentinan *A. baumannnii* isolates and another one in an ST25 *A. baumannii* isolate from Australia (131), however the location of *tetR-tetB* genes was different; they were located between *glmM* and *arsR* suggesting a possible later insertion of these two genes in different positions in the transposon (190). In addition, the same inverted repeats (IR) generated by the

insertion of the transposon were also found in the pMC1.1 plasmid, and with the similar backbone to pA297-3 suggest a common origin of these two plasmids. The genes *aac(3)-IIa* and *aac(6')-Ian* were associated with two IS6 family insertion sequences and bracketed by two IS*CR1* in an inverted orientation The IS*CR1* belong also to the IS91 family and have been described along with class 1 integrons and antimicrobial resistance genes (189), however to the best of our knowledge, these IS have never been described with *aac(3)-IIa* or *aac(6')-Ian*. Different conjugative protein encoding genes were also found in this plasmid, *traW, traY, traJ, traI*, *trbN* as well as genes involved in plasmid partition and replication *parB/repB* and *xerC*, that are related to segregational stability of plasmids. This plasmid encoded also for a system called BREX type 1 (bacteriophage exclusion) which has been described to be involved in phage resistance (191).

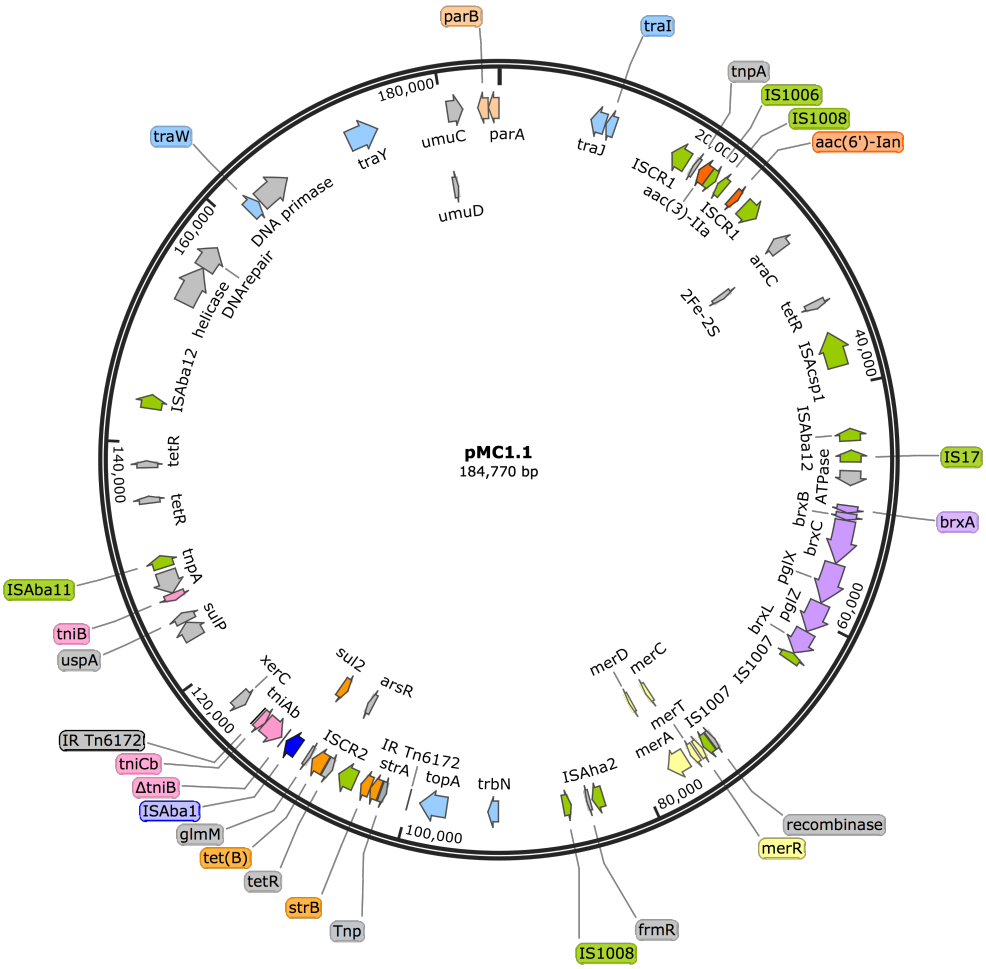

Figure 22. Map of plasmid pMC1.1.

The 8,7 Kb plasmids found in MC1 and MC23 (pMC1.2 and pMC23.2) were found to be identical, with a GC content of 34.3%. Annotation of this plasmid revealed ORFs encoding for a RepB replicon, a toxin-antitoxin system (BrnT-BrnA), that is involved in vertical stability; TonB-dependent receptor, related to the transmission of signals from the outside of the cell leading to transcriptional activation of target genes; septicolysin encoding gene, that is a cytolytic enzyme toward eukaryotc cells and is involved in pathogenesis, as well as *sel1* gene, that encodes for a protein that has been described in diverse prokaryotic genera and it has an important role in virulence. Some other

hypothetical proteins were also present. This small plasmid has been often found in IC1 *A. baumannii* isolates (128).

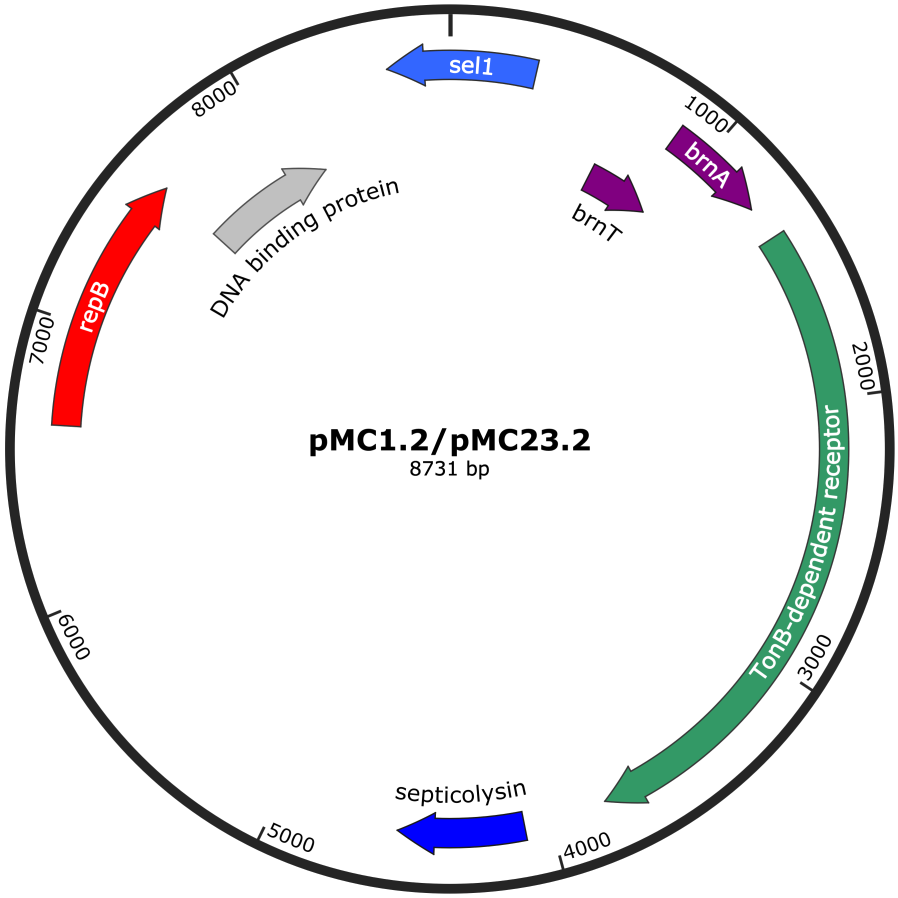

Figure 23. Map of plasmids pMC1.2/pMC23.2..

The biggest plasmid present in MC23 was pMC23.1, it belonged to GR6 according to its replicase, *repAci6*. Its GC content was 33.7% and almost all of its putative protein encoding genes were related to conjugative plasmid transfer in a *tra* locus, *traI* (*rlx*), that is a relaxase; the genes *traL* (*virB3*), *traE* (*virB5*), *traK* (*virB9*), *traB* (virB10), *traV* (*virB7*), *traC* (*virB4*), *trhF* (*Pep*), *traW* (*tivF8*), *traU* (*tivF7*), *trbC* (*tivF9*), *traN* (*tivF6*), *traF* (*tivF2*), *traH* (*tivF4*), *traG* (*tivF3*) and *traD*; that are part of type 4 (T4SS) secretion system. This T4SS is able to

secrete or take up both proteins and DNA, i.e. leading to natural competence (192). Two toxin encoding genes were present in the plasmid, *relE* and zeta toxin, but no antitoxins were found as in a very similar plasmid (pAC30c) in an *A. baumannii* isolate belonging to ST195 (IC2) (129); the stability genes *parA/parB* were also present.

The backbone of pMC23.1 and pAC30c was very similar, only a few differences were discovered. pMC23.1 lacked some hypothetical proteins present in pAC30c and the region encoding for tellurite resistance (*telA* gene and IS*66*) while *traD*, a cupin-like protein, (that is a superfamily of enzymes including dioxygenases, decarboxylases, hydrolases or isomerases); HlyD protein, that exports proteins from the cytosol to the outside of the cell, and an ABC transporter were not present in pAC30.

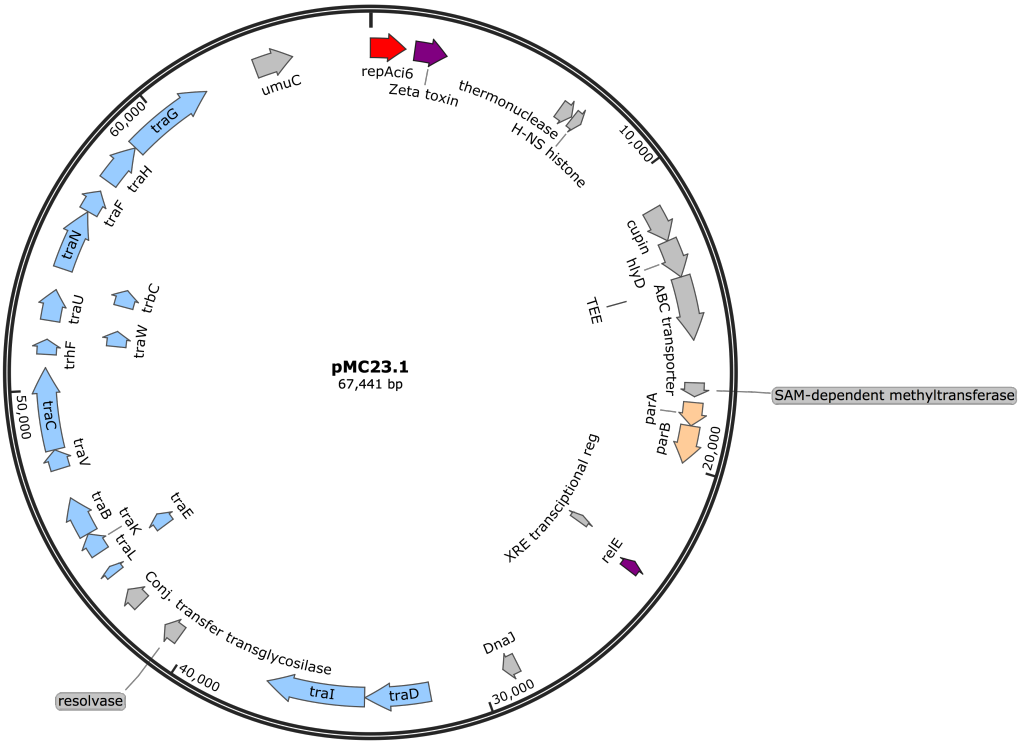

Figure 24. Map of plasmid pMC23.1.

Another small plasmid was present in the isolate MC23, pMC23.3 (39.2% GC content), and was found to have 100% similarity with an already described plasmid, pRAY, encoding resistance to gentamicin, kanamycin and tobramycin (*aadB* gene) together with *mobA* and *mobC* genes, which are thought to encode mobilization proteins (132). Many similar plasmids have been found in diverse *A. baumannii* ICs from different locations, suggesting a similar origin of all of them, and a subsequent diversification in their evolution. In accordance with other studies, no *rep* gene was found in the sequence, supporting the idea of the presence of a mechanism of replication relying on the host RNA polymerase (132).

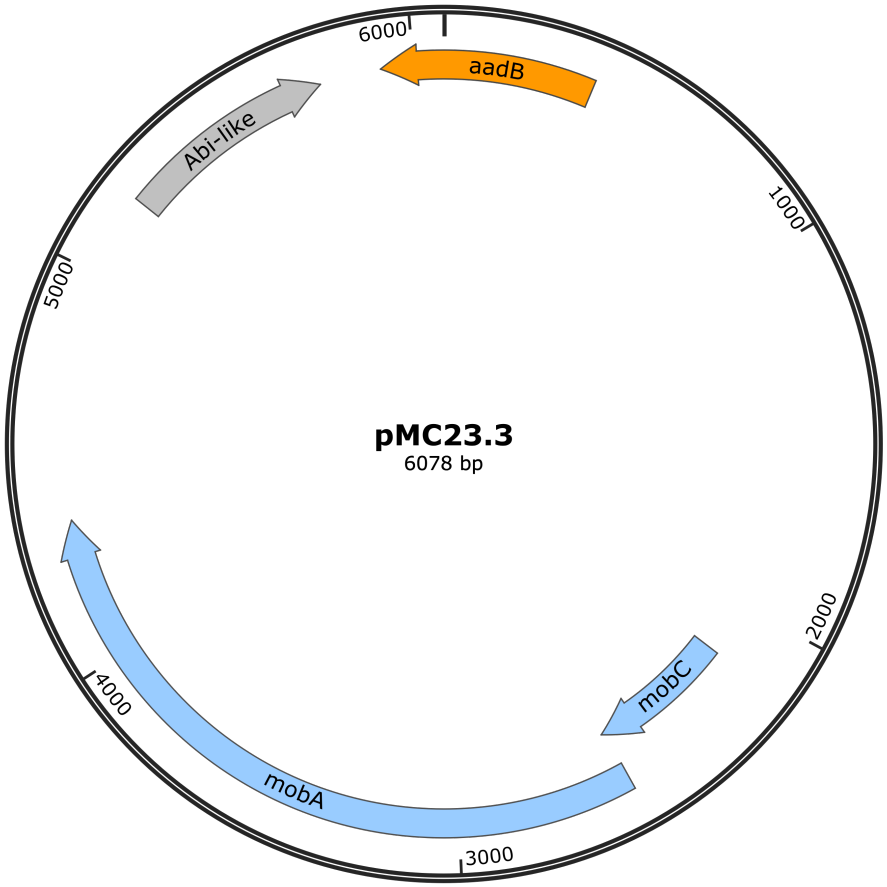

Figure 25. Map of plasmid pMC23.3.

Analysis of pMC75.1 revealed that it was 92% equal to pMC1.1, it also carried a Tn*6172,* in which antimicrobial resistance genes such as *sul2, strB* and *strA* were encoded but lacking *tet(B)* and *arsR* that were present in pMC1.1. The GC content of this plasmid was 37.49%. The mer operon was also found in this plasmid, *merR, merT, merP, merC, merA* and *merD* and many genes encoding conjugative transfer proteins, *traY, traW, traI, traJ, trbA* and *trbN.* Some genes of the BREX type 1 system were also present, which encode proteins involved in bacteriophage exclusión, *brxC, pglX, pglZ* and *brxL*. A *stbA* gene was found, the protein encoded by this gene has a role in plasmid stability as well as *parA/parB*. Several IS were also present, IS*Aba1*, IS*Aba125*, IS*Aba14*, IS*Aba42*, IS*1007* and IS*Aha2*. However, this plasmid lacked the transposon carrying *aac(3)-IIa* and *aac(6')-Ian*

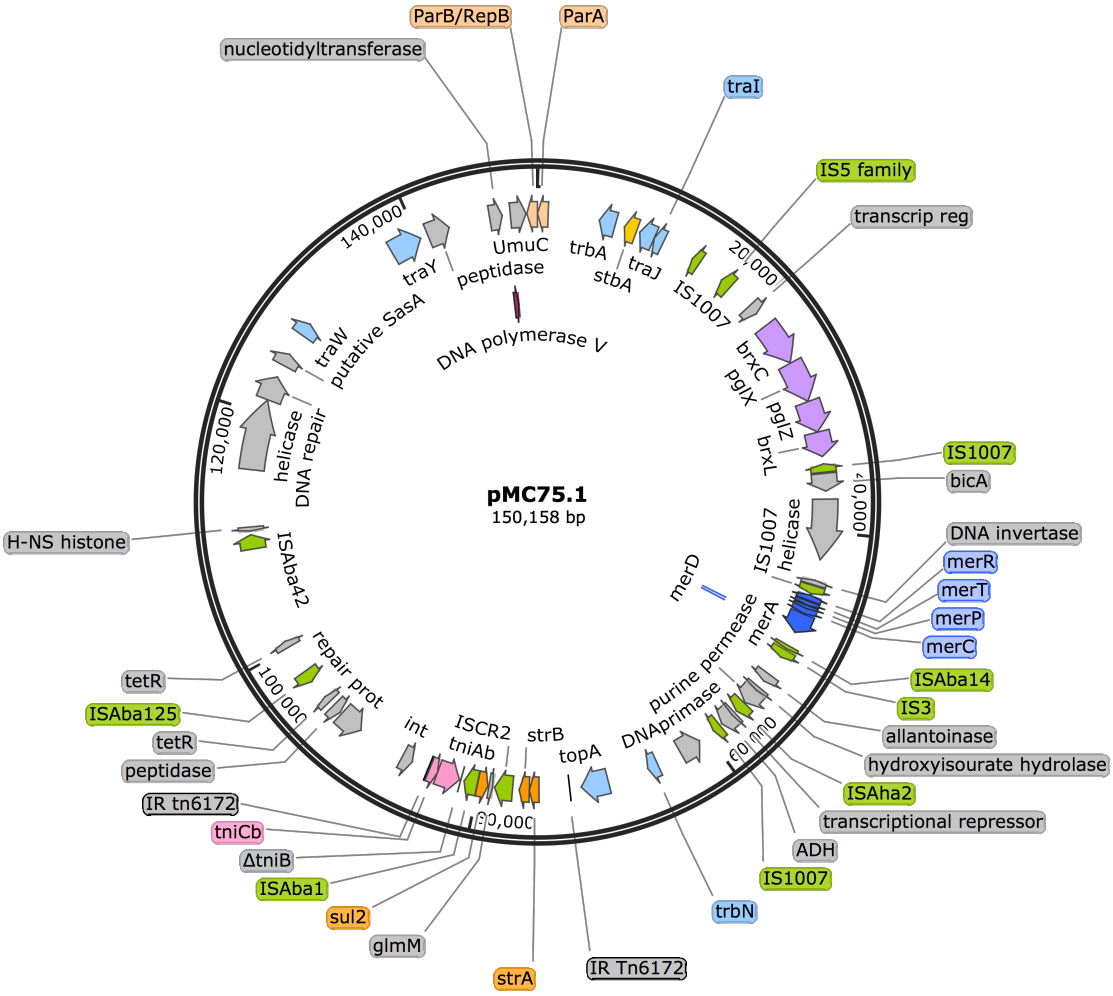

Figure 26. Map of plasmid pMC75.1.

The 13.9 Kb plasmid, pMC75.2 with a GC content of 40.3%, carried the extended-spectrum β-lactamase *bla*$_{\text{TEM-1B}}$ and the aminoglycoside resistance gene *aac(3)-IIa* flanked by two IS15DIV; a toxin-antitoxin system, *brnT/brnA*; a TonB-dependant receptor, a septicolysin gene and *mobA/mobS*, which are involved in plasmid mobility. The replicon of this plasmid belonged to the RepB (Rep-3 superfamily) with 100% homology.

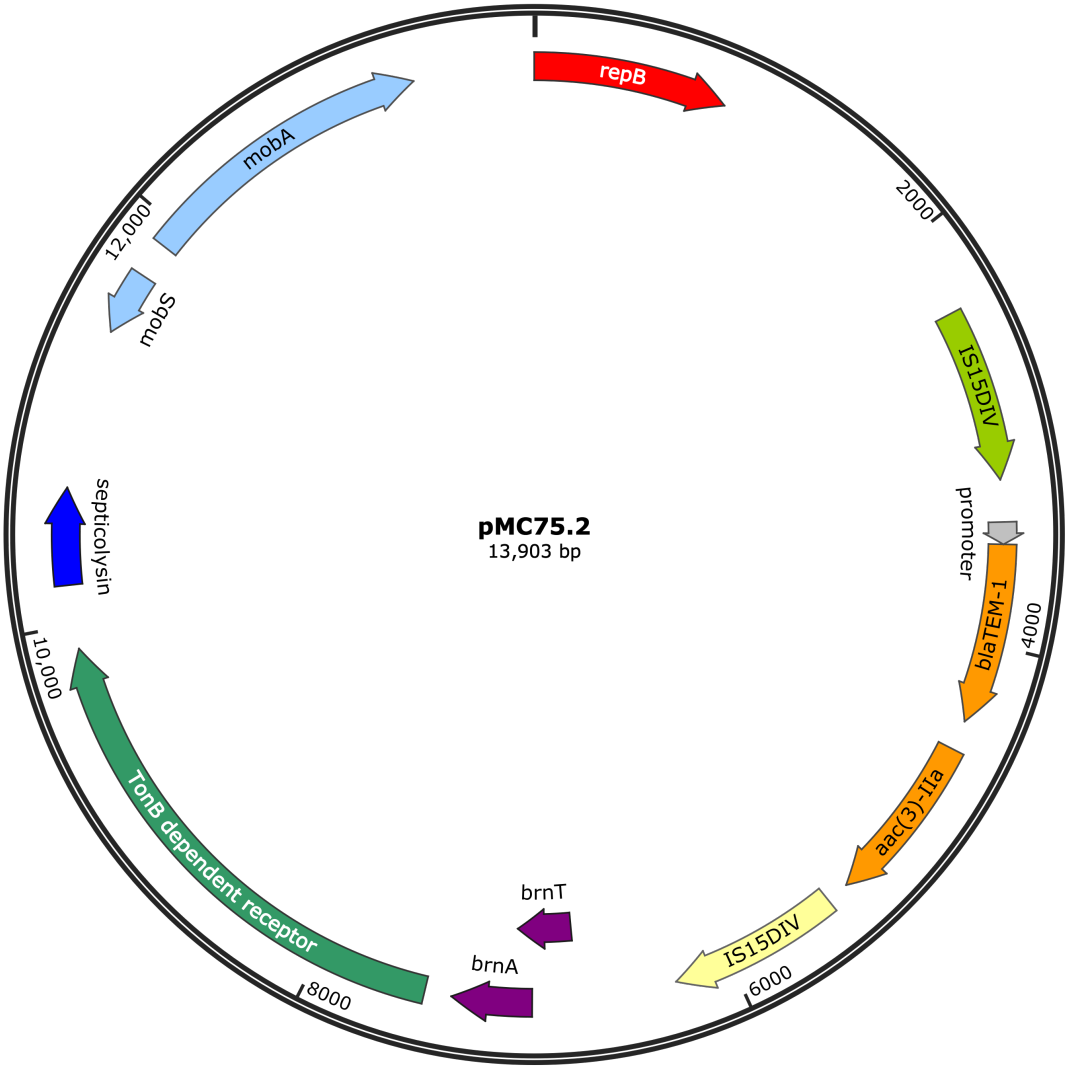

Figure 27. Map of plasmid pMC75.2.

1.1. Resistance islands

In contrast to what was found in MC1, *strA* was located in a resistance island in the chromosome in isolate MC23 (RI1.MC23), in addition, another resistance island was also found in this isolate (RI2.MC23).

The resistance island in which *strA* gene was found, carried other antimicrobial resistance genes such as *sul2, floR* and *strB.* Diverse IS were found as well, the resistance island was bracketed by two copies of a transposase of IS4 family in reverse orientation. Two genes involved in conjugation were also present in this structure, this finding suggests the possible insertion of this structure from a plasmid.

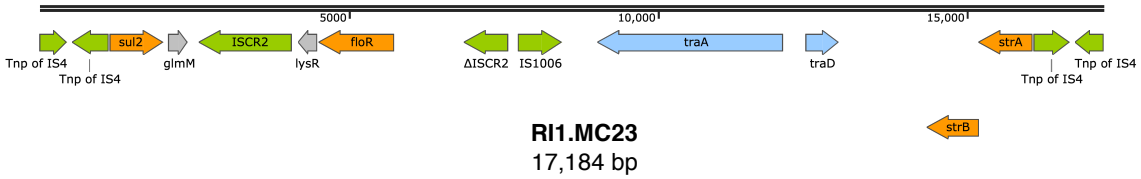

Figure 28. Map of resistance island RI1.MC23.

The second resistance island found in this isolate was RI2.MC23, that carried a typical structure from class 2 integrons, *dfrA-sat2-aadA1-ybeA-ybfA-ybfB-ybgA*, located between the Tn7 transposition module *tns*ABCDE and a non-functional IntI2 integrase. Additionally, a Tn3 transposon was found inserted in the Tn7 transposon, carrying three genes, *tnpA*, encoding for a Tn3 transposase; *tnpR* encoding a Tn3 resolvase and the antimicrobial resistance gene bla_{TEM-1A}.

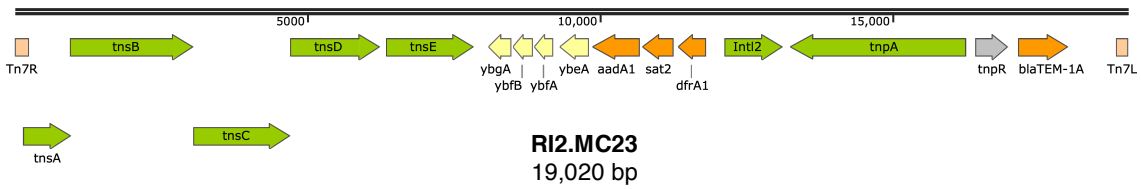

Figure 29. Map of resistance island RI2.MC23.

4. Identification of *Acinetobacter seifertii* isolated from Bolivian hospitals.

RESULTS

In three patients admitted to two different hospitals in Cochabamba, three isolates were recovered and identified as *Acinetobacter* sp. using phenotypical methods by the hospital laboratories according to the following criteria: Gram-negative by Gram stain; non-fermenter (K/K) by triple sugar iron agar; negative motility in brain heart infusion broth; negative oxidase; negative gelatin liquefaction; negative haemolysis. These data let to the assumption that the isolates were *A. baumannii,* and the patients were managed accordingly. The first isolate was recovered from a pressure ulcer (isolate MC37) in a 33 year-old man from Hospital Viedma in February 2016. The other two isolates were obtained from Hospital Materno-Infantil in March and April 2016, respectively. The first patient was a 59 year-old woman with an intraabdominal abscess from which the sample was taken (isolate MC13), the other isolate was recovered from a catheter in a 2 year-old child diagnosed with sepsis (isolate MC16).

All three isolates were susceptible to ciprofloxacin (MICs: 0.5-1 μg/mL), gentamicin (MICs: 0.5-1 μg/mL), imipenem (MIC: 0.125 μg/mL), meropenem (MIC: 0.25 μg/mL) and tigecycline (MIC: 0.5 μg/mL) as determined by agar dilution and they were also susceptible to colistin (MIC: 2 μg/mL) according to broth microdilution.

Semi-automated identification using the VITEK®2 GN ID card was only able to assign the isolates to the *A. baumannii* complex (containing also

Acinetobacter pittii, Acinetobacter calcoaceticus and *Acinetobacter nosocomialis*) while MALDI-TOF MS results showed a mixed pattern of *A. baumannii* (scores ranging from 2.02-2.34) and *A. pittii* (scores ranging from 1.89-2.08) (Table 15).

The band patterns obtained by *gyrB* multiplex PCR were inconclusive as well; they included bands specific for both *A. pittii* and *A. baumannii.* No bla_{OXA} genes were detected including the $bla_{OXA\text{-}51\text{-}like}$, which is intrinsic to *A. baumannii* (Table 15).

Because no conclusive results were obtained by these methods, whole genome sequencing was performed for the identification at the species level.

SpeciesFinder failed to resolve the species identification and *A. baumannii* was the first hit using KmerFinder, which we considered as a non-valid result because no $bla_{OXA\text{-}51\text{-}like}$ gene was found in any of the three isolates (Table 15). Partial *rpoB* analysis clustered the isolates with *A. seifertii,* therefore further analysis of the three isolates was carried out.

MLSA revealed that the percentage of similarity within species was above 97.8% while interspecies similarity was below 97.0%. The highest similarity values obtained positioned the three isolates together with *A. seifertii.* Furthermore, a neighbour-joining tree was constructed based on the seven concatenated housekeeping genes of the Pasteur scheme (MC13, ST994; MC16, ST994 and MC37, ST1064) and the three isolates fell within the cluster of *A. seifertii* strains (Figure 21).

Table 15. Results of species identification using different phenotypical and molecular methods.

Strain Sequence Type (Oxford/Pasteur)	**VITEK®2**	**MALDI-TOF MS**	***gyrB* PCR**	**SpeciesFinder**	**KmerFinder** (score, total template coverage)	***rpoB***	**MLSA** (similarity)	**ANIb**	**dDDH**
MC13* 1530/994	*A. baumannii* complex	*A. baumannii* (score 2.28-2.31) *A. pittii* (score 2.02-2.08)	*A. baumannii* *A. pittii*	FAILED	*A. baumannii* (2123, 26.14%)	*A. seifertii*	*A. seifertii* (98.82-99.56%)	*A. seifertii*	*A. seifertii*
MC16* 1530/994	*A. baumannii* complex	*A. baumannii* (score 2.33-2.34) *A. pittii* (score 2.05)	*A. baumannii* *A. pittii*	FAILED	*A. baumannii* (2122, 26.13%)	*A. seifertii*	*A. seifertii* (98.82-99.56%)	*A. seifertii*	*A. seifertii*
MC37 1559/1064	*A. baumannii* complex	*A. baumannii* (score 2.02-2.09) *A. pittii* (score 1.89)	*A. baumannii* *A. pittii*	FAILED	*A. baumannii* (2090, 24.19%)	*A. seifertii*	*A. seifertii* (98.86-99.60%)	*A. seifertii*	*A. seifertii*

*These two isolates belong to the same strain as determined by ANIb and dDDH

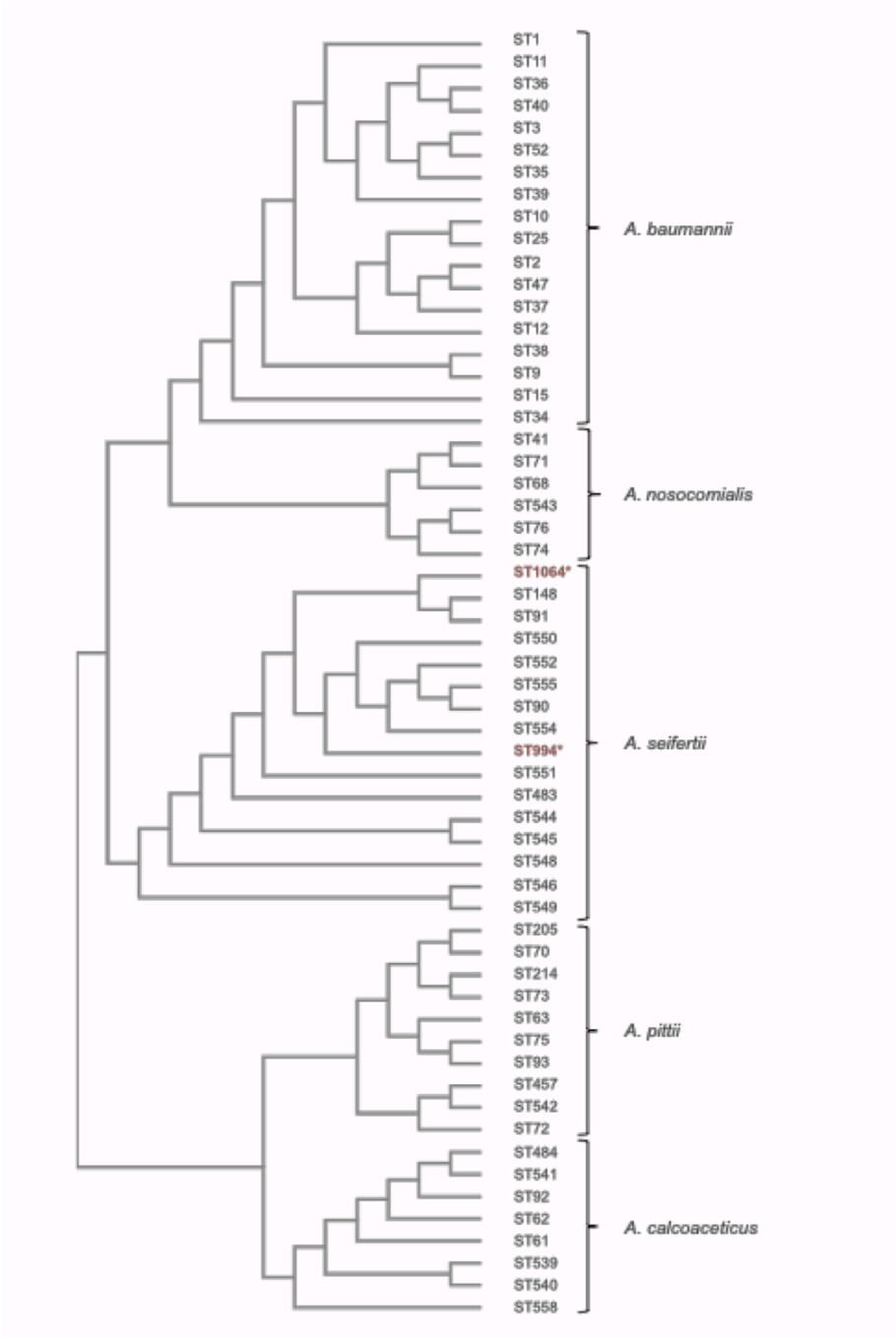

Figure 30. MLSA tree based on the seven concatenated housekeeping genes.

Additionally, dDDH results were congruent with MLSA, showing values above 70% between the three studied isolates and *A. seifertii* NIPH 973^{T} (Table 16).

Table 16. dDDH results.

		Formula 1			
Query genome	**Reference genome**	**DDH**	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**
MC13	*A. baumannii* CIP 70.34^T	60.70	[57.1 - 64.3%]	0.2355	44.71
MC13	*A. dijkshoorniae* JVAP01^T	64.20	[60.4 - 67.8%]	0.2155	56.88
MC13	*A. nosocomialis* NIPH 2119^T	74.10	[70.1 - 77.7%]	0.1622	82.98
MC13	*A. pittii* CIP 70.29^T	69.90	[66 - 73.6%]	0.1840	74.06
MC13	*A. calcoaceticus* CIP 81.8^T	65.80	[61.9 - 69.4%]	0.2065	62.18
MC13	*A. seifertii* NIPH 973^T	76.40	[72.4 - 79.9%]	0.1506	86.65
MC13	MC13	100.00	[100 %]	0.0000	99.62
MC13	MC16	99.90	[99.9 - 100%]	0.0033	99.59
MC13	MC37	79.30	[75.3 - 82.8%]	0.1359	90.3
		Formula 2			
		DDH	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**
MC13	*A. baumannii* CIP 70.34^T	39.70	[37.2 - 42.2%]	0.1005	2.52
MC13	*A. dijkshoorniae* JVAP01^T	33.40	[31 - 35.9%]	0.1253	0.38
MC13	*A. nosocomialis* NIPH 2119^T	46.00	[43.5 - 48.6%]	0.0815	9.95
MC13	*A. pittii* CIP 70.29^T	34.20	[31.7 - 36.7%]	0.1218	0.5
MC13	*A. calcoaceticus* CIP 81.8^T	31.50	[29.1 - 34%]	0.1341	0.19
MC13	*A. seifertii* NIPH 973^T	74.30	[71.3 - 77.1%]	0.0304	84.86
MC13	MC13	100.00	[100 %]	0.0000	98.3
MC13	MC16	100.00	[99.9 - 100%]	0.0001	98.29
MC13	MC37	73.20	[70.2 - 76%]	0.0318	83.38

Table 16. Continued.

		Formula 3				
Query genome	**Reference genome**	**DDH**	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**	**G+C difference**
MC13	*A. baumannii* CIP 70.34^T	56.20	[53.1 - 59.4%]	0.3123	12.35	0.62
MC13	*A. dijkshoorniae* JVAP01^T	56.00	[52.8 - 59.1%]	0.3138	11.78	0.41
MC13	*A. nosocomialis* NIPH 2119^T	69.30	[65.9 - 72.5%]	0.2305	71.14	0.32
MC13	*A. pittii* CIP 70.29^T	60.60	[57.3 - 63.8%]	0.2834	27.89	0.40
MC13	*A. calcoaceticus* CIP 81.8^T	56.10	[53 - 59.3%]	0.3129	12.11	0.23
MC13	*A. seifertii* NIPH 973^T	78.80	[75.3 - 81.9%]	0.1764	94.25	0.18
MC13	MC13	100.00	[100 %]	0.0000	99.99	0.00
MC13	MC16	100.00	[100 %]	0.0034	99.99	0.05
MC13	MC37	81.10	[77.7 - 84%]	0.1634	96.27	0.00

Table 16. Continued.

		Formula 1			
Query genome	**Reference genome**	**DDH**	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**
MC16	*A. baumannii* CIP 70.34^T	60.60	[56.9 - 64.2%]	0.2364	44.15
MC16	*A. dijkshoorniae* JVAP01^T	63.80	[60 - 67.4%]	0.2178	55.47
MC16	*A. nosocomialis* NIPH 2119^T	74.10	[70.1 - 77.7%]	0.1621	83.03
MC16	*A. pittii* CIP 70.29^T	69.50	[65.6 - 73.1%]	0.1863	72.98
MC16	*A. calcoaceticus* CIP 81.8^T	65.40	[61.6 - 69%]	0.2087	60.93
MC16	*A. seifertii* NIPH 973^T	76.40	[72.5 - 80%]	0.1502	86.75
MC16	MC16	100.00	[100 %]	0.0000	99.62
MC16	MC37	79.40	[75.4 - 82.8%]	0.1354	90.39
		Formula 2			
		DDH	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**
MC16	*A. baumannii* CIP 70.34^T	39.70	[37.3 - 42.3%]	0.1003	2.55
MC16	*A. dijkshoorniae* JVAP01^T	33.50	[31 - 36%]	0.1249	0.39
MC16	*A. nosocomialis* NIPH 2119^T	46.10	[43.5 - 48.6%]	0.0814	10.03
MC16	*A. pittii* CIP 70.29^T	34.20	[31.8 - 36.8%]	0.1214	0.51
MC16	*A. calcoaceticus* CIP 81.8^T	31.60	[29.2 - 34.1%]	0.1338	0.2
MC16	*A. seifertii* NIPH 973^T	74.30	[71.3 - 77.1%]	0.0304	84.88
MC16	MC16	100.00	[100 %]	0.0000	98.3
MC16	MC37	73.30	[70.2 - 76.1%]	0.0317	83.5

Table 16. Continued.

		Formula 3				
		DDH	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**	**G+C difference**
MC16	*A. baumannii* CIP 70.34^T	56.10	[53 - 59.3%]	0.3130	12.09	0.57
MC16	*A. dijkshoorniae* JVAP01^T	55.80	[52.6 - 58.9%]	0.3155	11.16	0.36
MC16	*A. nosocomialis* NIPH 2119^T	69.30	[65.9 - 72.6%]	0.2303	71.3	0.27
MC16	*A. pittii* CIP 70.29^T	60.30	[57.1 - 63.5%]	0.2851	26.72	0.35
MC16	*A. calcoaceticus* CIP 81.8^T	55.90	[52.7 - 59%]	0.3146	11.5	0.17
MC16	*A. seifertii* NIPH 973^T	78.80	[75.4 - 81.9%]	0.1760	94.32	0.13
MC16	MC16	100.00	[100 %]	0.0000	99.99	0.00
MC16	MC37	81.10	[77.8 - 84.1%]	0.1629	96.34	0.05

Table 16. Continued.

		Formula 1				
Query genome	**Reference genome**	**DDH**	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**	
MC37	*A. baumannii* CIP 70.34^T	59.20	[55.6 - 62.8%]	0.2446	39.25	
MC37	*A. dijkshoorniae* JVAP01^T	62.90	[59.2 - 66.5%]	0.2225	52.61	
MC37	*A. nosocomialis* NIPH 2119^T	71.10	[67.2 - 74.8%]	0.1775	77.04	
MC37	*A. pittii* CIP 70.29^T	65.70	[61.9 - 69.4%]	0.2067	62.09	
MC37	*A. calcoaceticus* CIP 81.8^T	66.00	[62.2 - 69.7%]	0.2050	63.06	
MC37	*A. seifertii* NIPH 973^T	72.60	[68.6 - 76.2%]	0.1699	80.15	
MC37	MC37	100.00	[100 %]	0.0000	99.62	
		Formula 2				
		DDH	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**	
MC37	*A. baumannii* CIP 70.34^T	39.50	[37.1 - 42.1%]	0.1010	2.41	
MC37	*A. dijkshoorniae* JVAP01^T	33.60	[31.1 - 36.1%]	0.1244	0.41	
MC37	*A. nosocomialis* NIPH 2119^T	46.00	[43.4 - 48.6%]	0.0816	9.87	
MC37	*A. pittii* CIP 70.29^T	34.10	[31.7 - 36.6%]	0.1220	0.49	
MC37	*A. calcoaceticus* CIP 81.8^T	31.70	[29.3 - 34.2%]	0.1331	0.21	
MC37	*A. seifertii* NIPH 973^T	74.10	[71.1 - 76.9%]	0.0306	84.69	
MC37	MC37	100.00	[100 %]	0.0000	98.3	
		Formula 3				
		DDH	**Model C.I.**	**Distance**	**Prob. DDH >= 70%**	**G+C difference**
MC37	*A. baumannii* CIP 70.34^T	55.00	[51.8 - 58.1%]	0.3209	9.43	0.62
MC37	*A. dijkshoorniae* JVAP01^T	55.20	[52.1 - 58.3%]	0.3193	9.93	0.41
MC37	*A. nosocomialis* NIPH 2119^T	66.90	[63.5 - 70.1%]	0.2446	60.07	0.32
MC37	*A. pittii* CIP 70.29^T	57.50	[54.3 - 60.7%]	0.3034	16.1	0.40
MC37	*A. calcoaceticus* CIP 81.8^T	56.40	[53.3 - 59.6%]	0.3109	12.9	0.23
MC37	*A. seifertii* NIPH 973^T	75.40	[72 - 78.6%]	0.1953	89.42	0.18
MC37	MC37	100.00	[100 %]	0.0000	99.99	0.00

In addition, ANIb also supported these results as all three isolates had values well above the 95% for *A. seifertii* (Table 17). Isolates MC13 and MC16 presented highly similar genomes as determined by ANIb and dDDH values, 99.9% and 100% respectively.

Table 17. ANIb results.

#ANIb and aligned percentage	*A. baumannii* CIP 70.34^T	*A. dijkshoorniae* JVAP01^T	*A. nosocomialis* NIPH 2119^T	*A. pittii* CIP 70.29^T	*A. calcoaceticus* CIP 81.8^T	*A. seifertii* NIPH 973^T	MC13	MC16	MC37
A. baumannii CIP 70.34^T	*	87.01 [73.76]	90.85 [74.16]	87.55 [73.81]	86.01 [70.55]	89.39 [71.40]	89.58 [72.19]	89.58 [71.83]	89.26 [71.37]
A. dijkshoorniae JVAP01^T	87.07 [81.27]	*	86.92 [81.61]	92.97 [86.80]	89.54 [82.50]	86.85 [77.58]	86.91 [79.37]	86.94 [78.99]	86.89 [78.22]
A. nosocomialis NIPH 2119^T	91.04 [78.49]	87.07 [79.03]	*	87.32 [78.32]	86.06 [76.04]	91.77 [82.37]	91.63 [81.57]	91.66 [81.04]	91.51 [79.15]
A. pittii CIP 70.29^T	87.58 [81.58]	93.09 [86.75]	87.16 [81.93]	*	89.49 [82.59]	87.50 [80.20]	87.44 [81.87]	87.46 [81.48]	87.30 [79.27]
A. calcoaceticus CIP 81.8^T	86.18 [77.25]	89.68 [81.99]	86.13 [78.93]	89.49 [82.18]	*	86.15 [78.05]	86.19 [79.73]	86.21 [79.34]	86.28 [78.96]
A. seifertii NIPH 973^T	89.43 [72.73]	86.87 [72.09]	91.71 [80.13]	87.45 [74.22]	85.95 [73.00]	*	96.59 [81.19]	96.59 [80.71]	96.47 [78.42]
MC13	89.36 [77.26]	86.77 [76.86]	91.47 [81.95]	87.21 [78.89]	86.03 [77.10]	96.58 [84.68]	*	99.99 [97.27]	96.37 [83.88]
MC16	89.25 [77.63]	86.78 [76.98]	91.35 [82.32]	87.23 [79.25]	86.02 [77.37]	96.52 [84.89]	99.98 [98.63]	*	96.34 [84.17]
MC37	89.32 [77.43]	86.89 [77.07]	91.46 [81.19]	87.26 [77.80]	86.15 [78.17]	96.66 [83.37]	96.60 [85.52]	96.60 [85.06]	*

DISCUSSION

While *A. baumannii* remains the most frequently recovered species of the genus *Acinetobacter* from clinical samples, there are increasing reports of other *Acinetobacter* species being involved in human infections (4, 103). To the best of our knowledge, this is the first report of *A. seifertii* in Bolivia causing different infections and the second report from South America (4).

A. seifertii infections and their outcomes are not well characterized, nonetheless in-vitro virulence-associated phenotypes such as increased biofilm formation, cell adherence and resistance to human serum were found in Korean isolates (193). Both cell adherence and biofilm formation could have contributed to the catheter-related infection, ulcer infection and abscess formation.

Although *A. seifertii* has now been reported to cause serious infections, it is in general susceptible to antimicrobials (103), but in some cases plasmids carrying antimicrobial resistance genes have been described within this species (4, 79). The acquisition of different antimicrobial resistance determinants is worrying because it narrows the therapeutic options. Furthermore, having the correct *Acinetobacter* species identification is important to understand the epidemiology and the clinical impact of the various *Acinetobacter* species. In our case, the standard tests used for species identification in the diagnostic laboratories were not adequate, reflecting that newer species such as *A. seifertii* are not yet included in the databases of these semi-automated identification methods. Although *rpoB* sequence analysis identified the isolates as *A. seifertii*, further analysis was necessary to confirm the result. In fact, as already stated

by Khosravi et al, identification based on a single gene sequence may not always be fully reliable (194), and underreporting of *A. seifertii* and other *Acinetobacter* species may be occurring due to the difficulties to identify them. Moreover, the limitations of using VITEK®2 and also *rpoB* sequences for identification of *Acinetobacter* spp. have already been described (83, 194). In addition, the necessity to include this species in the Bruker taxonomy database has been already discussed (82).

Nowadays, the most reliable methods for bacterial identification are based on the whole genome sequence [1,19,20], and our results based on these methods were congruent and allowed us to identify the species of the three isolates. It is important to be able to differentiate *Acinetobacter* species since the antibiotic susceptibilities and clinical outcomes can be very diverse among the different *Acinetobacter* species (82, 96).

In conclusion, the phenotypic and semi-automated identification methods are unreliable for identification of *A. seifertii*. In this study, identification of the three isolates to the species level was not possible until high-resolution molecular methods were used. *A. seifertii* appears to be a challenge as various studies have described misidentification of this species. Even if its prevalence, epidemiology, or virulence are not yet well known, an improvement in diagnostic laboratories should be made in order to include this organism of growing importance. Further studies should be carried forward in order to better characterize this species and analyse its incidence and clinical impact.

GENERAL DISCUSSION

GENERAL DISCUSSION

Infections caused by MDR *A. baumannii* have been increasingly reported worldwide together with the presence of carbapenem resistance. Carbapenems are the antimicrobials of choice to treat infections caused by MDR Gram-negative bacteria, but with the increase in carbapenem-resistance among *A. baumannii* isolates, the antibiotic therapy can be compromised. These carbapenem-resistance mechanisms can be found in very diverse structures such as an IS overexpressing the intrinsic $bla_{\text{OXA-51-like}}$ in *A. baumannii;* transposons carrying antimicrobial resistance genes located in both the chromosome or plasmids, or plasmids themselves carrying a wide variety of genes encoding antimicrobial resistance as well as virulence and other features; independently of the IC.

It is important to study the dynamics and resistomes of the bacterial populations in order to understand the situation in each hospital or unit, and be able to handle the infection control plan appropriately.

In this study the molecular epidemiology of a total of 87 *A. baumannii* isolates recovered from Bolivian hospitals was analyzed. The majority of these isolates belonged to different clones of IC7 (n=76), but there was also presence of IC4 and IC5 as sporadic cases. The epidemiological situation in these Bolivian hospitals differs from that in other Latin American studies. For example, IC4 ($CC15^{P}$) has been described in a study comprising 69.4% of the isolates between 2009-2011 in a Brazilian hospital, all these isolates carried the carbapenem-resistance gene $bla_{\text{OXA-23}}$ (142). This IC has been also found in

other Latin American countries such as Argentina and Chile, normally representing unrelated or sporadic cases (3). In the previously mentioned study, IC5 ($CC79^{P}$) comprised just 10% of the isolates, it appears to be the most prevalent among other studies carried in Latin American countries as well (147, 148). IC5 has been mainly isolated in North, Central and South America and it received the name Pan-American clone because of this (3). On the other hand, IC7 ($CC25^{P}$) has been reported worldwide and associated with diverse antimicrobial resistance determinants such as bla_{NDM-2}, bla_{OXA-72}, bla_{OXA-58} and bla_{OXA-23} (3, 139, 149). In Latin America, this clone has been reported in hospitals in Brazil, Paraguay, Bolivia, Argentina, Colombia, Mexico and Venezuela (3, 141, 142). Two sporadic *A. baumannii* isolates belonging to IC5 and IC7, respectively, have recently been isolated from neonates in a hospital in Brazil (150), while in Colombia most of the isolates recovered in a 2017 study belonged to $CC636^{Ox}$ (IC5) followed by $CC110^{Ox}$ (IC7) (151).

The prevalence of endemic IC7 *A. baumannii* isolates in these two hospitals suggests a change in their situation when compared to previous studies as well as a difference with other neighbouring countries (183).

All the carbapenem resistant isolates carried the bla_{OXA-23} gene in a Tn*2008,* which has been previously described in diverse ICs (177, 195) including IC7 isolates recovered from a Hospital in the same city (176). This Tn*2008* contributes to the overexpression of the carbapenemase gene and to its mobilization.

There was a high prevalence of genes conferring aminoglycoside resistance such as *aac(3)-IIa*, *strA* and *strB*; *tetB* conferring resistance to tetracycline or *sul2* conferring resistance to sulphonamide. These genes were found both in the chromosome and plasmids, demonstrating the plasticity of the *A. baumannii* genome and the mobility of these genes within mobile genetic elements such as transposons or plasmids.

The endemicity of IC7 isolates encoding the bla_{OXA-23} carbapenemase gene mirrors the importance of the epidemiological analysis and the establishment of adequate infection control measures to avoid the spread of the clones within the hospital and the possible mobilization of antimicrobial resistance mechanisms within *A. baumannii* or even to other species or genus.

In addition to the importance of studying the epidemiology and antimicrobial resistance mechanisms of *A. baumannii* in every hospital or ward, the relevance of correctly identifying the species within the genus and difficulties in this process have been also discussed.

As infections caused by *A. baumannii* have been increasingly reported in the last decades together with its resistance to antibiotics, specially carbapenems, special control measures are needed in order to prevent its spread causing outbreaks. Other species of the genus are being reported lately, and as well as in the case of *A. baumannii*, several carbapenem resistance mechanisms have been described related to these other species. It seems that the *Acinetobacter* genus has a great ability to acquire antimicrobial resistance determinants and become a threat in hospitals.

CONCLUSIONS

CONCLUSIONS

1. XDR *A. baumannii* isolates were prevalent in a Children Hospital causing diverse types of infections.
2. Tn*2008* was shown to be the main vehicle of bla_{OXA-23} in different ICs, contributing to its overexpression and mobilization.
3. IC7 *A. baumannii* is endemic in the two studied hospitals, this situation differs from that in other neighbouring countries. Sporadic cases of IC4 and IC5 were also present.
4. Antimicrobial resistance genes are encoded in mobile genetic elements contributing to their overexpression and spread. There was a high prevalence of aminoglycoside resistance genes found both in the chromosome and plasmids.
5. Endemicity of IC7 *A. baumannii* isolates and the genome plasticity of *Acinetobacter* spp. mirrors the importance of correctly establish infection control measures.

ABBREVIATIONS

ABBREVIATIONS

°C	Celsius degrees
μg	Microgram
μL	Microlitre
AAC	Acetyltransferase
Ab group	*Acinetobacter baumannii* group
ABC	ATP-Binding Cassette transporters
AFLP	Amplified Fragment Length Polymorphism
AMEs	Aminoglycoside modifying enzymes
ANI	Average Nucleotide Identity
ANT	Adenyltransferase
APH	Phosphotransferase
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ATCC	American Type Culture Collection
bla	β-lactamase gene
bla_{GES}	Guiana extended-spectrum β-lactamase
bla_{GIM}	German imipenemase
bla_{IMP}	Imipenemase metallo-β-lactamase
bla_{KPC}	*Klebsiella pneumoniae* carbapenemases
bla_{NDM}	New Delhi metallo- β-lactamases
bla_{SPM}	Sao Paulo metallo- β-lactamase
bla_{VIM}	Verone integron-encoded metallo- β-lactamase
BLAST	Basic Local Alignment Search Tool
BMD	Broth microdilution
bp	Base pair
CC	Clonal Complex
CDC	Centers for Disease Control and Prevention
CFU	Colony formation units
cgMLST	Core genome multi locus sequence typing
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
cm	Centimeter
COL	Colistin
cpn60	60-kDa chaperonin
CRAb	Carbapenem-Resistant *Acinetobacter baumannii*
CSB	Cell suspension buffer
dDDH	digital DNA-DNA hybridization
DDH	DNA-DNA hybridization
DLV	Double Locus Variant
DNA	Deoxyribonucleic acid
DR	Direct repeat
dsDNA	Double stranded DNA
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
ES	EDTA 0.5 M pH 9.0 + N-Lauroylsarcosine sodium salt 1%

ESBL	Extended spectrum β-lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
fusA	Elongation factor EF-G
GC	Guanine-Cytosine
gdhB	Glucose dehydrogenase B
GENTA	Gentamicin
gltA	Citrate synthase
gpi	Glucose-6-phosphate isomerase
g	Gram
gyrB	DNA gyrase subunit B
h	Hours
HCCA	α-Cyano-4-hydroxycinnamic acid
HGT	Horizontal Gene Transfer
IC	International Clone
ICU	Intensive Care Unit
IPM	Imipenem
IR	Inverted repeat
IS	Insertion sequence
Kb	Kilobase
KDa	Kilo Dalton
L	Litre
LB	Luria-Bertani
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix-Assisted Laser Desorption-Time Of Flight Mass Spectrometry
MATE	Multi-drug and toxic compound extrusion
MBL	Metallo-β-lactamase
MDR	Multidrug-resistant
MEM	Meropenem
MFS	Major facilitator superfamily
mg	Milligram
MIC	Minimum inhibitory concentration
min	Minute
MLSA	Multi locus sequence analysis
MLST	Multi Locus Sequence Typing
NaCl	Sodium chloride
NCTC	National Collection of Type Cultures
ng	Nanogram
nt	Nucleotide
O/N	Over-night
OMP	Outer membrane protein
OMV	Outer membrane vesicle
ORF	Open reading frame
OXA	Oxacillinase
PAHO	Pan American Health Organization

PBP	Penicillin-binding protein
PCR	Polymerase-chain reaction
PDR	Pandrug-Resistant
PFGE	Pulsed Field Gel Electrophoresis
pyrG	CTP synthase
RAST	Rapid Annotation Subsystem Technology
rDNA	Ribosomal DNA
recA	Homologous recombination factor
RI	Resistance island
RNA	Ribonucleic Acid
RND	Resistance-nodulation-cell division
rplB	50S ribosomal protein L2
rpoB	RNA polymerase subunit B
rpoD	RNA polymerase sigma factor
rRNA	Ribosomal RNA
RT	Room Temperature
s	Second
SLV	Single Locus Variant
SMR	Small multi-drug resistance families
SSC	Saline-sodium citrate buffer
ST	Sequence Type
T4SS	Type 4 Secretion System
TBE	Tris-borate-EDTA Buffer
TE	Tris-EDTA buffer
TIG	Tigecycline
tRNA	Transfer RNA
UV	Ultraviolet
V	Volts
vol	Volume
WGS	Whole Genome Sequencing
wt	Weight
XDR	Extensively drug-resistant

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APENDICES

Appendix 1. Primers and amplicons size.

Target	Primers	Sequence	Amplicon
***gyrB* multiplex PCR**			
A. calcoaceticus	D14 D19	5'-GACAACAGTTATAAGGTTTCAGGTG-3' 5'-CCGCTATCTGTATCCGCAGTA-3'	428 bp
A. pittii	D16 D8	5'-GATAACAGCTATAAAGTTTCAGGTGGT-3' 5'-CAAAAACGTACAGTTGTACCACTGC-3'	194 bp
A. baumannii	Sp2F Sp4R	5'-GTTCCTGATCCGAAATTCTCG-3' 5'-AACGGAGCTTGTCAGGGTTA-3'	490 bp
A. baumannii and *A. nosocomialis*	Sp4F Sp4R	5'-CACGCCGTAAGAGTGCATTA-3' 5'-AACGGAGCTTGTCAGGGTTA-3'	294 bp
***bla*$_{OXA}$ multiplex PCR**			
bla$_{OXA-51-like}$	OXA-51-F OXA-51-R	5'-TAA TGC TTT GAT CGG CCT TG-3' 5'-TGG ATT GCA CTT CAT CTT GG-3'	353 bp
bla$_{OXA-23-like}$	OXA-23-F OXA-23-R	5'-GAT CGG ATT GGA GAA CCA GA-3' 5'-ATT TCT GAC CGC ATT TCC AT-3'	501 bp
bla$_{OXA-24/40-like}$	OXA-24/40-F OXA-24/40-R	5'-GGT TAG TTG GCC CCC TTA AA-3' 5'-AGT TGA GCG AAA AGG GGA TT-3'	246 bp
bla$_{OXA-58-like}$	OXA-58-F OXA-58-R	5'-AAG TAT TGG GGC TTG TGC TG-3' 5'-CCC CTC TGC GCT CTA CAT AC-3'	599 bp
bla$_{OXA-143-like}$	OXA-143-F OXA-143-R	5'-TGGCACTTTCAGCAGTTCCT-3' 5'-TAATCTTGAGGGGGCCAACC-3'	149 bp
IS*Aba1*	ISAba1 F1 ISAba1 F3	5'-AGTTGCACTTGGTCGAATGAA-3' 5'-CTCTGTACACGACAAATTTCAC-3'	
Metalo-β-lactamases			
bla$_{VIM-1}$	VIM-1upv VIM-DIA-R	5'-GTCGCAAGTCCGTTAGCCCAT-3' 5'-AGGTGGGCCATTCAGCCAGA-3'	610 bp

Appendix 1. Continued.			
bla$_{GIM}$	GIM-F GIM-R	5'-AGAACCTTGACCGAACGCAG-3' 5'- ACTCATGACTCCTCACGAGG-3'	749 bp
bla$_{SPM-1}$	SPM1F SPM1R	5'-CCTACAATCTAACGGCGACC-3' 5'-TCGCCGTGTCCAGGTATAAC-3'	649 bp
bla$_{IMP}$	IMP-F IMP-R	5'-CTACCGCAGCAGAGTCTTTG-3' 5'-AACCGATTTTGCCTTACCAT-3'	587 bp
MLST, Oxford Scheme			
Citrate synthase (*gltA*)	Citrate F1 Citrate R12	5'-AATTTACAGTGGCACATTAGGTCCC-3' 5'-GCAGAGATACCAGCAGAGATACACG-3'	722 bp
DNA gyrase subunit B (*gyrB*)	gyrB_F gyr_R	5'-TGAAGGCGGCTTATCTGAGT-3' 5'-GCTGGGTCTTTTTCCTGACA-3'	594 bp
Glucose dehydrogenase B (*gdhB*)	gdhB 1F gdhB 775R gdh sec F* gdh sec R*	5'-GCTACTTTTATGCAACAGAGCC-3' 5'-GTTGAGTTGGCGTATGTTGTGC-3' 5'-ACCACATGCTTTGTTATG-3' 5'-GTTGGCGTATGTTGTGC-3'	774 bp
Homologous recombination factor (*recA*)	RA F RA R	5'-CCTGAATCTTCYGGTAAAAC-3' 5'-GTTTCTGGGCTGCCAAACATTAC-3'	425 bp
60-kDa chaperonin (*cpn60*)	cpn60_F cpn60_R	5'-GGTGCTCAACTTGTTCGTGA-3' 5'-CACCGAAACCAGGAGCTTTA-3'	640 bp
Glucose-6-phosphate isomerase (*gpi*)	gpi_F gpi_R	5'-GAAATTTCCGGAGCTCACAA-3' 5'-TCAGGAGCAATACCCCACTC-3'	456 bp
RNA polymerase sigma factor (*rpoD*)	rpo-F rpoD-R	5'-ACCCGTGAAGGTGAAATCAG-3' 5'-TTCAGCTGGAGCTTTAGCAAT-3'	672 bp
AMEs			
ant(2")-Ia/*aadB*	ANT(2")-Ia-F ANT(2")-Ia-R	5'-CGTCATGGAGGAGTTGGACT-3' 5'-CGCAAGACCTCAACCTTTTC-3'	303 bp

Appendix 1. Continued.

aac(3)-IIa	AAC-3'-IIa Fw AAC-3'-IIa R	5'-GGCAATAACGGAGGCGCTTCAAAA-3' 5'-TTCCAGGCATCGGCATCTCATACG-3'	563 bp
aph(3')-Ia/aphA1	APH(3')-Ia Fw APH(3')-Ia R	5'-CGAGCATCAAATGAAACTGC-3' 5'-GCGTTGCCAATGATGTTACAG-3'	624 bp
aac(3)-Ia/aacC-A1	AAC-3-Ia Fw AAC-3-Ia R	5'-GCAGTCGCCCTAAAACAAA-3' 5'-CACTTCTTCCCGTATGCCCAACTT-3'	464 bp
aph(3')-VIa/aphA-6	APH3'-VIa-F APH3'-VIa-R	5'-AAAGCGATCAATGCAAAACC-3' 5'-TATCCGTGATATCGCCATGA-3'	310 bp
aac(6')-Ih	aac(6')-Ih-F aac(6')-Ih-R	5'-ACACCACACGTTCAG-3' 5'-TGCCGATATCTGAATC-3'	408 bp
aac(6')-Ib/cr	AAC6'-Ib-cr-F AAC6'-Ib-cr-R	5'-TTGCGATGCTCTATGAGTGGCTA-3' 5'-CTCGAATGCCTGGCGTGTTT-3'	482 bp
aac(6')-IIa	AAC(6')-IIa-F AAC(6')-IIa-R	5'-GAACACTACCTGCCCAGAGC-3' 5'-TTCTCTCGAAGGCTTGTCGT-3'	397 bp
Methylases			
armA	armA-F armA-R	5'-CAAATGGATAAGAATGATGTT-3' 5'-TTATTTCTGAAATCCACT-3'	777 bp
rmtB	rmtB-F rmtB-R	5'-TCAACGATGCCCTCACCTC-3' 5'-GCAGGGCAAAGGTAAAATCC-3'	459 bp
rmtC	rmtC-F rmtC-R	5'-CGAAGAAGTAACAGCCAAAG-3' 5'-ATCCCAACATCTCTCCCACT-3'	711 bp
Carbapenemases multiplex 1			
VIM	J18 J43	5'-GATGGTGTTTGGTCGCATATC-3' 5'-CGTCATGAAAGTGCGTGGAG-3'	202 bp
KPC	J19 J20	5'-CGCCAATTTGTTGCTGAAGG-3' 5'-CAGGTTCCGGTTTTGTCTCC-3'	312 bp

Appendix 1. Continued.

bla$_{\text{OXA-24/40-like}}$	J21 J22	5'-AGAACCAGACATTCCTTCTTTCA-3' 5'-GCATTGTCAGCAGTTCCAGT-3'	402 bp
NDM	J23 J24	5'-GTTTGATCGTCAGGGATGGC-3' 5'-CTCATCACGATCATGCTGGC-3'	517 bp
bla$_{\text{OXA-48-like}}$	J25 J26	5'-GGTAGCAAAGGAATGGCAAGAA-3' 5'-CGACCCACCAGCCAATCTTA-3'	611 bp
bla$_{\text{OXA-23-like}}$	J27 J28	5'-TCTGGTTGTACGGTTCAGCA-3' 5'-GCATTTCTGACCGCATTTCC-3'	718 bp
Carbapenemase multiplex 2			
IMI	J31 J32	5'-AGACTCGATCGTTGGGAGTT-3' 5'-TCGCTTGGTACGCTAGCACG-3'	206 bp
bla$_{\text{OXA-58-like}}$	K29 K30	5'-ATCAAGAATTGGCACGTCGT-3' 5'-CCACATACCAACCCACTTGC-3'	303 bp
GES	J35 J36	5'-CTCAGATCGGTGTTGCGATC-3' 5'-TGTATCTCTGAGGTCGCCAG-3'	416 bp
GIM	J37 J38	5'-TTATCCTGGGCGACTGACAG-3' 5'-CAGCGGTCGGTTGCATTAAT-3'	508 bp
IMP	K31 K32	5'-GAAGGCGTTTATGTTCATAC -3' 5'-GTACGTTTCAAGAGTGATGC-3'	587 bp
IS*Aba1-bla*$_{\text{OXA-51-like}}$	K38 K39	5'-TGTGGTAAGCACTTGATGGG-3' 5'-ATTGCCATAACCAACACGCT-3'	704 bp
Primers designed for closing plasmids			
MC1 node 65/Spades	L64 L65	5'-TCGACAACTCTAGGGGATGC-3' 5'-TGCGGCCTACTTTTCCTGTA-3'	
MC1 node 79/Spades	L66 L67	5'-TAGCTGGGTGATTGAGGTCG-3' 5'-AGGTGCTCAAAGGAAAACGC-3'	

Appendix 1. Continued.

MC1 node 44/Spades	L68 L69	5'-ACATCTTCGTTCACTTCAGCAG-3' 5'-GAAGGAGGGTCTGAGGTTCC-3'
MC1 node 50/Spades	L70 L71	5'-GATCTCAAAGTCTTATCCGGCA-3' 5'-GTCACCAGCTTTACGACCTT-3'
MC1 node 73/Spades (primers in AAC(6')-Ian)	L72 L73	5'-GCCCGTAGATCACATCCTCA-3' 5'-ATTGGGTCTGGGTTCTTCGT-3'
MC1 node 83/Spades (primers in AAC(3)-IIa)	L74 L75	5'-ATTGATTCAGCAGGCCGAAC-3' 5'-CGATGCTTGGAAGAAACGGT-3'
MC1 node 51/Spades	L76 L77 (sul2)	5'-TGAACTGATGAGGAAGGCAC-3' 5'-AAAGAACGCCGCAATGTGAT-3'
MC1 node 62/Spades	L80 L81	5'-CGTTGGCGGTCTTTCTA-3' 5'-ATGATTGTCCACTTGCTGCC-3'
MC1 node 55/Spades	M1 M2	5'-CACGCACGTTCATTAGCTGA-3' 5'-TGGTTGGGGCTTATGGTCAT-3'
MC1 node 107/Spades (primers in AAC(3)-IIa)	M3 M7	5'-GCCTCATCGCTAACTTTGCA-3' 5'-CCTCCGTTATTGCCTTCCG-3'
MC1 node 26/Spades	M8 M9	5'-TTTGGTGGCGTAAAAGGTCG-3' 5'-TGATAGGGGTCGTCTCAGGA-3'
MC1 node 84/Spades	M10 M11	5'-CGTCTGAGTAGCGAATATGGC-3' 5'-GCCATATTCGCTACTCAGACG-3'
MC23 node 1/Spades	M4	5'-TACCCTGTGCTGGAATTCGT-3'
MC23 node 61/Spades	M5 M6	5'-CTGCCCCATCCAATCGAAAG-3' 5'-GACGAATTGTTAGGCCGCAT-3'
MC23 node 1/PlasmidSpades	M17 M18	5'-CCCAACGGAGGCCCTATTTA-3' 5'-TGTTTCCTCGTCCTCTGCAT-3'

*These primers were used to perform Sanger sequencing.

Apendix 2. Buffers

Buffers for *Apa*I-PFGE
TE, Tris-EDTA pH 8.0
10 mM TrisHCl
1 mM EDTA
PLUG LYSIS (1 mL/sample)
6mM Tris-HCl pH8
1M NaCl
0.1 M EDTA 0.5M pH8
0.2% Sodium deoxycholate
0.5 % N-Lauroylsarcosine sodium salt
0.5 % Brij®58
0.5 mg Lisozyme (50 mg/mL)
PROTEINASE K DIGESTION (1 mL/sample)
0.12 mg Proteinase K
20% ES (EDTA 0.5 M pH 9.0 + N-Lauroylsarcosine sodium salt 1%)

Buffers for S1 and *I-Ceu*I PFGE
CSB, Cell suspension buffer pH 8.0
100 mM Tris-HCl
100 mM EDTA
TE buffer pH 8.0
10 mM Tris-HCl
1 mM EDTA
Cell lysis buffer, CLB pH 8.0
50 mM Tris-HCl
50 mM EDTA
1 % N-Lauroylsarcosine sodium salt

Buffers for Southern blot
Denaturation solution
0.5 M NaOH
1.5 M NaCl
Neutralization solution
0.5 M NaOH
1.5 M NaCl
Hybridization buffer
50% formamide
5x SSC
2.5% blocking reagent
0.1% N-Lauroylsarcosine sodium salt
0.02% SDS
Low Stringency buffer
2x SSC
0.1% SDS
High Stringency buffer
0.1x SSC
0.1% SDS

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