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INVOLVEMENT OF HORIZONTAL GENE TRANSFER MECHANISMS IN ANTIBIOTIC RESISTANCE ACQUISITION IN *ACINETOBACTER BAUMANNII*

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Abstract

Antibiotic resistance is the ability of a pathogen to endure the effects of antibiotic agents. The increased use of antibiotics has led to a rise in the emergence of resistance, resulting in a major issue in modern society, which leads to high mortality rates due to resistant bacterial infections, with less treatment options. Horizontal gene transfer (HGT) is the acquisition of genetic material from bacteria of the same generation, which can include resistance genes, with consequent emergence of resistant strains. One of the pathogens known for its ability to quickly develop antibiotic resistance is *Acinetobacter baumannii*, which often acquires multidrug resistance (MDR). Amongst the HGT mechanisms used by pathogens to acquire antibiotic resistance, the most prominent is conjugation, however, several cases of natural transformation have been reported in *A. baumannii.* both HGT mechanisms have been evaluated to ascertain which is most prevalent in this species. *A. baumannii* A118 was the recipient strain and two strains of MDR *Salmonella enterica* and 1 strain of colistin-resistant *Escherichia coli* were used as donor bacteria in the natural transformation and conjugation assays. Natural transformation and conjugation occurred at similar frequencies, in the order of 10^{-8}, with transformants and transconjugants showing changes in the antimicrobial susceptibility profile. Among all the tested isolates, only one transconjugant, from the *S. enterica* serovar Typhimurium Sal25 donor strain, acquired the genes *mcr-1*, $bla_{\text{CTX-M-1}}$ and a class 1 integron, as confirmed by the changes in the antimicrobial susceptibility profile and by polymerase chain reaction of the respective genes and mobile genetic element.

The results of this thesis suggest that there are no significant differences between the prevalence of conjugation and natural transformation in *A. baumannii* A118.

Keywords

Antibiotic resistance | *Acinetobacter baumannii* | Horizontal gene transfer | Natural transformation | Conjugation

Resumo

A Resistência a antibióticos corresponde à capacidade de um agente patogénico suportar os efeitos dos antibióticos. O aumento do uso de antibióticos tem levado ao acréscimo do surgimento de resistência, resultando em consequências graves na sociedade moderna, com taxas de mortalidade elevadas devido à resistência dos agentes bacterianos infeciosos, com menos alternativas de tratamento. A transmissão horizontal de genes é a aquisição de material genético de bactérias da mesma geração, o que pode incluir genes de resistência, com o consequente surgimento de estirpes resistentes. Um dos agentes patogénicos conhecido pela sua capacidade de desenvolver rapidamente resistência a antibióticos é *Acinetobacter baumannii*, que frequentemente adquire multirresistência. Entre os mecanismos de transmissão horizontal de genes utilizados por agentes patogénicos para adquirir resistência a antibióticos, o mais proeminente é a conjugação, no entanto, vários casos de transformação natural têm sido reportados em *A. baummannii.* Ambos os mecanismos foram avaliados para determinar qual o mais prevalente nesta espécie. A estirpe recetora utilizada foi *A. baumannii* A118 e duas estirpes de *Salmonella enterica* multirresistentes e uma estirpe de *Escherichia coli* resistente à colistina foram utilizadas como dadoras nos ensaios de transformação natural e conjugação. Transformação natural e conjugação ocorreram em frequências semelhantes, na ordem de 10^{-8}, com transformantes e transconjugantes que apresentaram alterações nos perfis de susceptibilidade aos antimicrobianos. Entre todos os isolados testados, um único transconjugante, da estirpe dadora *S. enterica* serovar Typhimurium Sal25, adquiriu os genes *mcr-1*, $bla_{\text{CTX-M-1}}$ e um integrão de classe 1, confirmado pelas mudanças no perfil de susceptibilidade a antibióticos e por reação em cadeia da polimerase dos genes respetivos e do elemento genético móvel.

Os resultados desta tese sugerem que não existem diferenças significativas entre a prevalência da conjugação e da transformação natural em *A. baumannii* A118.

Palavras-chave

Resistência a antibióticos | *Acinetobacter baumannii* | Transmissão horizontal de genes | Transformação natural | Conjugação

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AMEs |Aminoglycoside-modifying enzymes

CAZ |Ceftazidime

CFU |Colony forming unit

COL |Colistin

CR |Carbapenem-resistant

CRAB |Carbapenem-resistant *Acinetobacter baumannii*

CT |Colistin

CTX |Cefotaxime

DNA |Deoxyribonucleic acid

ESBL |Extended-spectrum β-lactamases

EUCAST |European Committee on Antimicrobial Susceptibility Testing

GC |Guanine-Citosine

HGT |Horizontal gene transfer

ICU |Intensive care unit

KAN |Kanamycin

KPC |*Klebsiella pneumoniae* carbapenemase

LB |Luria Bertani medium

LPS |Lipopolysaccharide

MATE |Multidrug and toxic compound extrusion

MBLs |Metallo-β-lactamases

MCR |Mobile colistin resistance

MDR |Multidrug resistance

MFS |Major facilitator superfamily

MGEs |Mobile genetic elements

MH |Mueller-Hinton

MIC |Minimum inhibitory concentration

OD |Optical density

OMVs |Outer membrane vesicles

OXAs |Oxacillinases

PBS |Phosphate-buffered saline

PCR |Polymerase chain reaction

PDR |Pan-drug resistance

pDNA |Plasmid DNA

rRNA |Ribossomal ribonucleic acid

RNA |Ribonucleic acid

RND |Resistance nodulation cell division

S3 |Compound sulphonamides

SH |Spectinomycin

SMR |Small multidrug resistance

STR |Streptomycin

T4SS |Type IV secretion system

T6SS |Type VI secretion system

TIG |Tigecycline

UV |Ultra-violet

VAP |Ventilator-acquired pneumonia

XDR |Extensive drug resistance

WHO |World Health Organization

Chapter 1 | State-of-the-art

1.1 | Antimicrobial resistance

Antimicrobial resistance, the ability of a pathogen to withstand the effects of antimicrobial agents, and infections by these resistant pathogens can achieve lethal consequences with the lack of effective measures against them, and can hamper common surgical procedures, as well as other medical treatments (Mestrovic *et al*., 2022).

Antimicrobial resistance is expected as an evolutionary outcome in bacteria with the use of antimicrobials and has been a recurring problem in later years as a result of the widespread use of antibiotics in the healthcare department, which has accelerated the process, with an increase in resistant infections in hospitals and community-acquired infections. This issue is prevalent in both Gram-negative and -positive bacteria, with cases of multidrug resistance (MDR), which is defined as the lack of susceptibility to at least one microbial agent in three or more antimicrobial classes (Magiorakos *et al*., 2012). Several Gram-negative bacteria species, such as Acinetobacter baumannii, are currently resistant to virtually all available antibiotics, both in non-hospital environments (El-Kazzaz *et al*., 2020), in community-acquired infections, namely community-acquired pneumonia (Ling *et al*., 2006) and community-acquired bacteraemia (Chusri *et al*., 2019), and hospital environments, especially in ventilator-associated pneumonia (VAP) (Čiginskienė *et al*., 2019) and hospital acquired bacteraemia (Chusri *et al*., 2019), amongst others. Resistance to antimicrobials has been considered not only a public health problem on the global scale, but also a societal issue (Gajdács *et al*., 2021). The spread and emergence of antimicrobial resistance is higher in medium and low-income countries, with the highest estimated to be Southeast Asia within all the WHO regions (Chereau *et al*., 2017). In Europe, nosocomial infections associated with MDR microorganisms lead to costs of nearing 12.000 euros per patient, related with accommodation and antibiotic drugs for infection treatment (Giraldi *et al*., 2019). With drug resistance came a reduction of treatment options and higher mortality caused by infection, with casualties totalling to about 60.000 per year between the US and Europe, and approximately $6 billion in health care costs for both regions, the increase of antimicrobial-resistant pathogens is becoming an imminent

threat to human health, with future mortality predicted to exceed that of cancer (WHO, 2014; Cassini *et al*. 2019; O'Neill, 2014).

Gram-negative bacilli, such as *A. baumannii*, are also the most common cause of hospital-acquired infections in intensive care units (ICUs), including pneumonia, surgical site infection, urinary tract infection, and bloodstream infection (Weinstien, 1991; Weinstein *et al*., 2005), with varying frequencies amongst them and the increase in incidence of *Acinetobacter* species (Weinstein *et al*., 2005). Amongst hospital-acquired infections, it is detected low susceptibility to common antibiotics by *A. baumannii* (Mamishi *et al*., 2019). Prolonged hospital stay is one of the main risk factors in inducing colonization and/or infections with MDR *A. baumannii* (Eliopoulos, Magarakis & Perl, 2008)

If no suitable actions are taken in present time, it has been estimated that antimicrobial resistance will lead to 10 million deaths annually by 2050 (O'neill, 2014, Mestrovic *et al*., 2022).

1.2 | Pathogens of high priority in novel therapy development and antibiotic research for human health

Regarding the emergence and spread of antibiotic resistance, healthcare facilities worldwide have cases where disease-causing bacteria escape the antimicrobial effects, in spite of the advancements of modern medicine. A group identified with the acronym ESKAPE, encompassing *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (Rice, 2008), are virtually resistant to all available antimicrobials. Caused by the non-susceptibility to existing antimicrobial therapies, these species are considered of extreme concern, leading to pressure in the coordinated global development of new antibiotics or antibacterial therapies. *A. baumannii*, despite having a lower infection rate when compared with the other ESKAPE pathogens (Oliveira *et al*., 2020), has a high tendency to develop resistance against even last-resort clinical antibiotics. Between the years of 2011 and 2016, an increase of 30% of resistance to β-lactam class antibiotics, specifically carbapenems, in *A. baumannii* has been seen globally (Harding, Hennon & Feldman, 2018) and international clonal lineages have been associated with the spread of multidrug and carbapenem resistant isolates. Even last-resort

carbapenem and polymyxin antibiotics are ineffective against pandrug-resistant isolates (PDR), which are microorganism resistant to all antibiotics routinely tested (Xie at. Al, 2018; Qureshi *et al*., 2015).

The World Health Organization (WHO) ranked antibiotic-resistant bacteria according to priority in the discovery, research and development of new effective drugs. Carbapenem-resistant *A. baumannii* (CRAB) was placed at the top of the highest rank of priority pathogens that present the greatest risk to human health, especially in healthcare environments, nursing homes, and in patients dependent on ventilators and blood catheters (Davies & Bennett, 2017; Tacconelli *et al*, 2018). The rank of critical priority is also applied to carbapenem-resistant (CR) *P. aeruginosa* and CR and 3rd generation cephalosporin resistant *Enterobacteriaceae*, and there are two more levels of priority, including the high and the medium priority pathogens. All of these priority pathogens have some form of antibiotic resistance to either methicillin, fluoroquinolone, vancomycin, clarithromycin, 3rd generation cephalosporin, ampicillin and/or penicillin.

The criteria measured to assign priority took into account mortality, healthcare and community burden, prevalence and trend of resistance, transmissibility, preventability in the healthcare community setting, treatability, and possibility of development of new antibiotics in the future (Tacconelli *et al*., 2018). CRAB scored highest amongst all in the trend to develop resistance, and reached the max values attained by other pathogens on all other criteria, proving to be the most threatening to human health and that it deserves to be one of the main focuses in future research and development for new antibiotic therapies (Tacconelli *et al*., 2018).

1.3 | Genus *Acinetobacter*

Acinetobacter is a genus that took several years to achieve its current taxonomic position. It was first described in 1911 by Beijerink, who isolated it from a soil sample with calcium-acetate enrichment (Beijerink, 1911) and named it *Micrococcus calco-aceticus*. Due to its immotile nature, which differentiated the microorganism from others, Brisou and Prévot designated the genus *Acinetobacter* (from the Greek, “Akinetos”, non-motile) (Brisou & Prévot, 1954). Only in 1968 was *Acinetobacter* a truly accepted genus by Baumann, with his

group's comprehensive study, where several previously studied species were declared to be from the same genus (Baumann, Doudoroff & Stainer, 1968).

Bacteria in the genus *Acinetobacter* are Gram-negative bacilli, non-fermenting, strictly aerobic, non-fastidious, non-motile, catalase-positive, and oxidase-negative, with a GC content of 39-47% (Howard *et al*., 2012). Identifying the genus *Acinetobacter* has proven challenging, due to its difficulty in de-staining, being sometimes wrongly identified as a Gram-positive. Identification to the genus level used to be attained through the transformation assay of Juni (Juni, 1972), but currently it is done with gene sequencing (Howard *et al*., 2012).

This genus can be found widely in nature, with a ubiquitous distribution, both in soil and water (Baumann *et al*., 1968), but currently the habitats of interest are the hospital environments (Peleg *et al*., 2008), due to its clinical relevance, and where usually MDR strains of *A. baumannii* tend to emerge. The ubiquitous distribution suggested there were nospecific reservoirs (Berlau *et al*., 1999), however, drug-resistant strains have been found in vegetables and thus they can be considered gateway for *A. baumannii* (Eveillard *et al*., 2013) and can be referred as one of the routes for this bacterium to enter hospital environments, since in such cases infection control would have lesser implications (Berlau *et al*., 1999). It has been known that this bacterium can survive for long periods of time in the hospital environment, as a result of its capacity to survive desiccation and to form of biofilms (Harding, Hennon & Feldman, 2018; Al-Kadmy *et al*., 2018).

1.4 | The species *Acinetobacter baumannii*

Acinetobacter baumannii is an opportunistic pathogen that has emerged in recent years and has posed a great threat to human health, due to its MDR profile and ability to be a source of nosocomial infections, especially in ICU. Inside the genus *Acinetobacter*, *A. baumannii* belongs to the *A. calcoaceticus-A. baumannii* complex, since the differentiation between species was quite difficult before gene sequencing methodologies (Gerner-Smidt, Tjenberg & Ursing, 1991), when phenotypical identification was the only method; due to the phenotypic similarities between species of the *A. calcoaceticus-A. baumannii* complex, such as the non-fastidious nature, no metabolic tests are available to differentiate between this non-fermenting Gram-negative bacteria (Peleg *et al*., 2008). In the end, molecular methods are the only reliable ones to identify *A. baumannii* to the species level.

MDR *A. baumannii* strains can be found in hospitals and are usually detected in outbreaks (Kurihara *et al*., 2020), in patients who received broad-spectrum antibiotics alongside either having a long hospitalization, recent surgeries or with comorbidities. When analysing strains originated form hospital outbreaks and comparing them with sporadic strains, the outbreak strains were observed to be less heterogenous than the sporadic ones, with a possibility that the outbreak strains were of common clonal origin, implying the presence of clonal strains along different hospitals is not limited by geographic distribution (Dijkshoorn *et al*., 1996).

A. baumannii strains have been responsible for hospital outbreaks, and some of them reflected the varied degree of survivability of this genus in dry, immobile surfaces found in nosocomial environments, independent of the surface material and lasting from several months to even years, making it an important species to search for in cases of hospital bacterial outbreaks (Wendt *et al*., 1997; Holton, 1992; Sherertz & Sullivan, 1985; Weernink *et al*., 1995)

A. baumannii is often isolated from respiratory tract, blood culture, wound and urine samples (Hakyemez *et al*., 2013), with most isolates obtained from ICUs. Contamination of surfaces in the healthcare environment, such as door handles, mechanical ventilators, hemofiltration machines, hospital beds, and computer keyboards have been reported during outbreaks; even with the specific guidelines created to detect and control transmission, nosocomial outbreaks still occur (Hakyemez *et al*., 2013).

Type VI secretion system (T6SS), an effective weapon in bacterial warfare, provides the tools necessary to uptake DNA released by the destroyed competitor cells, and its presence has a close relationship with antimicrobial resistance in *A. baumannii* (Dong *et al*., 2022). T6SS-mediated killing leads to the release of DNA contents into the surrounding media, and when paired with natural competence results in acquisition of antibiotic resistance islands, so the T6SS killing can contribute to a higher rate of horizontal gene transfer (HGT), consequently bringing about the high antimicrobial resistance of *A. baumannii* (Dong *et al*., 2022).

Another unique characteristic to this pathogen is the presence of acinetobactin: a siderophore, molecular mechanism for iron uptake, was recovered from *A. baumannii*, leading to believe to be exclusive to this species, increases the uptake of iron and is contribute to the bacteria overall survivability (Chapartegui-González *et al*., 2021; Peleg *et al*., 2012; Yamamoto, Okujo & Sakakibara, 1994).

1.5 | Nosocomial infections

A. baumannii is the most common microorganism in burn victims interned in ICUs (Chim, Tan & Song, 2007), where patients are more sensitive to infection due to the loss of the protective barrier of the skin and decreased function of the cellular and humoral immune system, with isolates showing highly multiresistant profiles. The presence of wound, bloodstream, and VAP infections is higher in burn victims in ICU than others (Chim, *et al*., 2007). It has been recorded a seasonal variation in the incidence of *A. baumannii* infections, with an increase in the months of greater heat and humidity (Oliveira *et al*., 2020). Surgical site nosocomial infections account for 14-16% of hospital acquired infections, and the colonization of microorganisms in the closing wounds following surgery occurs only if primary wound healing fails to occur, so the most debilitated are at higher risk of developing this type of infections (Petca *et al*., 2021). Other risk factors for surgical site nosocomial infections include diabetic status, alcohol and smoking status, obesity, immunosuppression, age, recent radiotherapy and even sex, being higher in males, with risk factors regarding the procedure itself being ventilation, poor sterilization and surgery duration. Even if the guidelines specific for these situations are thoroughly followed, they can still be of no avail (Ban *et al*., 2017) due to the dependency of the state of health of the patient after surgical procedure.

VAP, although not exclusive, is frequently associated with *A. baumannii*, and is the most common infection associated with this species (Fragkou *et al*., 2019) in the ICU, with varying mortality rates of 25% to 75%, posing a great healthcare issue (Penwell *et al*., 2015), with worldwide geographical distribution (Wood *et al*., 2018).

1.6 | History of antibiotic resistance

Recently, ampicillin-sulbactam and imipenem have been the most reliable agents for the treatment of *A. baumannii*, with cefepime, amikacin, and the fluoroquinolones providing variable activity (Shafer *et al*., 2007). With the recent emergence of carbapenem resistance in *A. baumannii*, the American and European guidelines for treatment are ampicillin-sulbactam, with alternatives being inocycline, tigecycline, polymyxins, or cefiderocol, and in cases of moderate to severe infections, high dosage of ampicillin-sulbactam in combination with minocycline, tigecycline, polymyxin B, extended-infusion meropenem, or cefiderocol (Domingues *et al*., 2023). In some strains resistant to colistin, colistin combinations with other

antibiotics appear to be the best treatment option for the majority of patients in these cases (Hakyemez *et al*., 2013; Almutairi *et al*., 2022). For the nosocomial *Acinetobacter* spp. strains, the effectiveness of antibiotics appears to be higher with the use of colistin, tigecycline, imipenem and meropenem, but resistance to imipenem and meropenem has been observed to have increased over the years (Hakyemez *et al*., 2013). In some cases of severe urinary tract infections there is specifically high prevalence of carbapenem resistance amongst *A. baumannii* (Girija, Priyadharsini & Paramasivam, 2019).

A. baumannii antibiotic resistance can occur not only against a single antimicrobial agent, and in some cases is non-susceptible to at least 1 agent in 3 or more antimicrobial categories, as previously mentioned. Another situation of resistance to several antibiotics is extensive drug resistance (XDR), which includes strains non-susceptible to at least one or more antimicrobial agents amongst all antimicrobial classes excepting of two (As, 2019). Pandrug-resistant (PDR) *A. baumannii* are non-susceptible to all antibiotics routinely tested, and were first discovered in 1998, with cases occurring still in recent years (Karakonstantis, Kritosotakis & Gikas, 2020). The use of synergistic combination of antibiotics has proven fruitful *in vitro* against PDR, MDR and XDR *A. baumannii* (Durante-Mangoni, Utili & Zarrilli, 2014), overcoming the issue with newly obtained resistances, although *in vivo* studies should be performed in order to confirm these findings.

The *in vitro* combinations of imipenem, tigecycline, and amikacin proved to have a synergistic effect, as well as combination of tigecycline with colestimethate, which resulted in lower MIC values in *A. baumannii* clones, and imipenem was revealed to have a synergistic effect with colestimethate as well (Sopirala *et al*., 2010).

1.6.1 Antibiotics with potential activity against *Acinetobacter baumannii*

Since *A. baumannii* commonly presents resistance to multiple antibiotics, there are limited options for treatment of *A. baumannii* infections, with variations of the susceptibility patterns that are specific to their geographical areas (Michalopoulos & Falagas, 2010). Currently, there is a small amount of antibiotics normally effective at dealing with infections caused by *A. baumannii*, which include carbapenems, polymyxins E and B, sulbactam, piperacillin/tazobactam, tigecycline and aminoglycosides.

Aminoglycosides:

Aminoglycosides are a large class of antibiotics first obtained in 1943, with the isolation of streptomycin, and are used to treat infections caused by Gram-negative bacteria or used in synergy against Gram-positive infections, inducing a bactericidal effect by inhibiting protein synthesis (McDermott *et al*., 2022). There can be some nefarious side effects that are dose-dependent and only observed in cases where administration occurs in high dosages, such as nephrotoxicity, cochleotoxicity, which is sensorineural hearing loss, and vestibulotoxicity, which is ototoxicity that affects the balance organs in the inner ear (McDermott *et al*., 2022), and some patients appear to have predisposition of hearing loss caused by aminoglycoside administration.

Resistance to aminoglycosides has emerged in *A. baumannii*, accompanied by resistance to carbapenems, polymyxins and tigecycline, with reports worldwide (Karakonstantis, Kritsotakis & Gikas, 2020). The apparent solutions for this co-resistance cases are treatment with synergistic combinations of numerous antibiotics, although they have yet to find clinical benefit (Karakonstantis, Kritsotakis & Gikas, 2020), as research has the significant differences between *in vivo* and *in vitro* conditions that influence the effectiveness of this solution.

Resistance to aminoglycosides by *A. baumannii* is mediated by aminoglycoside-modifying enzymes and a methylase for the 16S ribosomal sub-unit, where resistance rates varied from 68 to 100% against aminoglycoside-class antibiotics and an increase in the minimum inhibitory concentration (MIC) of isolates with both resistance determinants present (Jouybari *et al*., 2021), proving the importance of the genes coding for aminoglycoside-modifying enzymes (AMEs) and the methylase in aminoglycoside resistance in *A. baumannii* (Jouybari *et al*., 2021).

Carbapenems:

Carbapenems are β-lactams that are protected from the effect of pathogen enzymes that have specific effect on this class of antibiotic, and thus is used to treat severe infections caused by extended-spectrum β-lactamase (ESBL) producing bacteria (Codjoe & Donkor, 2018), and used against MDR bacteria. These broad-spectrum antimicrobial agents act against both Gram-negative and Gram-positive bacteria, including anaerobes. The mechanism of action allows to halt bacterial cell wall synthesis, leading to cell death via autolytic action, like most β-lactams. They are usually used to treat invasive or life-threatening infections because their bactericidal effect on the infecting bacteria is concentration-independent (Codjoe & Donkor, 2018).

Cases of resistance against carbapenems in *A. baumannii* have become more frequent in recent years in many countries (Castanheira *et al*., 2014), with carbapenemase-producing strains of *A. baumannii*.

Mediation of carbapenem resistance in *A. baumannii* occurs mainly due to carbapenemases of the Ambler class D family, known as oxacillinases, and there is an intrinsic oxacillinase gene in *A. baumannii*, $bla_{\text{OXA-51}}$, conferring resistance to carbapenems, which can be commonly found to be upregulated and has several variants emerging since its discovery (Turton *et al*., 2006; Castanheira *et al*., 2014).

Susceptibility in *A. baumannii* can vary between several carbapenems, and carbapenemases, namely serine-β-lactamases, that have poor activity against carbapenems are primarily produced by this bacterium and *Pseudomonas aeruginosa* (Cojoe *et al*., 2017). The resistance to carbapenems associated with resistances to other antibiotics is predominantly present in *A. baumannii* and other two ESKAPE pathogens (Karakontatis, Kritsotakis & Gikas., 2020). Carbapenemase-producing *A. baumannii* isolates are normally clonal, with only a small number of international clones responsible for most dissemination of these strains in the healthcare environment (Castanheira *et al*., 2014).

Colistin:

Colistin, also known as polymyxin E, is an antibiotic that kills bacteria by causing permeability to increase in the outer membrane of bacteria, by impairing the 3-dimentional structure of the lipo-polysaccharide (LPS) structure in the outer membrane (Andrade *et al*., 2020). Although it has seen some use since the 80s, in spite of nefarious side effects, such as neuro- and nephrotoxicity, colistin (COL) has been classified as an antibiotic of critical use and used only as last resort against MDR Gram-negative pathogens in humans (Trebosc *et al*., 2019), including *A. baumannii* (Falagas *et al*., 2010, Catchpole *et al*. 1997; Michalopoulos *et al*., 2005, Cai *et al*. 2012.), due to the lack of newly developed antibiotics (Trebosc *et al*., 2019). It has also proven effective against VAP caused by PDR resistant *A. baumannii* and *P. aeruginosa* (Kallel *et al*., 2007). Although colistin resistance in *A. baumannii* was quite rare, some isolates have been reported in Europe and North and Latin America, and one extremely resistant strain in Korea (Ko *et al*., 2007). Presently the increase in prevalence of colistin-resistant strains on different continents requires treatment alternatives to be developed (Novović & Jovčić, 2023).

In 2006, Li *et al*. reported heterorresistance, a type of resistance where sub-populations of bacteria have higher levels of resistance than the rest of the populations in the same culture in MDR strains of *A. baumannii*, that grew on COL. It was also noted that patients subjected to COL treatment had higher resistance rates in *A. baumannii* isolates when compared to isolates not exposed beforehand (Hawley, Murray & Jorgensen, 2008). Some sub-populations of COL-resistant strains grew in concentrations above the MIC observed, a situation called heteroresistance, as well as beyond the breakpoint, which are the result of standard colistin administration (Li *et al*., 2006).

Piperacillin/tazobactam:

Piperacillin-tazobactam is a broad-spectrum antibiotic commonly prescribed to treat moderate to severe infections. Piperacillin is a β-lactam and acts accordingly, interfering with the process of peptidoglycan synthesis for the bacterial cell wall, affecting both Gram-negative and Gram-positive, both aerobes and anaerobes (Hayashi *et al*., 2010). Tazobactam is a β-lactamase inhibitor, with a facilitated binding to the enzyme and inactivating it, allowing the effect of β-lactams on the target pathogen, it works on class A serine β-lactamases, while having no effect on C cephalosporinases, such as AmpC, and class D serine oxacillinases, coded by the bla_{oxa} genes (Hayashi *et al*., 2010).

Even though it was considered a viable treatment option for *A. baumannii*, it is generally considered to be ineffective against infections caused by the bacterium, due to mechanisms of resistance, namely production of non-class A β-lactamases, to which the antibiotic has no effect (Hayashi *et al*., 2010), however, it was observed to be a viable treatment option in some cases of MDR *A. baumannii* (De Francesco *et al*., 2013)

Susceptibility to piperacillin-tazobactam has decreased over the years, rendering the antibiotic combination no longer viable for *A. baumannii* infections, namely urinary tract infections (Jiménez-Guerra *et al*., 2018). Resistance was also observed in PDR *A. baumannii*, where extremely high resistance rates were detected (Sobouti *et al*., 2020; AlAmri *et al*., 2020).

Polymyxin B:

One of the two commercially available polymyxins and with clinical use, polymyxin B acts in the same way as COL destabilizing the LPS on the outer membrane of Gram-negative bacteria, functioning as a detergent and disrupting it (Zavascki *et al*., 2007). Like COL, it has some nephrotoxicity associated and is used as a last resort, but it has been demonstrated and suggested to be lesser (Phe *et al*., 2014), for this reason it is the main polymyxin used in several centers (Avedissian *et al*., 2019). With a similar spectrum of activity to colistin, it is effective against *Acinetobacter* spp., which are intrinsically susceptible to polymyxin B.

Resistance can occur in *A. baumannii*, that must have a MIC value above 2 mg/L to be considered resistant (Nurtop *et al*., 2019), but fortunately it is mostly geographically limited, with prevalence and rate of increased colistin resistance being higher in South-East Asia and Eastern Mediterranean than other regions of the world (Pormohammad *et al*., 2020).

Polymyxin B can be used against MDR strains of *A. baumannii*, when used in combination with nisin, an antimicrobial food preservative, producing a synergistic effect, allowing the reduction in the dosage of polymyxin B, and thus leads to a decrease of the nephrotoxic side effects (Thomas *et al*., 2019). Another synergistic effect with polymyxin B was observed *in vitro* with the joint administration with cannabidiol, where a higher killing curve against *A. baumannii* has been observed, when compared to polymyxin B monotherapy, which suggests a positive effect if used in the clinical setting in the future (Hussein *et al*., 2022).

Reports of resistance to polymyxins have increased over the years amongst Gram-negative bacteria, including *A. baumannii*, particularly after monotherapy with polymyxins, where the most common causes for resistance are complete loss of LPS or modification of lipid A (Hussein *et al*., 2022).

Sulbactam:

Sulbactam is a β-lactamase inhibitor, the enzyme produced by bacteria that renders β-lactams useless, and it is administered with β-lactam antibiotics to complement and guarantee antibiotic activity. Its complementary use with carbapenems has been reported to be more effective than alternative antibiotic therapies with polymyxins (Oliveira *et al*., 2008).

The use of sulbactam-ampicillin has been suggested as a reliable therapeutic treatment for nosocomial infections of MDR *A. baumannii* (Levin *et al*., 2003). Sulbactam efficacy, both by itself and in combination with ampicillin has proven efficient in treating non-life-threatening

multirresitant *A. baumannii*, with a bacteriostatic effect, halting bacterial activity (Corbella *et al*., 1998). It has also been verified a higher *in vitro* efficiency of sulbactam when combined with other antibiotics against multi-resistant strains, proving the benefits of drug synergism when faced with the emergence of new resistant strains (Temocin *et al*., 2015). In a pneumonia model for *A. baumannii* infection, the combination of imipenem and sulbactam resulted in the highest bactericidal effect in susceptible and intermediate-resistant strains (Montero *et al*., 2002).

Although historically the sulbactam-ampicillin was effective at treating nosocomial infections caused by *A. baumannii*, clinical resistance has started emerging in the past (Penwell *et al*., 2015). It has been suggested resistance to sulbactam activity derives from an increase in drug efflux or loss of a penicillin-binding protein activity, as well as an establishment of a clear relation between TEM-1, a β-lactamase, expression and decrease in sulbactam activity (Penwell *et al*., 2015; Seifert *et al*., 2020). The confirmation of resistance to sulbactam in MDR clinical isolates of *A. baumannii* proved the main factors for resistance were mutations in genes coding for penicillin-binding proteins, as well as inhibition of one of those genes, with varying degrees of resistance recorded amongst them (Penwell *et al*., 2015).

Tigecycline:

Tigecycline (TIG) is a glycylcycline, a tetracycline derivative that inhibits protein synthesis, which is directed towards tetracycline-resistant pathogens; it has a high excretion rate (59%) (Bhattacharya, Parakh & Narang, 2009), and it has been used for the treatment of MDR strains of *A. baumannii* in cases of VAP or bacteremia infection (Shafer *et al*., 2007).

In some cases, resistance to TIG may arise during therapy, increasing the MICs after only a brief exposure to the drug (Reid *et al*., 2007) with a six-fold MIC increased, from to 2.00 to 12.00 μ g/mL, over the course of 14 days on a tracheal aspirate isolate previously susceptible (Anthony *et al*., 2008). Bloodstream infection by TIG-non-susceptible *A. baumannii* has occurred while receiving TIG treatment (Peleg, Adams & Patterson, 2007). Due to low achievable concentrations in serum, TIG treatment for bloodstream infection of *A. baumannii* can fail, even for low MICs. Its use should be avoided in cases of bacteraemia in sites of poor antibiotic penetration, such as urinary tract, where chances of the therapy failing and resistance development are greater (Anthony *et al*., 2008).

Resistance to TIG has also been reported via efflux pumps, and a gene which codes for this resistance mechanism, is present in both susceptible and non-susceptible strains, with different rates of expression, with a recorded increase in the MIC of the former after exposure to TIG (Peleg, Adams & Patterson, 2007)

It should be noted that the reported MICs can also be controversial, since TIG has special conditions for correct MIC evaluation, namely Mueller Hinton agar quality and anaerobic storage conditions (Petersen & Bradford, 2005, Hope *et al*., 2005).

1.7 | Antimicrobial resistance mechanisms:

There are several known mechanisms of antibiotic resistance, including change of the antibiotic target, which decreases the affinity of the antibiotic; decreased concentration of the antibiotic at the target site, due to efflux pumps, where the antibiotic is pumped out of the bacterial cytosol, or reduced membrane permeability, which prevents the entrance of the antibiotic inside the bacterial cell; and enzyme inactivation of the antibiotic molecules, where the antibiotics are cleaved or complexed with other components, rendering them useless (Nikaido, 2009).

Bacteria that show resistance to antimicrobial agents have either innate mechanisms of resistance or have gone through acquisition of those resistance mechanisms, via the internalization and integration of foreign genetic material that conferred resistance or selection of mutants throughout generations exposed to selection factors that allowed the emergence of resistance (Nikaido, 2009).

Acquisition of resistance can be obtained via mutation of the target of the antibiotic, preventing it from taking effect. For example, high-level streptomycin resistance in *E. coli* is caused by a mutation of the ribosomal proteins. Another case is fluoroquinolone resistance, which is mainly caused by mutations in target enzymes (Hooper, 2000).

A mutation responsible for the resistance of imipenem, a carbapenem, frequently used to treat *A. baumannii* infections, is in the CarO protein, a porin that plays a key role in the influx of imipenem into *A. baumannii,* where the mutations alter the structure or abolish or decrease the protein expression, reducing antibiotic entry into the bacteria, thus reducing its effect

(Zhu, Chen & Hou, 2019). This mutation can also increase resistance to most common antibiotics except tigecycline, minocycline, and cefoperazone/sulbactam.

Another case of resistance via mutation in *A. baumannii* is resistance to colistin, where the initial binding target for the antibiotic, the lipid A component of LPS, is lost or changed. The mutation in one of the three genes *lpxA*, *lpxC*, and *lpxD* causes loss of LPS production, with the first report of LPS-deficient Gram-negative bacterium in the clinical setting in 2010 (Moffatt *et al*, 2010). It was also verified that non-colistin antibiotic sensitivity was increased in the strains that lacked LPS production, altering the antibiotic resistance profile, due to the increased permeability to the outer membrane of the bacterium (Moffatt *et al*., 2010).

Multidrug efflux pumps can pump out more than one antibiotic type, and thus are a source of multidrug resistance. One of the largest families of efflux pumps is the Major Facilitator Superfamily (MFS), some with expression negatively regulated by repressor, whose action is relieved by the binding of substrates to the repressor itself (Schumacher & Brennan, 2002). This superfamily is expressed in *A. baumannii*, where resistance is mediated by MFS transporters (Sharma *et al*., 2016) and expressed in MDR clinical isolates in the presence of antibiotics (Khaledi *et al*., 2016). There are also other families expressed in *A. baumannii*, namely the resistance nodulation cell division (RND), small multidrug resistance (SMR) and multidrug and toxic compound extrusion (MATE) families (Abdi *et al*., 2020), with the former the most important one in MDR *A. baumannii*. Within the RND superfamily, the AdeABC pump causes resistance to several antibiotic classes, amongst them aminoglycosides, and has increased expression in clinical isolates.

Antibiotic enzyme inactivation confers resistance, for instance, to β-lactams, including cephalosporins, such as cefotaxime, and aminoglycosides, such as streptomycin. Resistance to aminoglycosides can occur via enzymatic phosphorylation, acetylation, and adenylation.

In the case of β-lactams, specific enzymes named β-lactamases, which hydrolyse the β-lactam ring, were detected just a few years after the introduction of penicillin in clinical use. This issue was solved with the use of compounds that resist enzymatic hydrolysis, such as methicillin, but another enzyme, TEM β-lactamase, was reported in Gram-negative bacteria in MDR strains (Datta & Kontomichalou, 1965). This enzyme made penicillin with Gram-negative activity nearly useless throughout the world, after its universal spread. Another type of important β-lactamase present in Gram-negative bacteria is the chromosomally encoded AmpC. With the spread of β-lactam resistance, various β-lactams were developed to fight this

issue, with second generation cephamycin cefoxitin and third generation cephalosporin cefotaxime reported to be resistant to both TEM β-lactamase and AmpC enzymes. Even with the effectiveness of third generation cephalosporins, soon emerged new resistant strains, with the overproduction of the chromosomal enzyme AmpC, even with the low affinity with these antibiotic substrates (Vu & Nikaido, 1985). With the proven effectiveness of third generation cephalosporins, mutants for the *ampC* gene were selected over time. Other examples of β-lactamases present in *A. baumannii* include VEB and PER extended-spectrum β-lactamases, where the former can spread over countries and the latter has emerged in *A. baumannii* strains in Belgium (Naas *et al*., 2006). In Belgium and France another type of β-lactamases was discovered to have emerged in *A. baumannii*, Ambler class A GES β-lactamase, found mainly in *K. pneumoniae*, *E. coli*, and *P. aeruginosa* on an international scale (Bogaerts *et al*., 2010), which have extended-spectrum properties, hydrolysing oxyimino-cephalosporins.

There have been reports on the presence of resistance islands for heavy-metal resistance in *A. baumannii* strains endemic to hospitals in Europe (Fournier *et al*., 2006). As it has been reported in the past causing infection in injured soldiers in conflict zones (CDC, 2004), multidrug resistance has been identified in medical facilities in Iraq and Afghanistan, in several isolates.

One of the widely disseminated *A. baumannii* resistance gene is $bla_{\text{OXA-23}}$, detected in the species in 1985, and is naturally occurring in another *Acinetobacter* species, which has a high probability of having transferred the gene to *A. baumannii* via HGT (Da Silva & Domingues, 2016), and can be inserted in both chromosome and plasmids, and can be surrounded by transposons, genomic islands and insertion sequences.

As an example of the tendency to develop resistance, over the span of just over a year, dozens of unique clinical isolates of MDR *A. baumannii* have been identified in a medical center in the United States of America. Among the MDR isolates, resistance to imipenem reached 18%, while the highest resistance frequency was to ciprofloxacin, as high as almost 96%, and amikacin, almost reaching 37% (Adams-Haduch *et al*., 2008). With the presence of the carbapenemase gene $bla_{\text{OXA-23}}$ and 16S rRNA methylase gene *armA*, resistance at high levels was predicted for imipenem and amikacin, respectively. The increased expression of the carbapenemase gene $bla_{\text{OXA-23}}$ was believed to be caused by the insertion sequence IS*Aba1* that precedes it, working as a strong promotor.

1.7.1 | Resistance genes in *Acinetobacter baumannii*:

Aminoglycoside resistance genes:

In *Acinetobacter baumannii* integron-positive strains, there have been reported, amongst others, resistance IntI1- associated genes *aac*1 and *aad*1*A*, which were proven to confer resistance to gentamycin, streptomycin and spectinomycin. Resistance to streptomycin was present as well in strains with an absent integron, where genes *strA* and *strB* were detected (Mak *et al*., 2008).

The *aphA1* gene is responsible for the resistance to kanamycin and neomycin and can exist in the bacterial genome both in the presence and absence of an integron (Jennifer *et al*., 2009).

The presence of AMEs is also notable in clinical MDR *A. baumanii*, through the expression of the genes *aac(3)-Ia* and *aac(6')-Ib*, *ant(2")-Ia* and *ant(3")-Ia*, and *aph33ib*, *aph(3')-Ia* and *aph(6)-Id* (Wang *et al*., 2017). The expression of the AMEs is the major cause of aminoglycoside resistance in many bacteria, and there have been several reports on the presence of multiple AMEs genes in *A. baumannii* isolates (Wang *et al*., 2017).

A. baumannii strain AYE, which had the aminoglycoside resistance genes *aac3*, coding for acetyltransferase, specific against gentamycin, *aad*A1 and *aad*DA1, coding for adenylyltransferase against streptomycin and spectinomycin; *aad*B, coding for adenylyltransferase against gentamycin, kanamycin and tobramycin; a putative adenylyltransferase gene.

Another gene, *aadB* has been observed in a clinical isolate of *A. baumannii* 064 (Domingues, Nielsen & Sa Silva, 2012), which codes for aminoglycoside adenyltransferase, responsible for resistance against gentamicin, kanamycin and tobramycin.

β-lactams resistance genes:

β-lactamases confer resistance against penicillin, cephalosporins, and carbapenems, with the most common group found in *A. baumannii*, oxacillinases (OXAs). The OXAs can be grouped in six main homology groups with different targets: OXA-51, chromosomic and intrinsic, the biggest subgroup; OXA-23, chromosomic or plasmidic; OXA-40/24, acquired carbapenemases; and OXA-58, OXA-143, and OXA-48, more recently described as having low hydrolytic activity (Nowak & Paluchowska, 2016). ESBL and metallo-β-lactamase (MBL) genes present in isolates from ICU wards are $bla_{\text{CTX-M}}$, which codes for resistance to cefotaxime, and bla_{SHV}, coding for

enzymes that have evolved from narrow to extended spectrum of hydrolysing activity, affecting monobactams and carbapenems (Safari *et al*., 2015; Liakopoulos, Mevius & Ceccarelli, 2016).

There is a natural occurring resistance gene in this species, the *bla*$_{\text{OXA-51}}$, which is classified as an intrinsic gene to *A. baumannii*, with different variants found in the species, referred collectively as *bla*$_{\text{OXA-51}}$-like (Turton *et al*., 2006). This gene codes for a carbapenemase and is responsible for the resistance to imipenem and other carbapenems in *A. baumannii*, (Hu *et al*., 2007; Zhu, Chen & Hou, 2019). On the strains with the resistance phenotype there is an upstream insertion sequence IS*Aba1* that provides a promoter to increase gene expression, hence augmenting resistance to β-lactams and creating the naturally resistant *A. baumannii* strains (Evans *et al*., 2008). Genes encoding for carbapenemases have been verified to be present in cases of MDR *A. baumannii*, namely *bla*$_{\text{OXA-23}}$-like, *bla*$_{\text{OXA-51}}$-like, *bla*$_{\text{OXA-24}}$-like and *bla*$_{\text{OXA-58-}\textit{like}}$, as well as MBL resistance genes *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP,}}$ that have been detected in ESBL-producing *A. baumannii* (Safari *et al*., 2015).

ESBL are enzymes that have proven troublesome, since they are capable of degrading later generation cephalosporins, such as cefotaxime and ceftazidime (Jaisankar *et al*., 2020, Zhanel *et al*., 2013). ESBL-producing *A. baumannii* isolates resistant to ceftazidime, cefipime and cefotaxime have the *csgA* gene present, which has a major role in adhesion to surfaces, cell aggregation, and biofilm formation (Jaisankar *et al*., 2020).

The insertion sequence (IS) IS*Aba1*'s presence upstream of *bla*$_{\text{OXA-51}}$-like has been verified to increase the expression of the oxacillinases by acting as a strong promoter and inducing resistance to carbapenems in *A. baumannii* (Vijayashree Priyadharsini, Smiline Girija & Paramasivam, 2018). The gene *bla*$_{\text{ADC 1}}$, named ADC because it is an *Acinetobacter* derived cephalosporinase, codes for an AmpC β-lactamase, which is overproduced when in the presence of IS*Aba1* (Vijayashree Priyadharsini, Smiline Girija & Paramasivam, 2018). The *bla*$_{\text{OXA-23}}$ is the most globally spread in the world in *A. baumannii*, with some variants of the *bla*$_{\text{OXA}}$ genes dominant in specific regions, having been detected in several nations (Hamidian & Nigro, 2019).

Other important reported carbapenemases are the *Klebsiella pneumoniae* carbapenemase (KPC), a class A serine carbapenem-hydrolyzing β-lactamases firstly described in 2001 (Ramirez, Bonomo & Tolmasky, 2020). Variants of this carbapenemase have been detected in carbapenem resistant strains of *A. baumannii* in South America (Ramirez, Bonomo &

Tolmasky, 2020). *bla*$_{KPC}$ is minimally inhibited by common β-lactamase inhibitors, such as clavulanate, tazobactam and sulbactam (Porreca *et al*., 2018). The origin of this resistance gene in *K. pneumoniae* proves the dissemination of resistance genes via HGT, namely to *A. baumannii*.

bla$_{NDM}$ is another MBL firstly described in 2009 (Pfeifer *et al*., 2011), with international detection soon after its discovery and related genes found in *A. baumannii* in Germany (Pfeifer *et al*., 2011). Having observed its location on conjugative plasmids it can be suggested this MBL has a rapid transfer and dissemination rate (Kumarasamy *et al*., 2010). It was detected in China in *A. baumannii* clinical isolates where there was resistance to carbapenems and cephalosporins, while some isolates remained susceptible to fluoroquinolones, aminoglycosides and colistin. These isolates were clonally different and had the *bla*$_{NDM}$ in different plasmids, which were successfully transferred via conjugation into an *E. coli* strain (Chen *et al*., 2011).

Other β-lactamase genes found in clusters in resistant strain of *A. baumannii*, AYE, are *bla*$_{VEB-1}$, a coding gene for class A beta-lactamases, specific against all β-lactams, except carbapenems; *bla*$_{oxa-10}$, coding gene for class D beta-lactamases, specific against all β-lactams, except carbapenems, and *bla*$_{oxa-69}$ (Fournier *et al*., 2006).

Rifampicin can be used in combination with colistin in cases of MDR *A. baumannii* (Mohammadi *et al*., 2017), and a resistance gene *arr-2* was detected in an *A. baumannii* isolate that presented a cluster of resistance genes (Fournier *et al*., 2006).

Colistin resistance genes:

The most widely known plasmidic colistin-resistance genes are from the *mcr* group to which 10 variants have been detected up to date (Hussein *et al*., 2021). Although these genes have been reported worldwide in Gram-negative bacteria from environmental, human and animal samples, no occurrences of the gene in *A. baumannii* have been reported in the past (Da Silva & Domingues, 2017). However, in colistin resistant strains of *A. baumannii* in Baghdad, it was detected the *mcr-1*, *mcr-2*, and *mcr-3* genes in isolates from clinical and environmental samples, which has been suggested to be causes by the presence of mobile genetic elements (Al-Kadmy *et al*., 2020). Mutations in the targets of colistin in *A. baumannii* have also been responsible for reported of resistance, where the lipid A component of LPS's expression is altered, which are usually inserted into the outer leaflet of the outer membrane. The

mutations are either in one of three genes *lpxA*, *lpxC*, and *lpxD* (Moffatt *et al*, 2010), which causes prevention complete loss of LPS production, or mutation of the *pmrA*/*pmrB* two-component system, modifying the lipid A, affecting the binding of colistin to the surface of the bacteria, possibly leading to alterations in virulence (Da Silva & Domingues, 2017).

Glycylcycline resistance genes:

Tygecycline resistance via two-component regulatory systems is the result of the genes *baeR* and *baeS* being co-transcribed and *adeA* and *adeB* genes expression and has been observed that the deletion of the *baeRS* genes causes a reduction in the expression of the *adeAB* genes. With the deletion of the *baeRS* genes it was observed reduction in the MIC of tigecycline, likewise the overexpression of the *baeRS* genes was observed to have to opposite effect, increasing the MIC to tigecycline. (Lin *et al*., 2014).

The genes *tet*A, coding for an efflux pump, and *tet*R, coding for a tetracycline repressor, were detected and were able to concede resistance to all tetracyclines in the MDR strain *A. baumannii* AYE (Fournier *et al*., 2006).

A study performed in Spain found the resistance genes *tetA* and *tetB*, which are responsible for antimicrobial resistance via efflux pumps, and belong to the MFS. *tetB* causes resistance against minocycline and tetracycline, and the *tetA* only to tetracycline; while *tetA* gene was verified to be highly prevalent in *A. baumannii*, *tetB* had minimal prevalence on the bacterial isolates of *A. baumannii* analysed. (Martí, Fernández-Cuenca, Pascual *et al*., 2006; Coyne, Courvalin, & Périchon, 2011) However, in more recent studies, it was detected a high prevalence of both *tetA* and *tetB* among *A. baumannii* isolates obtained in Ahvaz, Iran (Moosavian *et al*., 2020).

Quinolone resistance genes:

Resistance to quinolones in *A. baumannii* is the result of mutations in the *gyrA*, *parC* and/or *gyrB* genes, where single nucleotide changes induce resistance to this class (Vila *et al*., 1995; Park *et al*., 2011; Fournier *et al*., 2006). There are other cases as well, for example *qnrA*, a plasmid-mediated quinolone resistance gene, among *A. baumannii* isotates obtained in Iran (Moosavian *et al*., 2020). Isolates resistant to ciprofloxacin, a fluoroquinolone, have been found to have substitutions in the genes *gyrA* and *parC* (Adams-Haduch *et al*., 2008; Fournier *et al*, 2006). Quinolone resistance in *A. baumannii* is also mediated by efflux pump, where the

abaQ gene, which codes for an MFS transporter that is similar to other MFS transporters involved in drug efflux in *A. baumannii* and whose presence increases resistance to trimethoprim and novobiocin, with the highest recorded resistances against quinolone-type antibiotics, such as ciprofloxacin, levofloxacin, and nalidixic acid (Pérez-Varela *et al*., 2018).
Other quinolone resistance genes have been detected in China in *A. baumannii* where isolates harboured *qnrB* and *qnrS*, as well as mutations in the quinolone resistance–determining regions of the *gyrA* gene, encoded in plasmids (Yang *et al*., 2016).

Sulphonamide resistance genes:
Four *sul*1 genes, with predicted specificity against all sulfonamide drugs, via production of dihydropteroate synthetase, were identified in a comparative genomics study, with two genes matching with *A. baumannii*, and two matching with *Acinetobacter* spp. (Fournier *et al*., 2006). The genes were also identified in another clinical MDR *A. baumannii* strain, specifically *sul1* and *sul2* (Wang *et al*., 2017). A gene conferring resistance to trimethoprim *dhrfl* was also found in this *A. baumannii* strain.

Within its genus, *A. baumannii* strains exhibit the main antimicrobial resistance mechanisms in Gram-negative bacteria.

1.8 | Horizontal gene transfer

HGT is the non-sexual movement of genetic information between genomes (Burmeister, 2015), where new genetic material can replace existing genes, or simply add to the genome (Keeling & Palmer, 2008). HGT is responsible for the spread of antibiotic resistance genes among bacteria of the same generation, in contrast to vertical gene transfer, which occurs from parent to offspring, affecting different generations.
Proof of HGT is found in most bacterial genomes, and although not all transfer events may be significant, they represent the product of the evolutionary process that may sometimes have a benefit (Arnold, Huang & Hanage, 2022). The acquisition of genetic material that is relevant according to the circumstances, such as resistance genes in the presence of antibiotics, makes a selection of the cells that will form resistant colonies in the future (Arnold *et al*., 2022),

assuring the permanence of those genes in the population. This leads to the concentration of resistance genes in the nosocomial setting and the rapid dissemination of resistant strains, making HGT prevention one of the strategies that must be included in the fight against the emergence of resistant organisms in the healthcare environment (Lerminiaux & Cameron, 2019). The genes passed onto by vertical inheritance can be liberated by HGT into unrelated pathogens (Lerminiaux & Cameron, 2019). Even with this being a major problem, the best data obtained on the subject in the clinical setting are the reservoirs in hospitals and the transmission between these reservoirs, while the rates of horizontal transfer in clinical environments and the impacts of HGT on the frequency of diseases remains speculative or unknown, as well as the uniqueness of the aspects of each clinical environment (Lerminiaux & Cameron, 2019). There is also a barrier in the scientific experimentation with antimicrobial resistance gene transmission in the clinical setting, due to ethical considerations and privacy rules, which reduces the simplicity of conducting targeted studies to track HGT (Lerminiaux & Cameron, 2019)

HGT can be responsible for inducing major pathogenicity in harmless bacteria (Seitz & Blokesch, 2013) and has been found to contribute to the acquisition, integration and maintenance of pathogenicity islands in genomes of bacteria, leading to the spread of virulence factors.

There are four mechanisms of HGT in bacteria: conjugation, where direct contact between bacterial cells occurs; natural transformation, where DNA fragments are internalized and incorporated into the recipient's genome; transduction, which is mediated by bacteriophages that transfer genetic material between bacterial cells following its bacteriophage life cycle; and vesicle-mediated transformation, where vesicles that are naturally released by bacteria transfer DNA between bacterial cells.

1.8.1 | Conjugation

Conjugation is usually the mechanism most related to the spread of resistance, and it is universally conserved amongst bacteria, occurring in an ample array of environments. It consists in the transfer of genetic material, usually plasmids, via direct contact between live bacteria, where genetic information can flow one way or both ways and is the major driver for evolution amongst bacterial genomes (Virolle *et al*., 2020).

The first discovery of conjugation occurred in 1946 by Edward Tatum and Joshua Lederberg, where the unidirectional transference of DNA from and to bacteria through a fertility (F) factor led to the realization that the F factor is a replicative extra-chromosomal genetic element, later named “plasmid” that could cross cell membranes of the parental strains (Lederberg & Tatum, 1946).

Not only are resistance genes transported between bacteria via conjugation, but also genes for adapting to various conditions, altering the metabolism, both in biofilms and bacterial communities, in several environments ranging from soil, water, sewage, or associated with plant or animal hosts (Virolle *et al*., 2020). The adaptations can vary from changing to a symbiotic lifestyle, acquiring virulence factors, and resistance to heavy metals and/or antimicrobials.

This HGT method is mediated by conjugative plasmids or, less frequently, by integrating conjugative elements (Goessweiner-Mohr *et al*., 2015). Conjugative plasmids are mostly secreted onto the recipient cell via a macromolecular membrane-associated machinery called Type IV secretion system (T4SS), responsible for the intercellular protein substrate transport, from which a subgroup evolved to additionally translocate DNA-protein complexes. T4SS is not only responsible for the spread of antibiotic resistance, but also virulence factors amongst pathogenic bacteria (Elena *et al*., 2015). In Gram-negative bacteria the structure that mediates conjugation is named the mating pair formation (MPF) complex, constituted by the conjugal pilus, the structure that establishes contact between cells, and the mating channel, through which the DNA intermediate is transferred (Christie, 2001). This HGT process is also used in pathogenicity when some Gram-negative bacteria interact with eukaryotic cells (Christie, 2001).

The ability to perform conjugation of the donor strain depends on the expression of the transfer genes clustered in the *tra* region of the plasmid, that code for the protein factors necessary for the conjugative pilus and the T4SS of the MPF, along with the components for the processing of the plasmid. The rate at which the F plasmid is transferred depends on the phase of growth of the bacteria cells, with a maximum at the exponential phase and mostly halted in the stationary phase, which has been observed in Gram-negative bacterium (Headd & Bradford, 2020). In some cases it can occur genetic mutations that drastically increase the efficiency of conjugation of conjugative plasmids from diverse compatibility groups, called superspreader mutations (Virolle *et al*., 2020).

Plasmid-mediated conjugation can occur in several conditions, having even been indicated to occur in space flight conditions, with no negative effect of microgravity on the results (De Boever *et al*., 2007).

There can be some genetic barriers when it comes to different species mainly due to recombination issues, where the mismatch repair system's enzymes act as inhibitors of recombination between species; nonetheless a triggered SOS response, the general response to DNA damage, stimulates interspecies recombination (Matic, Rayssiguier & Radman, 1995). There can also be limitations with the intake of foreign DNA due to the defence mechanisms of the host bacteria, however transferable plasmids have evolved adaptation mechanisms against these host defence systems (Virolle *et al*., 2020). The stabilization of the MTF is also an important factor in the efficiency of plasmid transfer that is dependent on one of the porins or the LPS' structure.

1.8.2 | Natural transformation

Natural transformation is the uptake of naked DNA from the surrounding environment by bacteria, DNA that was released due to the death of other bacteria or secreted by living cells (classified as donors); the uptake of DNA is followed by the incorporation of this DNA into the genome of the recipient bacteria that is naturally competent, and the expression of the uptaken DNA. It can occur on many environments, ranging from human fluids, soil, water and food products (Domingues Nielsen & Da Silva, 2012).

A prime example of natural transformation was described for the first time in 1928 by Frederick Griffith (Griffith, 1928), where the inoculation of non-virulent strain of *Streptococcus pneumoniae* into mice proved harmless, while mice inoculated with the virulent strain resulted into its death. The real proof of natural competence for transformation occurred when the non-virulent strain was inoculated with the heat-killed virulent strain, inoculated into the mice, and the mixed culture killed the animal models, as well as was also possible to re-isolate live virulent bacteria from the infected animals. This proved natural competence for genetic transformation leads to the spreading of virulence traits.

Natural transformation can only occur when the recipient bacterium is competent for transformation (Seitz & Blokesch, 2013). Natural competence is the state necessary for the bacterium to intake external DNA and incorporate it into its genome, and environmental

factors contribute to the development of natural competence. Those factors include genotoxic stress, bacterial cell-cell communication, starvation of preferred carbon sources and presence or absence of certain sources of carbon (Seitz & Blokesch, 2013). The entry into the state of natural competence is sometimes accompanied in some bacteria by the halt of bacterial growth, which can represent a strategy of adaptation to changing conditions of the environment (Blokesch, 2016). The onset of competence can be induced in bacteria through high cell density, antibiotic stress, DNA damage, intracellular growth, absence of preferred carbon sources and general starvation, or can be constitutively expressed (Blokesch, 2016).

Motility has also been related to DNA uptake in *A. baumannii*, being influenced mainly by media viscosity, as no transformation occurs in solid media, and disabling the movement proteins has a negative impact on natural transformation competence (Wilharm *et al*., 2013). Some strains of *Acinetobacter* spp., specifically *A. baumannii* and *A. nosocomialis* have been reported to be naturally competent, specifically those found in clinical environments, which have also been in circulation for several years when they were discovered, suggesting natural transformation is the most effective method of resistance acquisition in harsh environments (Domingues *et al*., 2019). For example, in the clinical setting, where disinfection is common practice, natural transformation is facilitated due to the abundance of foreign antibiotic resistance DNA resulting from the destroyed bacteria, which become donors for the surviving disinfectant-injured populations (Jin *et al*., 2020), falling out of line with the resistance acquisition methods majorly reported on other multi-resistant species, namely conjugation.

Since it involves the uptake of DNA from the surrounding environment, death of bacteria caused by human activity, such as disinfection with chlorine, can lead to an increase in transformation frequency (Jin *et al*., 2020). This genetic transfer can occur between closely related bacteria, in the case of chromosomal DNA (Lorenz & Wackernagel, 1994), where recombination is likely to occur or, can transfer the DNA in the absence of recombination if inserted into a broad-host-range plasmid (Thomas & Nielsen, 2005) or by transposition (Domingues *et al*., 2012). The mobile genetic elements (MGEs) such as integrons, gene cassettes and transposons, have been successfully acquired via natural transformation on *Acinetobacter baylyi* BD413 (Domingues, Nielsen & Da Silva, 2012).

Horizontal gene transfer via natural transformation has been observed to be a good factor for conversion to a higher state of fitness into new environments, with a general advantage to

naturally transformable strains, with genetic exchange accelerating the rate of adaptation to a completely novel environmental setting (Baltrus, Guillemin & Phillips, 2008).

1.8.3 | Transduction

Transduction is characterized by the transfer of bacterial DNA from the donor to the recipient cells by a bacteriophage. During the infection process, bacteriophages, virus that infect bacteria, encapsulate bacterial genetic information and upon a new infection on a different target, move the genetic information to the newly infected bacteria. Just like natural transformation, this HGT mechanism can occur without direct cell to cell interaction (Wachino *et al*., 2019). Transduction can be generalized or specialized, and the packaging of host DNA in lieu of the viral genome has the potential of transferring large blocks of bacterial DNA in an isolated particle (Schneider, 2021). Many bacterial virulence factors are encoded by phage or phage-like elements and can present the major source of variation amongst bacterial strains. There is a distinct type of transduction denominated lateral transduction, which is known to occur in *Staphylococcus aureus*. The difference between transduction and lateral transduction is on the frequency of transference of chromosomal regions, which is extremely high, and the size of those chromosomal regions, which are larger (Humphrey *et al*., 2021). This results in a more powerful mode of DNA transfer mediated by phages, which exceeds the mobility of core genes in the bacterial chromosome of any plasmid or transposable element, at a rate of 1000-fold higher than (Humphrey *et al*., 2021).

Although transduction is a well-known driving factor in HGT between bacteria, bacteriophages have not been reported to be relevant in HGT in *A. baumannii* (López-Leal *et al*., 2020). However, recent studies have shown that antibiotic resistance genes can be transferred via transduction in *A. baumannii* and it has been demonstrated diversity of prophages, the genetic material of bacteriophages integrated into a bacterium's genome, is responsible for encoding many antibiotic resistance genes in this bacteria, and found in many lineages of *A. baumannii* (López-Leal *et al*., 2020; Wachino *et al*., 2019). Detection of the intrinsinc oxacillinases from *A. baumannii* in prophages proves prophage-encoded antibiotic resistance genes are a common occurrence in this pathogen (López-Leal *et al*., 2020).

1.8.4 | Vesicle-mediated transformation

This mechanism is mediated by outer membrane vesicles (OMVs), which are constantly released during growth by a range of bacterial species (Fulsundar *et al*., 2014; Dell'Annunziata *et al*., 2021), and have other functions besides HGT, associated with bacterial physiology and pathogenesis (Fulsundar, Domingues & Nielsen, 2019), such as modulation of host immune response, formation of the biofilm matrix, and intracellular communication, as they can transfer a myriad of biomolecules, protecting them from extracellular degradation and dilution (Dell'Annunziata *et al*., 2021). The OMVs have been recognized as genetic transfer vectors, carrying plasmids, chromosomal DNA fragments, bacteriophage DNA and RNA fragments (Pérez-Cruz *et al*., 2013; Velimirov & Ranftler, 2018; Gaudin *et al*., 2014; Medvedeva *et al*., 2014).

OMVs are produced by various Gram-negative bacteria and are formed by the outer membrane of the cells, being apparently common in the *Acinetobacter* genus and has been demonstrated to occur in *A. baumannii*, where proteins and nucleic acids related to virulence and antibiotic resistance have been detected (Fulsundar, Domingues & Nielsen., 2019).

Vesicle mediated transformation is another mechanism of HGT where no direct contact between cells must occur to have DNA transfer across bacteria.

This type of mechanism has been verified to occur in other *Acinetobacter* spp., such as *Acinetobacter baylyi*, and there are different interactions of OMVs depending on the recipient species for the vesicles (Fulsundar *et al*., 2014), as well as the resistance genes $bla_{\text{NDM-1}}$ and $bla_{\text{oxa-23}}$ present in plasmids can be transferred into *A. baumannii* via vesicle-mediated transformation (Chatterjee *et al*., 2017; Rumbo *et al*., 2011).

1.8.5 | Mobile genetic elements

MGE are elements that promote intra- and intercellular movement of DNA and play a crucial role in HGT of resistance genes. MGEs with intracellular DNA movement include insertion sequences, transposons, integrons and gene cassettes, and those that are transferred between bacteria include plasmids and integrative conjugative elements (Partridge *et al*., 2018).

Plasmids are circular DNA or RNA molecules found in bacteria, amongst other organisms. Plasmids can disassociate from chromosomal DNA and replicate independently; plasmids in

A. baumannii can range in size from 2 kb to more than 150 kb (Cerezales *et al*., 2020), with some genes associated with antibiotic resistance. They can be horizontally transferred between bacteria via conjugation and natural transformation (San Millan, 2018; Domingues *et al*., 2012). There can be specific associations of plasmids and bacterial clones, reaching the point of a strong link between specific antibiotic resistance plasmids and particular bacterial lineages, such as *K. pneumoniae* sequence type (ST) 11 or ST405 and plasmid pOXA-48 (San Millan, 2018), *E. coli* ST131 clade C2/H30Rx and IncFII plasmids carrying $bla_{\text{CTX-M}}$ (San MIllan,2018).

Plasmids in *A. baumannii* belong to a restricted number of plasmid lineages, which was verified by the whole gene content of the evaluated plasmids that were interconnected (Salgado-Camargo *et al*., 2020). These plasmid lineages were observed to have stable gene content, even though they were isolated from each other over many years (Salgado-Camargo *et al*., 2020). Plasmids found in other Gram-negative species are not similar to those found in *A. baumannii*, as they do not appear to be as steadily maintained as those found in *Acinetobacter* species, and *Acinetobacter* plasmids are not detected in other Gram-negative bacteria (Lam *et al*., 2023). After the integration of a new plasmid in a host cell, the resistance genes can be further mobilized by plasmid-borne transposable element copying them into different plasmids or the chromosome, increasing the potential of the resistance genes to transmit to more bacterial recipients (Lerminiaux & Cameron, 2019).

The regulation of the T6SS is mediated by genes contained in a self-transmissible resistance plasmid which negatively regulate the expression of T6SS, resulting in a bacteria-killing phenotype when absent, activating it only under specific circumstances, such as being under attack from another bacterium or environmental conditions that lead to membrane perturbations. The plasmid, which regulates antibiotic resistance, can be lost in a subset of the population, and so is the antibiotic resistance of that sub-population, resulting in killing of other bacteria, and the duality of the expression of this plasmid, with antibiotic-resistant and bacteria-killing subpopulations represents a novel survival strategy for *A. baumannii* (Weber *et al*., 2015). This secretion system specialized for elimination of competitors is only expressed in part of the population of resistant strain of *A. baumannii*, making the bacteria that express the T6SS more susceptible to antibiotics, while the sub-population that remains with the secretion system silenced persists against antibiotic activity (Weber *et al*., 2015), and that sub-

population is responsible for the differentiation into the killing cells, making it able to out-compete other bacteria.

Foreign genes can also be inserted into the plasmid sequence and will be replicated with the plasmid. Plasmids conferring a survival in the presence of antibiotics can have a fitness cost, that can be later alleviated by compensatory mutations (San Millan, 2018).

First described in 1989 (Stokes & Hall, 1989), integrons are genetic units capable of capturing different resistance genes, present in gene cassettes, and expressing the genes carried by them. Integrons have been found responsible to be important not only in the spread of antibiotic resistance, but also for bacterial genome evolution (Mazel, 2006). When they were first described, they were believed to be mobile, but the mobility is dependent on the elements where they are incorporated, such as transposons. Integrons contain in-site specific recombination systems that capture and mobilize the genes contained in gene cassettes, as well as promoter sites for those genes. The structure of a class 1 integron consists of 2 conserved segments separated by a variable region, where the cassettes are integrated, often including antibiotic resistance genes. The essential genetic components of the class 1 integron are the *intI* gene, which codes for a site-specific recombinase from the integrase family, an adjacent site *attI*, recognized by the integrase and the receptor for the gene cassettes and a correctly oriented promotor P_C for the expression of the cassettes (Hall & Collis, 1995; Bouvier, Demarre & Mazel, 2005). They can be grouped in chromosomally located integrons in the bacterial chromosome and mobile integrons (Escudero *et al*., 2015), and the latter is divided in at least 8 classes, differentiated by the sequence of the integrase gene, and all integrons found in antibiotic-resistance clinical isolates belong to those classes and are mobile, while the ones found in environments non-related with antibiotic use had features similar to the cases of antibiotic resistance and pathogenic bacteria, suggesting integrons are more prevalent in bacteria than previously thought (Escudero *et al*., 2015; Nield *et al*., 2001).

Mobile integrons are the result of the association with transposable elements and conjugative plasmids. They have a small capacity, reaching eight cassettes (Escudero *et al*., 2015), encoding almost exclusively antibiotic resistance genes for most of the clinically relevant antibiotics.

The class 1 integron is the most disseminated amongst commensals and pathogens in humans and animals (Kang *et al*., 2005). With the selective antibiotic pressure in the clinical setting, mobile integrons represent such an adaptive advantage that they are more commonly found

in Gram-negative clinical isolates (Labbate, Case & Stokes, 2009). Even amongst Gram-negative bacteria, the class 1 integron was reported to have the highest prevalence in *Acinetobacter* spp. (Pormohammad *et al*., 2019). The class 1 integron has been associated with over 130 gene cassettes that have antibiotic resistance genes for almost all the antibiotic families, including β-lactams, aminoglycosides, trimethoprim, chloramphenicol, streptothricin, fosfomycin, macrolides, rifampin, quinolones and antiseptics of the quaternary ammonium-compound family (Partridge *et al*., 2009).

Gene cassettes are small MGEs which contain one gene and a specific recombination site used to insert themselves into integrons, the *attC*, that are located in the terminations of the cassettes and are essential to their mobility, sometimes forming multi-cassette arrays, with a varying number between integrons (Recchia & Hall, 1995; Escudero *et al*., 2015). Resistance genes encoded in cassettes have been discovered to be responsible for the resistance to virtually all antibiotics (Xu *et al*., 2009; Rowe-Magnus & Mazel, 2001), and integrons have the genetic machinery to convert gene cassettes in functional and expressible genes (Mazel, 2006). Integron cassettes appear ubiquitous, being recovered from several different environments (Escudero *et al*., 2015).

1.9 | Horizontal gene transfer in *Acinetobacter baumannii*

Acinetobacter spp. environmental and clinical isolates have been known to be able to undergo HGT through natural transformation (Domingues *et al*., 2019; Leungtongkam *et al*., 2018), conjugation (Leungtongkam *et al*., 2018), (with various degrees of success), transduction (López-Leal *et al*., 2020) and vesicle mediated transformation (Fulsundar, Domingues & Nielsen, 2019), with natural transformation with more representation in *A. baumannii* resistance acquisition (Godeux *et al*., 2022).

The main force that drives HGT in *A. baumannii* seems to be natural transformation, as recombination events have been detected to spontaneously occur both at the intra and interstrain level between *Acinetobacter* pathogenic species and *A. baumannii*, that can result in the acquisition of resistance to carbapenems in the clinical setting (Godeaux *et al*., 2022).

In 2010, only a single isolate of *A. baumannii* has been shown to be naturally competent for transformation (Ramirez, 2010), but currently several cases of natural competence occurring in *A. baumannii* are known (Domingues *et al*., 2019; Le *et al*., 2021).

HGT in *A. baumannii* via natural transformation is not a rare occurrence (Domingues *et al*., 2019), and natural competence is essential to facilitates the occurrence of this HGT mechanism (Hu *et al*., 2019), which can vary with the growth phase of cultures. The risk of importing deleterious alleles and the metabolic expense resulting from the transcription of the new genetic material can affect the transformation rates over short evolutionary timescales (Hu *et al*., 2019). The uptake of DNA has reached high values in International Clone II *A. baumannii* (Hu *et al*., 2019). Rifampicin resistant individuals were able to acquire apramycin resistance via natural transformation with isolates that originally had their resistance as a result of mutation (Godeux *et al*., 2022). Imipenem-resistant transformants were obtained after acquisition of the $bla_{\text{oxa-23}}$ gene from a donor strain of *A. baumannii* into a strain of *A. nosocomialis*. (Godeux *et al*., 2022)

Conjugation in *A. baumannii* has led to an increase of pathogenicity, due to the transfer of virulence factors carried in a plasmid pAB5, which belongs to a family of plasmids that contain a resistance island that encodes multiple putative antimicrobial resistance genes and genes related to the repression of the T6SS in other *A. baumannii* strains, as well as carrying the genetic machinery necessary for conjugation (Di Venanzio *et al*., 2019). It was verified that a strain lacking the pAB5 was susceptible to multiple antibiotics and the presence of T6SS, proving the passage of this conjugative plasmid impacts the pathogenicity of a MDR *A. baumannii* urinary isolate. (Di Venanzio *et al*., 2019). Conjugation experiments where carbapenem-resistant *A. baumannii* isolates successfully transferred plasmids containing several *bla* genes into a bacterial strain of *E. coli, E. coli* J53 (Zhao *et al*., 2019).

Resistance to antibiotics can be efficiently acquired between strains of *A. baumannii* and between *A. baumannii* and another *Acinetobacter* spp. It has been demonstrated that in a mixed population gene transfer occurs rapidly through natural transformation in communities of sessile cells. Acquisition of large resistance islands in *A. baumannii* was previously attributed to transduction, as acquisition via natural transformation was presumed to be limited by their size, but it was supported by genetic evidence that HGT of the resistance islands can occur through homologous recombination after uptake by natural transformation, although these resistance islands were originally introduced to *A. baumannii* via conjugation, followed by insertion in the chromosome (Godeaux *et al*., 2022).

The transference of genetic material mediated by prophages can entail chromosomal antimicrobial resistance genes between *A. baumannii* strains, as the resistance genes in this

pathogen are primarily harboured on the chromosome rather than transferable plasmids (Wachino *et al*., 2019). It has been verified that the resistance gene *armA*, coding for aminoglycoside resistance methylase, was passed between *A. baumannii* strains via transduction in a transposon that has been frequently found on the chromosomal/plasmid genomes of isolates of *A. baumannii* belonging to different geographical locations (Wachino *et al*, 2019). Another relevant gene coding for a β-lactamase, $bla_{\text{NDM-1}}$, was transferred between two nosocomial *A. baumannii* strains and it was concluded that transduction was the HGT mechanism that allowed the dissemination of antibiotic resistance genes in *A. baumannii* (Krahn *et al*., 2016).

It has been observed the release of OMVs with high levels of class D β-lactamases by non-susceptible *A. baumannii* has a sheltering effect on carbapenem-susceptible *A. baumannii* cells in the same media, proving this mechanism effective at transporting antibiotic resistance enzymes between strains of *A. baumannii* (Liao *et al*., 2015).

In cases of resistance to tigecycline in *A. baumannii*, it was detected an increase in production of OMVs in tigecycline-resistant strains when compared to tigecycline susceptible strains (Cho, Sondak & Kim, 2023). It was also observed selective transference of the OMVs that mediated tigecycline resistance to other pathogens, while being able to induce antibiotic resistance more potently than antibiotic exposure in *A. baumannii*, to a rate of over 500-fold (Cho *et al*., 2032).

1.10 | Main objectives

All the different HGT mechanisms have been reported in *A. baumannii*. Overall, conjugation is assumed to be the main HGT mechanism responsible for antibiotic resistance in bacteria. However, conjugation does not seem very common in *A. baumannii*, as genes responsible for the transfer (*tra*) or mobilization (*mob*) of plasmids are missing on most sequenced *A. baumannii* plasmids (Fondi *et al*., 2010) and several studies fail to demonstrate conjugation (Bergogne-Bérézin & Towner, 1996; Fu *et al*., 2014; Héritier *et al*., 2005; Towner *et al*., 2011; Da Silva & Domingues, 2016). On the other hand, *A. baumannii*'s ability to undergo natural transformation is increasingly reported (Wilharm *et al*, 2013; Domingues *et al*, 2019; Hu *et al*. 2019) and may present as an alternative to contribute to resistance acquisition is this species.

The main aim of this work is to determine the antimicrobial resistance transfer rate by conjugation and natural transformation and unravel which is the major contributor for the MDR profile of *A. baumannii*.

To address this question, the naturally competent *A. baumannii* A118 will be used as recipient in conjugation and natural transformation assays with donor DNA from different bacterial species, namely *Escherichia coli* 189 and *Salmonella enterica* serovar Typhimurium 490 and Sal25.

The knowledge of the mechanisms that facilitate the movement of resistance genes is an important step to develop effective measures to control antibiotic resistance. Through the findings of this study, we hope to determine which of the HGT mechanisms is the preferential target to be inhibited to prevent, or at least slow down, the emergence of multirresistance in *A. baumannii*.

Chapter 2 | Materials and Methods

2.1 Protocol materials

Luria-Bertani (LB) medium is a rich medium used commonly to culture *Enterobacteriaceae*, but useful with the non-fermenting *A. baumannii* since it has no specificities for its growth media, broadly used in recombinant DNA assays and other molecular biology procedures (MacWilliams & Liao, 2006). The LB medium was made with LB broth according to Lennox in powdered form (MP Biomedicals, USA) and agar powder for bacteriology (VWR Chemicals, USA) at 1,5%. The LB broth was made using only the powdered media. In this work, sterile antibiotic solutions were added to the sterilized media to select for resistant isolates, namely kanamycin (Sigma Aldritch, UK) 30 mg/ml, streptomycin (Duchefa, the Netherlands) 30 mg/ml and cefotaxime (Fisher Scientific, USA) 30 mg/ml and colistin (Thermo Scientific, USA) 10 mg/ml stock solutions.

Semisolid media was proven to be effective at allowing the internalisation of naked DNA contained in the environment surrounding the recipient bacterial cells, with the proper viscosity for successful natural transformation in *Acinetobacter baumannii* (Wilharm *et al*., 2013). It was prepared with agar 0,5%, sodium chloride (VWR Chemicals, USA) at 2,5 g/l, tryptone (Bacto™, BD, USA) at 5g/l and yeast extract (Bacto™, BD, USA) at 2,5 g/l.

The Mueller-Hinton (MH) agar is the recommended medium for antimicrobial susceptibility testing by the disc-diffusion method, with acceptable reproducibility in susceptibility testing, low level of inhibitors for antibiotics, and it allows proper growth of most non-fastidious pathogens (Biemer, 1973). MH agar was made using broth in powdered form (Oxoid, United Kingdom) and 1,5% agar.

The Phosphate-buffered saline (PBS) used during the natural transformation protocol was obtained by mixing 8 g/l of sodium chloride, 0,2 g/l of potassium chloride (Merck, Germany), 1,44 g/l of sodium phosphate dibasic (VWR Chemicals, USA) and potassium phosphate monobasic (VWR Chemicals, USA) using miliq H_2O as solvent.

The broth microdilution for MIC determination media includes a cation-adjusted MH broth (25 mg/ml Ca^{2+} and 12,5mg /ml Mg^{2+}), where the calcium and magnesium were added in the form of chloride solutions, namely $CaCl_2$ and $MgCl_2$, both in solutions at 10 mg/ml. The MH

broth cation concentration has to be balanced to prevent discrepancies in the interpretation of the colistin MIC results and maintain accuracy of the procedure (Etowski & Beckwith, 1981). The antibiotic colistin for the broth microdilution was prepared in a microcentrifuge tube at a concentration of 1024 µg/ml from a stock of 10 mg/ml.

2.2 Bacterial strains:

2.2.1 Recipient strains

- *A. baumannii* A118 (Ramirez *et al*., 2010): clinical isolate susceptible to antimicrobials. It is competent for natural transformation and was used as recipient in transformation assays.

- *A. baumannii* A118k: isogenic of A118, it was acquired by natural transformation of A118 with naked DNA from *A. baumannii* 121-1 (Domingues *et al*., 2019). It contains a kanamycin resistance marker and was used as recipient in conjugation assays.

2.2.2 Donor strains

- *E. coli* 189 (Lima *et al*., 2022): strain isolated from swine organs, resistant to colistin, due to the presence of the mobile colistin resistance gene *mcr-1*, and to cefotaxime, due to the $bla_{\text{CTX-M-1}}$ gene.

-*S. enterica* serovar Typhimurium Sal25 (Figueiredo *et al*., 2015): strain isolated from a porcine processed food, resistant to cefotaxime, due to the $bla_{\text{CTX-M-1}}$ gene, to colistin, associated with the presence of the *mcr*-1 gene, and to streptomycin and spectinomycin, due to the *aadA1* gene cassette embedded into a class 1 integron; these resistance genes are co-carried in a IncHI2 plasmid of 150 Kb. Sal25 also carries a $bla_{\text{TEM-1}}$ resistance gene and shows low susceptibility to heavy metals (Figueiredo *et al*., 2019). The *mcr-1* gene (Figueiredo *et al*., 2016) was transferred to *E. coli* strain J53, where co-transference of the $bla_{\text{CTX-M-1}}$ was observed (Figueiredo *et al*., 2016; Figueiredo *et al*., 2015). The insertion sequence IS*ECp1* is responsible for the capture and mobilization of several antibiotic resistance genes, and it was located upstream of the $bla_{\text{CTX-M-1}}$ gene (Figueiredo *et al*., 2015).

- *S. enterica* serovar Typhimurium 490S (Domingues *et al*., 2012): strain isolated from a pork hamburger, with a class 1 integron inserted in an intact Tn*21* transposon, where there are present the bla_{OXA-30} and *aadA1* gene cassettes, conferring resistance to β-lactams, as well as spectinomycin and streptomycin, respectively. These MGEs are inserted into a plasmid.

- *A. baumannii* strain 121-1 (Domingues *et al*., 2019): transformant acquired by natural transformation of *A. baumannii* strain 121 with a kanamycin resistance marker; it carries the kanamycin marker inserted into the chromosome.

2.3. Antimicrobial susceptibility testing

Three methods were employed to assess the susceptibility of the strains against the antibiotics used in the HGT assays: Kirby-Bauer disc diffusion, E-test and broth microdilution. All methods were performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

For the disc diffusion method, the susceptibility of the donor strains Sal25 and 490S were tested for streptomycin and spectinomycin, sulphonamides compounds, cefotaxime, ceftazidime and colistin ; a suspension of the strains with turbidity of 0.5 McFarland, measured in a densitometer (Grant-bio, Den-1B, England), was prepared in sodium chloride 0,9 % solution for both the strains and it was spread with a cotton-tipped sterilized swab on the surface of MH agar, followed by application of the antibiotic discs, and incubation at 37°C for 18-24 hours. The diameter of the bacterial inhibition growth was measured in millimetres. The same procedure was performed to determine the MIC of donor strains Sal25 and 490S to streptomycin, with the difference that an Etest strip containing a gradient of antibiotic concentrations was applied instead of discs and the MIC value was read at the intersection of the edge of the inhibition ellipse with the side of the strip.

Antibiotic discs:

The following antibiotic discs were used in the Kirby-Bauer disc-diffusion assays: streptomycin 10 µg, spectinomycin 10 µg, cefotaxime 30 µg, ceftazidime 30 µg, kanamycin 30 µg, colistin 10 µg, compound sulphonamides 300 µg and colistin 10 µg. (Oxoid, UK)

Colistin susceptibility testing was performed via broth microdilution (Chew *et al*., 2017), and the disc diffusion method utilized was solely for the purpose of assessing any change in susceptibility. The broth microdilution assay allows to obtain the MIC of colistin, by performing incubation in wells that have decreasing concentrations of colistin ranging from 512 µg/ml to 0,5 µg/ml, over 11 wells on each line of the 96-well plate, up to the 12th well which had no colistin, but miliq H_2O for a control where no antibiotic activity would occur. The assessment of the concentrations of colistin to which the transformants or transconjugants were resistant was solely done by the observing the presence or absence of bacterial growth in in the wells at different concentrations. If the well had visible growth would be considered below the MIC of colistin, and if had no visible growth, it would be considered equal to or above the MIC. The MIC was determined by the smallest concentration of colistin in which there was no growth.

2.4. Bacterial DNA extraction

2.4.1 Boiled total DNA

DNA extraction to use as template in PCR tests was done via transfer of the bacterial cells into 200 µl of MilliQ H_2O, boiled for 10 minutes on a dry block thermostat (Biosan, Bio T DB-100, Latvia) at 100°C and centrifuged (Thermo Fisher Scientific, Expresso, China) at 14000 rpm for 2 minutes, recovering the supernatant where the DNA is contained.

2.4.2 Plasmidic DNA

Plasmidic DNA for the transformation protocol was extracted and purified with an adapted methodology from Birnboim & Doly (Birnboim & Doly, 1979), where open circular DNA and linear DNA is removed and covalently closed circular DNA, such as plasmids, remains from the samples used. The principle of this extraction method is the range of pH where linear DNA suffers denaturation, but covalently closed circular DNA does not.

Firstly, a colony-forming unit (CFU) from plasmid-containing donor bacteria grown in solid LB agar medium was inoculated into 5ml of LB broth with the respective selective antibiotic, streptomycin for *S. enterica* serovar Typhimurium strains Sal25 and 490S, and colistin for colistin-resistant *E. coli* strain 189 and incubated overnight at 37°C in the controlled environment incubator shaker (New Brunswick Scientific co., USA) at 100 rpms.

From the 5 ml of the bacterial suspension, 3 ml were centrifuged (Sigma, 3-18K, Germany) at 5500 rpm for 10 minutes, the supernatant discarded, and the pellet homogenized with 100µl of elution buffer 1X buffer (Thermo Scientific, Lithuania), to weaken the cell wall, followed by the addition of 100 µl of sodium dodecyl sulphate (SDS) 20% (Merck, Germany), which leads to complete lysis of the cells, and the same volume of NaOH 0,4M (Panreac, EU), to achieve the needed pH for linear DNA denaturation. The tubes were obligatory to be gently inverted to mix the components and not affect negatively the consistency of the mix, that became milky and viscous in appearance, and if mixed too energetically would form lumps separated by a liquid phase. The mixture rested 5 minutes on ice.

Afterwards 150 µl of potassium acetate 5M (Merck, Germany) at 4°C (pH = 4.8) were added, to both neutralise the solution and to allow renaturation and aggregation of the high molecular weight chromosomal DNA, forming an insoluble network. Additionally, both protein-SDS complexes and high molecular weight RNA precipitate with the high concentration of sodium acetate. The tubes were quickly inverted and placed on ice for a minimum of 10 minutes.

To remove the contaminating macromolecules, the mix was centrifuged at 4000g for 5 minutes at room temperature. 200 µl of phenol (Thermo Scientific, USA) and 200 µl of chloroform (Thermo Scientific, USA) were added to 400 µl of the supernatant solution, which aid in the separation of proteins from DNA. The tubes were gently inverted, and a milky solution was formed.

This new solution was centrifuged at 4000 rpm for 5 minutes, where 2 immiscible solutions of different densities could be distinguished. 700 µl of ethanol 100% kept at -20°C were added to 350 µl of the supernatant to precipitate the plasmid DNA, and residual low molecular weight RNA. To mix, the Eppendorf tubes were gently inverted and then kept still for 5 minutes at room temperature.

Centrifugation of the tubes at 4000rpm for 5 minutes was done, followed by removal of the supernatant, and washing of the pellet with 1ml of ethanol 70% at room temperature. Re-centrifugation at 4000rpm for 5 minutes was performed.

To obtain the dry pellet containing the plasmid DNA, the pellet was dried in the centrifuge tubes in an incubator at 37°C. The filtered and dried pellet was resuspended in 20 µl of elution buffer 1X buffer and 1 µl of RNase 1mg/ml was added.

2.4.3 Total genomic DNA

Total genomic DNA was acquired by extraction with Anion-Exchange Resin columns (Genomic DNA kit, QIAGEN, Germany), according to the manufacturer's protocol and resuspended in EB buffer, pH 8.5 (QIAGEN, Germany).

2.4.4 Determination of DNA concentration

The concentration of the genomic and plasmidic DNA was determined in the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, USA).

2.5 Natural transformation assays

Natural transformation was performed as described by Wilharm *et al*., 2013. A short description of the transformation protocol follows.

The recipient strain was susceptible to all the used antibiotics.

Day 1: The recipient strain A118 was inoculated and incubated overnight (24h) on LB agar at 37°C. The sterility control was performed with PBS, MilliQ H_2O and NaCl 0.85%, which are necessary during the protocol, where they were also incubated in LB medium.

Day 2: One CFU of the recipient strain was suspended in 20 µl PBS 1X in a microcentrifuge tube of 1,5 ml and the 4 µg of DNA prepared in MilliQ H_2O, either with plasmidic DNA (pDNA) or total DNA; in the negative control, the DNA was replaced with H_2O. Both the bacteria and the DNA were mixed in a 1:1 ratio and seeded onto the semisolid media, stabbed 7 times, in equally spaced points in the medium, and injecting 2 µl of the bacteria-DNA suspension just below the surface of the semisolid media. The plates were sealed with parafilm, to prevent dehydration, and incubated at 37°C for 18-24 hours.

Day 3: The bacterial growth was collected from the surface of the medium via one of the two methods:

- Flushing: 1 ml of PBS 1X was used to flush and collect the bacterial growth from the surface of the medium.
- Removal with the inoculation loop: a 10 µl loop was used to remove the cell growth from the semisolid medium plates and suspended in 1ml PBS 1X.

A 10^{-1} dilution in PBS 1x was performed and the optical density (OD) was measured at 600nm in a spectrophotometer (Thermo Fishing Scientific, Genesys 10UV, USA). The collection tubes were centrifuged for 5 minutes at 6000 rpm and the supernatant discarded. The volume necessary to obtain an OD of 10 in the undiluted sample was calculated for each sample and the correspondent PBS 1x volume was added.

Successive dilutions were performed on a 96 well plate on NaCl 0,85% up to the dilution factor of 10^{-8}, followed by plating of 100 µl and inoculation on LB and LB + kanamycin 30 µg/ml media in the assays with the *A. baumannii* 121-1 DNA, LB and LB + streptomycin 10 µg/ml for the assays with DNA from Sal25 and 490S, LB and LB + cefotaxime for the assays with DNA from Sal25, and LB and LB + colistin 12 µg/ml for the assays with DNA from *E. coli* 189, according to the table 1, and incubate the samples overnight at 37°C.

Table 1 - Dilutions plated in natural transformation assays

Natural transformation mixture	Dilutions inoculated in LB + antibiotic plates	Dilutions inoculated in LB plates
A118 + 121.1 DNA	10^{-1}	10^{-10}
A118 + Sal25 DNA/pDNA	10^{0} and 10^{-1}	10^{-7} and 10^{-8}
A118 + Sal25 DNA/pDNA	10^{0} and 10^{-1}	10^{-7} and 10^{-8}
A118 + 490S DNA	10^{0} and 10^{-1}	10^{-7} and 10^{-8}
A118 + 189 DNA/pDNA	10^{0}	10^{-7} and 10^{-8}
A118 + H_2O	10^{0}	10^{-7} and 10^{-8}

pDNA- plasmidic DNA

Day 4: The CFUs were enumerated. If CFUs were not between 30 and 300, new dilutions were plated.

The colonies considered possible transformants, from a phenotypical point of view, were the ones that grew in the media with antibiotic, streptomycin for the transformants that received DNA from Sal25 and 490S, and colistin for the transformants that received DNA from 189.

Day 5: CFUs were counted and registered, when in the range of 30-300 CFUs per plate, or new dilutions were plated. The CFUs grown in the media without antibiotic were necessary to account for the total number of bacteria CFUs. Pure CFUs were obtained by two consecutive isolations in LB with antibiotic pressure.

Acquisition of transformants was confirmed when there was growth in LB + Kanamycin 30 μg/ml together with absence of growth in Simmons citrate agar (Pronadisa, Spain).

For the positive control of transformation, total DNA from *A. baumannii* 121-1, which has homologous DNA to the recipient A118, was used as donor DNA in the transformation assay.

The negative controls, in which instead of DNA, H_2O was used, were used to verify whether or not the resistance to the tested antibiotics was a direct result of the transformation assay or the result of possible random mutations that lead to emergence of resistance.

2.6 Conjugation assays

Conjugation was performed with live bacterial strains incubated with each other.

Day 1: The donor and recipient strains were isolated and incubated in LB agar overnight at 37°C.

Day 2: One CFU of each strain was suspended on 5ml of LB broth and incubated overnight at 37°C and aeration at 150 rpm.

Day 3: 500 μl of the of recipient strain and 50 μl of the donor strain were mixed. For the controls, only the 50 μl of the broth with the donor strain were used, with the remaining volume of broth with the recipient strain replaced with 500 μl of MilliQ H_2O, and vice-versa for the controls that had only the recipient cell.

After homogenisation of this suspension with the vortex, 100 μl of each mixture was applied onto a LB agar plate. Incubation was done overnight at 37°C.

Day 4: The growth of each plate was collected and suspended in 1 ml NaCl 0,85% and successive dilutions were performed in a 96 well plate on NaCl 0,85% up to the dilution factor of 10^{-8}. In order to select the transconjugants, and enumerate recipient, donor and transconjugant cells, different antibiotics were used in the solid LB medium. For the recipient strain A118k, LB with kanamycin 30 μg/ml, for the donor strains Sal25 and 490S, LB with streptomycin 10 μg/ml, and for the donor strain 189, LB with 12 μg/ml colistin. For the transconjugants' selection with Sal25 or 490S as the donors, LB with kanamycin 30 μg/ml and streptomycin 10 μg/ml were used, while for the transconjugants that had 189 as the donor, LB with kanamycin 30 μg/ml and 12 μg/ml colistin.

The inoculation was performed by adding 100 μl of each suspension at the respective dilution factor on the culture medium with the appropriate antibiotic according to the table 2.

Table 2 – Dilutions plated in conjugation assays

Conjugation mixture	LB + Kanamycin	LB + Streptomycin	LB + Kanamycin + Streptomycin	LB + Colistin	LB + Kanamycin + Colistin
A118 + Sal25	10^{-8}	10^{-1}	10^{0}	-	-
A118 + 490S	10^{-8}	10^{-1}	10^{0}	-	-
A118 + H_2O	10^{-8}	10^{0}	10^{0}	-	-
Sal25 + H_2O	10^{0}	10^{-1}	10^{0}	-	-
490S + H_2O	10^{0}	10^{-1}	10^{0}	-	-
A118 + 189	10^{-8}	-	-	10^{0}	10^{0}
A118 + H_2O	10^{-8}	-	-	10^{0}	10^{0}
189 + H_2O	10^{0}	-	-	10^{-1}	10^{0}

Day 5: CFUs were counted and registered, when in the range of 30-300 CFUs per plate, or new dilutions were plated.

Pure CFUs were obtained by two consecutive isolations in LB with antibiotic pressure.

2.7 Characterization of transformants and transconjugants:

Phenotypic and genotypic traits of transformants and transconjugants were evaluated by different methods.

After selected transformants/transconjugants were isolated, the antimicrobial susceptibility profile was determined by the Kirby-Bauer disc diffusion method for each of the antibiotics the genes present on the donor strains conferred resistance to. The resistance profile was compared with the profile of the recipient and donor strains.

Genotypic characterization of the transformants/transconjugants were performed by Polymerase Chain Reaction (PCR) in isolates that showed a phenotypic profile consistent with specific donor DNA genes acquisition.

2.7.1 Statistical analysis

In order to assess if there were significant differences between the use of different DNAs for the transformation assays, namely plasmid or whole-DNA, a T-test of independent samples was preformed, with the null hypothesis of no significant differences would be found between the transformation rates with the different DNAs of the same donor that were used.

To compare the frequencies of natural transformation and conjugation, statistical analysis was performed also via a T-test of independent samples, with a null hypothesis of no significant differences between the two HGT mechanisms approached in this experiment in *A. baumannii* A118 and A118k. The statistical analysis software used was STATISTICA (Version 7, StatSoft©). The null hypothesis was that there was a lack of significant differences between the two studied HGT mechanisms and would be rejected if the *p* value obtained in the T-test was below 0,05, which would imply the two HGT mechanisms had indeed statistically significant differences in their frequencies.

2.8 Polymerase chain reaction:

For the preparation of the PCR, an 18 µl PCR mixture of reagents was prepared per sample, including controls, where it was used 10 µl of DreamTaq™ Green PCR Master Mix 2x (Thermo Scientific, USA), 7 µl of DNAse-free water (Thermo Scientific, USA) and 0,5 µl of each of the two primers at 10 µM. 2 µl of the boiled DNA sample were added, and the tubes were loaded in the thermocycler (Biometra®, T1, Germany) for the PCR. The ladder used to assess the amplicon size after electrophoresis was Gene Ruler 1kb (Thermo Scientific, USA).

Table 3 – Primers and targeted genes for the PCR

Target genes	Primers	Amplicon size (bp)	Reference
bla$_{CTX-M-1}$	CTX-M_fw: 5'- TTTGCGATGTGCAGTACCAGTAA-3' CTX-M_rv: 5'- CGATATCGTTGGTGGTGCCATA-3'	544	Edelstein *et al*., 2003
Class 1 integron	In5'CS: 5'-GGCATCCAAGCAGCAAG-3' In3'CS: 5'-AAGCAGACTTGACCTGA-3'	2000 (490S) 1000 (Sal25)	Lévesque *et al*., 1995
mcr-1	mcr1_320bp_fw: 5'- AGTCCGTTTGTTCTTGTGGC-3' mcr1_320bp_rev: 5'- AGATCCTTGGTCTCGGCTTG-3'	320	Rebelo *et al*., 2018
Kanamicin resistance marker	Mu179F: 5'- ATCGGTAATAAAGCCGATATGCG-3' Mu179R: 5'- TCAGCAGCTGATTAATCAACGAG-3'	2400	G. Wilharm, personal communication
IS*Aba1*	IS*Aba1*a: 5'- ATGCAGCGCTTCTTTGCAGG-3' IS*Aba1*b: 5'-xAATGATTGGTGACAATGAAG-3'	389	Héritier, Poirel & Nordmann, 2006

For the detection of insertion sequences located near the resistance genes, the transformants of the A118 with the donor total-DNA from Sal25 that were incubated on media with the presence of cefotaxime 30 µg/ml went through PCR where the insertion sequence IS*Aba1* was targeted to possibly explain the odd properties of the CFUs of the resulting transformants. There are 4 different programs (Tables 4-8) for the thermocycler for the PCR depending on the genetic elements to be amplificated.

2.8.1 Detection of DNA after PCR

After the PCR, an 1% agarose (NZYTech, Portugal) gel was prepared, in Tris-acetate-EDTA 1X buffer (TAE) (Thermo Scientific, Lithuania) and 2 µl of ethidium bromide (Applichem, Germany) at 0,5 µg/ml to allow the DNA bands to be visualized under ultra-violet (UV) light. The electrophoresis was run at 80 mV and 60 mA for 1h15 (GE Healthcare, EPS 301, Sweden) on a horizontal electrophoresis system (Amersham Biosciences, HE 38, UK) and the amplified DNA that migrated in the electrophoresis gel was observed over a UV light, attainable by placing the agarose gel on a long wavelength ultraviolet transilluminator at 300 nm (Vilber Lourmat, TFX-20M, France).

Table 4 – PCR program for the amplification of the *mcr-1* gene

PCR program for *mcr-1* gene	Temperature	Duration	Repetition
Initial Denaturation	94°C	15m 0s	1x
Denaturation	94°C	0m 30s	25x
Annealing	58°C	1m 30s	
Extension	72°C	1m 0s	
Final Extension	72°C	10m 0s	1x
Cooling	4°C	pause	

Table 5 – PCR program for the amplification of the class 1 integron

PCR program for class 1 integron	Temperature	Duration	Repetition
Initial Denaturation	94°C	5m 0s	1x
Denaturation	94°C	1m 0s	35x
Annealing	55°C	1m 0s	
Extension	72°C	5m 0s	
Final Extension	72°C	16m 0s	1x
Cooling	4°C	pause	

Table 6 – PCR program for the amplification of the *bla*$_{\text{CTX-M-1}}$ gene

PCR program for *bla*$_{\text{CTX-M-1}}$ gene	Temperature	Duration	Repetition
Initial Denaturation	94°C	5m 0s	1x
Denaturation	94°C	1m 0s	31x
Annealing	56°C	1m 0s	
Extension	72°C	1m 0s	
Final Extension	72°C	10m 0s	1x
Cooling	4°C	pause	

Table 7 – PCR program for the amplification of kanamycin resistance marker

PCR program for kanamycin resistance marker	Temperature	Duration	Repetition
Initial Denaturation	94°C	5m 0s	1x
Denaturation	94°C	1m 0s	
Annealing	60°C	1m 0s	30x
Extension	72°C	3m 0s	
Final Extension	72°C	5m 0s	1x
Cooling	4°C	pause	

Table 8 – PCR program for the amplification IS*Aba1*

PCR program for kanamycin resistance marker	Temperature	Duration	Repetition
Initial Denaturation	94°C	5m 0s	1x
Denaturation	94°C	1m 0s	
Annealing	52°C	1m 0s	30x
Extension	72°C	1m 0s	
Final Extension	72°C	3m 0s	1x
Cooling	4°C	pause	

Chapter 3| Results

3.1 Characterization of recipient and donor bacteria

The MICs determined by E-test to streptomycin were 12 µg/ml for A118, > 256 µg/ml for Sal25 and > 256 µg/ml for 490S, which corresponds with resistance, since resistance is considered when the MIC vales are of > 32 µg/ml, which makes it a highly resistant phenotype (Kidsley *et al*., 2018) *E. coli* strain 189 was already described as being colistin-resistant, with resistance marked by the MIC of 32 µg/ml (Lima *et al*., 2022).

3.2 Donor DNA concentration

After the plasmid DNA extraction protocol of strains Sal25, 490S and 189, successful extraction was confirmed via agarose gel electrophoresis. The concentration of the plasmid and total DNA was determined with NanoDrop spectrophotometer analysis, with results shown in Table 9.

Table 9 – Concentration of DNA

Donor strains	DNA concentration (ng/ µl)
121-1	1290 (whole DNA)
Sal25	2882.5 (plasmid DNA)
490S	2499.1 (plasmid DNA)
189	3815.4 (plasmid DNA)

3.3 Horizontal gene transfer assays

3.3.1 Natural transformation frequency

After the transformation assays with the recipient strain *A. baumannii* A118, the number of CFUs on the plates with the antibiotic pressure, which represents the transformants, and the number of CFUs on the plates with no antibiotic, to account the total amount of bacteria, were used to obtain the transformation frequency, following the formula: $\frac{CFUs\ on\ LB\ with\ antibiotic}{CFUs\ on\ LB\ without\ antibiotic}$ (Table 10).

For the obtention of the A118k strain used in the conjugation assays, where homologous DNA from kanamycin-resistant strain of *A. baumannii* 121-1 was used, the calculated transformation frequency was $1.03*10^{-6}$ or $2.82*10^{3}$ CFUs/µg of DNA, which is similar to the transformation frequencies previously obtained with *A. baumannii* A118 (Ramirez *et al*., 2010).

In the assay where the recipient strain was subjected to transformation with Sal25 genomic DNA, the transformation frequency was $1.38*10^{-8}$ when selected with streptomycin, with an average of $5.68*10^{2}$ CFUs/µg of DNA, and $1.80*10^{-7}$ under cefotaxime selective pressure, with an average of $3.25*10^{4}$ resistant CFUs/µg of DNA. In transformation with plasmid DNA from the same donor strain, the transformation frequency obtained with streptomycin selection was $4{,}79*10^{-8}$, resulting in an average of $3.54*10^{2}$ resistant CFUs/µg of pDNA. When the donor strain was 490S, where only the plasmidic DNA was used, it was obtained a transformation frequency of $7.45*10^{-8}$, with an average of $5.18*10^{2}$ resistant CFUs/µg of pDNA. When the donor DNA used was plasmid DNA from 189 donor strain, the natural transformation frequency obtained was $3{,}05*10^{-8}$, with an average of $2.22*10^{2}$ colistin resistant CFUs/µg of pDNA.

Table 10 - Natural transformation frequencies of *A. baumannii* A118 with different donor DNA

	Transformation frequency			
Donor DNA	STR 30 µg/ml	CTX 30 µg/ml	COL 12 µg/ml	KAN 30 µg/ml
121.1 total DNA	-	-	-	$1.03*10^{-6}$ $2.82*10^{3}$ CFUs/µg of DNA
Sal25 total DNA	$1.38*10^{-8}$ $5.68*10^{2}$ CFUs/µg of DNA	$1.80*10^{-7}$ $3.25*10^{4}$ CFUs/µg of DNA	-	
Sal25 pDNA	$4.79*10^{-8}$ $3.54*10^{2}$ CFUs/µg of pDNA	-	-	
490S total DNA	$7.45*10^{-8}$ $5.18*10^{2}$ CFUs/µg of pDNA	-	-	
189 pDNA	-	-	$3.05*10^{-8}$ $2.22*10^{2}$ CFUs/µg of pDNA	

STR - streptomycin**; CTX -** cefotaxime**; COL –** colistin**; KAN –** kanamycin; **pDNA -** plasmid DNA

3.3.2 Conjugation frequency

Following the conjugation with the A118k and donor strains, the CFUs were enumerated on the solid media with both kanamycin and each selective antibiotic (AB), as well as on the solid media with individual antibiotics. The conjugation frequency was calculated according to the following formula: $\frac{CFUs\ on\ media\ with\ antibiotics\ 1\ \&\ 2}{CFUs\ on\ AB1\ media + CFUs\ on\ AB2\ media}$ (Table 11).

In the assay where the recipient strain A118k went through conjugation with the donor Sal25, the conjugation frequency was $1.57*10^{-8}$. When the donor strain for the conjugation was 490S, a $1.72*10^{-8}$ conjugation frequency was observed. The conjugation of the recipient strain A118k with donor 189 resulted in a conjugation frequency of $2.06*10^{-8}$.

Table 11 – Conjugation frequency of *A. baumannii* A118k with different donor strains

Donor strain	Conjugation frequency
Sal25	$1.57*10^{-8}$
490S	$1.72*10^{-8}$
189	$2.06*10^{-8}$

3.3.4 Statistical analysis of natural transformation and conjugation frequencies

For the natural transformation assays where the recipient strain A118 went through transformation with donor Sal25's total and plasmidic DNA (Table 12), there were no significant differences amongst the transformation frequencies when either type of DNA was used (T-test of independent samples; $p > 0,05$).

The transformation and conjugation frequencies did not present significant differences (Table 12) between the HGT mechanism used (T-test of independent samples; $p > 0,05$) when the donor strain used was Sal25.

The transformation and conjugation frequencies did not present significant differences (Table 12) between the HGT mechanism used (T-test of independent samples; $p > 0,05$) when the donor strain used was 490S.

The transformation and conjugation frequencies did not present significant differences (Table 12) between the HGT mechanism used (T-test of independent samples; $p > 0,05$) when the donor strain used was 189.

It is possible to conclude the frequencies of natural transformation and conjugation were not significantly different from one another, all carrying *p* values above the 0,05 value.

Table 12 – Statistical analysis where two frequencies were compared, and their *p* values displayed.

Frequencies	*p* value
Sal25 DNA vs Sal25 pDNA	0.35
Sal25 Natural transformation vs Sal 25 Conjugation	0.22
490S Natural transformation vs 490S Conjugation	0.11
189 Natural transformation vs 189 Conjugation	0.46

3.4 Phenotypic characterization of transformants and transconjugants

Some transformants/transconjugants were randomly selected for detailed analysis from the solid LB media with the respective antimicrobials. The antimicrobial susceptibility profile determination was performed by the disk diffusion method, with the exception of colistin, which was determined by broth microdilution method, where the MIC was assessed. The antibiotics used on the disc diffusion test were selected based on the resistance genes present on the donor strains for the transformation assay and both the recipient and donors for the conjugation assay, with streptomycin, spectinomycin, and compound sulphonamides to evaluate the acquisition of the class 1 integron carrying the *aadA1* gene, cefotaxime and ceftazidime for the $bla_{\text{CTX-M-1}}$ gene, and colistin to verify if there was any change in the inhibition growth that may be related with the acquisition of the *mcr-1* gene.

The transformants obtained with Sal25 donor DNA in the media with cefotaxime 30 µg/ml had a consistence that made them very mucoid, with high water-retention and increased difficulty in collenting them with the innoculation loops. This only happened with the transformants incubated in media with cefotaxime, so a PCR was done to detect insertion sequence IS*Aba1*

close to the intrinsic *bla*$_{OXA51}$ and *ampC* genes that may have been acquired and increased the genes expression.

Acquisition of IS*Aba1* did not occur, as verified by the absence of a visible band on the agarose gel after UV light exposure.

3.4.1 Characterization of transformants via disk diffusion susceptibility method

In the case of the assay with the donor strain Sal25's (Table 13) genomic DNA, with selection on solid LB media with cefotaxime 30 µg/ml, there was bacterial growth close to the antibiotic discs with spectinomycin, with higher inhibition diameters observed in the discs with streptomycin, and largest diameters in the compound sulphonamides antibiotic discs. There was high frequency of bacterial growth close to and touching the antibiotic discs with cefotaxime and ceftazidime, with a steady small diameter of growth around the discs with colistin. In the disc diffusion assays where the Sal25 plasmid DNA was used (Table 14), with selection on LB media with streptomycin 30 µg/ml, there was a high frequency of bacterial growth close to and touching the antibiotic discs of streptomycin and spectinomycin, with no significant changes in the large diameters observed in the discs with compound sulphonamides. On the antibiotic discs with cefotaxime and ceftazidime the diameters of growth measured were significantly higher than the ones in the transformants where genomic DNA of Sal25 was used. In the discs with colistin, there was a mild increase in the diameter measured.

The transformants from Sal25 donor DNA, which were isolated on medium with cefotaxime, showed changes in the susceptibility profile for all tested antimicrobials; however, the CFUs showed a mucous phenotype that occurred specifically in the isolates grown in this media, with no similarities between this and the transformants isolated in media with streptomycin.

In the assay with 490S where it was used plasmid DNA (Table 15), the majority of the bacterial isolates had growth close to and touching the antibiotic discs with streptomycin and spectinomycin, with some cases where greater diameters were observed in the discs with spectinomycin, and with large diameters of growth measured on the discs with sulphonamides compounds. There were no cases of growth close to or touching the antibiotic discs that contained cefotaxime and ceftazidime.

Table 13 – Antimicrobial susceptibility of transformants obtained in natural transformation of *Acinetobacter baumannii* A118 with *Salmonella enterica* serovar Typhimurium Sal25 genomic DNA.

	Antimicrobial susceptibility diameter (mm)					
Bacteria	S (10 µg)	SH (10 µg)	S3 (300 µg)	CTX (30 µg)	CAZ (30 µg)	CT (10 µg)
1	19.1	15.2	31.8	9.6	12.2	11.7
2	16.9	13.3	25.5	12.2	17	11.3
3	15.5	13.5	24.3	12	10.2	11.4
4	18.2	14	24.6	9.5	12.8	10.2
5	16.6	16.6	24.5	0	0	11.1
6	21.1	9.7	18.4	0	0	11.3
7	19.2	9.1	19	6.5	0	11.4
8	20.2	10.6	19.9	0	0	11.2
A118	6.5	13.1	23.3	20.8	21.9	11.3
Sal25	0	0	0	0	15.1	10.3

S - streptomycin**; SH -** spectinomycin**; S3 –** compound sulphonamides**; CTX -** cefotaxime**; CAZ -** ceftazidime**; CT -** colistin

Table 14 – Antimicrobial susceptibility of transformants obtained in natural transformation of *Acinetobacter baumannii* A118 with *Salmonella enterica* serovar Typhimurium Sal25 plasmid DNA.

	Antimicrobial susceptibility diameter (mm)					
Bacteria	S (10 µg)	SH (10 µg)	S3 (300 µg)	CTX (30 µg)	CAZ (30 µg)	CT (10 µg)
1	0	14	22.8	19.1	19.9	11.4
2	0	13.7	22.8	17.3	18.6	10.9
3	0	14.8	22.4	19.2	20.2	11.1
4	9.1	0	24.2	24.1	24.6	12.1
5	0	0	20.6	18.5	19.9	11.8
6	8.4	0	21.8	19.5	21.3	11.3
7	7.3	0	24.3	20.1	22.8	12.2
8	8.3	12.8	13.5	15.8	20.5	12.8
9	12	12.8	21.5	15.6	19	10.8
10	9.5	0	22.1	23.4	24.7	12.9
11	0	0	22.1	19.8	18.8	11.7
12	8.4	11.5	14.2	17.2	20.6	13.8
13	8.6	0	21.5	21.4	22.3	13
14	10.4	12.8	23.7	21.9	25	13.6
15	12.1	13.4	17.2	21.5	26.3	13.2
16	0	0	10.4	17.8	24.2	13.1
17	8.7	10.4	24.7	24.4	23.3	14.2
18	0	0	17.7	23.6	25.9	13.7
19	0	10.6	20.2	23	22.8	12.3
20	9.5	9.8	21.6	24.8	23.7	13.8
21	8.3	10.6	13.5	17.7	21.9	12.4
A118	6.5	13.1	23.3	20.8	21.9	11.3
Sal25	0	0	0	0	15.1	10.3

S - streptomycin**; SH -** spectinomycin**; S3 –** compound sulphonamides**; CTX -** cefotaxime**; CAZ -** ceftazidime**; CT -** colistin

Table 15 – Antimicrobial susceptibility of transformants obtained in natural transformation of *Acinetobacter baumannii* A118 with *Salmonella enterica* serovar Typhimurium 490S plasmid DNA.

Antimicrobial susceptibility diameter (mm)					
Bacteria	S (10 µg)	SH (10 µg)	S3 (300 µg)	CTX (30 µg)	CAZ (30 µg)
1	0	0	16.9	18.9	17.9
2	0	0	20	18.5	19.6
3	0	0	20.5	17	18.6
4	0	0	17.5	19.2	19.5
5	7.3	0	24.9	22.8	24.1
6	12	13.5	22.9	16.7	19.3
7	0	0	20	18.8	17.6
8	9.5	11.3	16.5	15.8	21.7
9	12.3	13.6	23.1	16.4	19.5
10	12.1	13	24.2	17.2	19.7
11	0	13.3	23.2	16.6	20
12	0	0	0	24.5	23.6
13	10.5	21.9	20.6	18.7	21.5
14	0	23	22.9	20.5	23.3
15	0	24.6	23.4	22.2	22.4
16	14.2	16.9	28.5	23.5	26.5
17	0	22.8	20	18.8	24
18	0	21.8	20.9	16.5	19.5
19	12.5	21.6	22.5	17.4	20.1
20	12.6	23.1	20.8	16.6	19.1
A118	6.5	13.1	23.3	20.8	21.9
490S	12.5	0	0	30.1	27.5

S - streptomycin**; SH -** spectinomycin**; S3 –** compound sulphonamides**; CTX -** cefotaxime**; CAZ -** ceftazidime

3.4.2 Characterization of transformants via broth microdilution determination of the minimum inhibitory concentration

With the broth microdilution procedure performed on the 21 isolated transformants that grew in the solid LB media with colistin 12 μg/ml, the MIC values for colistin were determined (Table 16). The MIC of the donor strain was 32 μg/ml of colistin, with the recipient being 0.5 μg/ml. The colistin MICs of the transformants were varied and ranged between 1 and 512 μg/ml.

Table 16 – Minimum inhibitory concentration of transformants obtained in natural transformation of *Acinetobacter baumannii* A118 with *Escherichia coli* 189 plasmid DNA.

Bacteria	Colistin minimum inhibitory concentration (μg/ml)
1	32
2	32
3	256
4	256
5	256
6	64
7	8
8	8
9	128
10	512
11	64
12	128
13	128
14	128
15	64
16	64
17	64
18	64
19	1
20	64
21	16
189	32
A118	0,5

3.4.3 Characterization of transconjugants via disc diffusion susceptibility method

The transconjugants isolated in solid LB media with kanamycin and streptomycin 30 μg/ml for selection, were resistant to those antibiotics, regardless of which *S. enterica* donor strains were used.

On the transconjugants obtained with donor strain Sal25 (Table 17), the diameter of the inhibition of the bacterial growth varied greatly among transconjugants on the discs with spectinomycin, with predominant small growth halos and some cases of bacterial growth making contact with the disc. For the compound sulphonamides discs most of the transconjugants had large inhibition halos around them, with 5 out of the 17 transconjugants with the bacterial growth touching the disc. Cefotaxime and ceftazidime discs had similarly large growth halos among them, with 3 exceptions where the transconjugants grew in contact with the cefotaxime antibiotic discs. Transconjugants growth around the colistin discs were different from the previous transformants, with relatively larger inhibition growth halos.

For the transconjugants obtained through conjugation with 490S as the donor (Table 18), predominantly smaller inhibition growth halos were observed in the spectinomycin antibiotic discs, with some exceptions of large halos. Compound sulphonamides had overall large diameters of bacterial growth around its antibiotic discs. Cefotaxime and ceftazidime had similar results in the bacterial growth among each other, with relatively large halos. Like the situation of the transconjugants of Sal25 and the transformants with the same donor strain, the transconjugants of 490S had relatively greater diameters of the growth halos of the discs with colistin when compared with the transformants that went through natural transformation with 490S DNA.

Table 17 - Antimicrobial susceptibility of transconjugants obtained in conjugation of *Acinetobacter baumannii* A118k with donor *Salmonella enterica* serovar Typhimurium Sal25.

Antimicrobial susceptibility diameter (mm)							
Bacteria	K (30 µg)	S (10 µg)	SH (10 µg)	S3 (300 µg)	CTX (30 µg)	CAZ (30 µg)	CT (10 µg)
1	0	0	26.1	24	22.5	28	17.5
2	0	0	12.5	28	24	25	15.5
3	0	0	17	27	20	23.6	15
4	0	0	19.9	27.2	21	23.9	14.5
5	0	0	17.5	28	20.5	24.2	15.1
6	0	0	11	27	24	25	17.1
7	0	0	26	28.1	20	25.1	15.1
8	0	0	13.9	26.3	20.2	22.5	14.9
9	0	0	9	20	26	26	15
10	0	0	0	12	23	22.3	16
11	0	0	9.4	23.8	26	25.1	16
12	0	0	0	21	22	23	15
13	0	0	12.3	0	20.5	22.5	13.9
14	0	0	0	0	0	25	12
15	0	0	9	0	0	21.5	13.5
16	0	0	0	0	0	13.5	13.6
17	0	0	0	12	12	27	12.4
A118k	0	6.5	13.1	23.3	20.8	21.9	11.3
Sal25	21.5	0	0	0	0	15.1	10.3

K - kanamycin; **S** - streptomycin; **SH** - spectinomycin; **S3** – compound sulphonamides; **CTX** - cefotaxime; **CAZ** - ceftazidime; **CT** - colistin

Table 18 - Antimicrobial susceptibility of transconjugants obtained in conjugation of *Acinetobacter baumannii* A118k with donor *Salmonella enterica* serovar Typhimurium 490S.

	Antimicrobial susceptibility diameter (mm)					
Bacteria	K (30 µg/ml)	S (10 µg)	SH (10 µg)	S3 (300 µg)	CTX (30 µg)	CAZ (30 µg)
1	0	0	14	24.6	22	23
2	0	0	14.2	26.4	21	24
3	0	0	28	24.2	21.9	26
4	0	0	11	26.1	22	23.2
5	0	0	12	29.1	20	22
6	0	0	13.2	28.1	23	24.8
7	0	0	26	25.1	20.2	25.5
8	0	0	27.2	34.8	24	27.4
9	0	0	9.5	25.2	21.2	25.1
10	0	0	9.8	19	23.1	24.3
11	0	0	22	14	22.5	28
12	0	0	21.9	19	23	28
13	0	0	18	23.5	21.5	24
14	0	0	0	20	22	22
15	0	0	10	21	22	22
16	0	0	0	21	19.5	21
17	0	0	0	24.5	24	24.5
A118k	0	6.5	13.1	23.3	20.8	21.9
490S	21	12.5	0	0	30.1	27.5

K - kanamycin; **S** - streptomycin; **SH** - spectinomycin; **S3** – compound sulphonamides; **CTX** - cefotaxime; **CAZ** - ceftazidime

3.4.4 Characterization of transconjugants via broth microdilution determination of the minimum inhibitory concentration

The transconjugants obtained with the donor strain 189 that grew in solid LB media with colistin 12 µg/ml went through an assay to determine the MIC via broth microdilution (Table 12). The majority of the transconjugants' MIC values were similar to the MIC of the donor strain 189, with two transconjugants with higher MICs, specifically 64 and 128 µg/ml.

Table 19 - Minimum inhibitory concentration of transconjugants obtained in conjugation of *Acinetobacter baumannii* A118k with *Escherichia coli* 189.

Bacteria	Colistin minimum inhibitory concentration (µg/ml)
1	32
2	32
3	32
4	128
5	64
6	32
7	32
189	32
A118k	0,5

3.5 Genotypic characterizations of transformants and transconjugants

In order to evaluate the acquisition of resistance genes and elements, boiled DNA was extracted from the isolates that showed altered antimicrobial susceptibility profile consistent with the acquisition of donor genes and used as template in PCR.

3.5.1 Characterization of transformants

Acquisition of resistance the class 1 integron was not confirmed by PCR in any of the selected transformants obtained with both Sal25 and 490S donor strains, both plasmidic DNA. The transformants where Sal25 whole DNA was used rendered no results that would need a PCR

to be performed. The positive control for the class 1 integron was donor Sal25, since it is present in both donor strains. No follow up PCR reactions were performed, as the lack of the amplicon for the class 1 integron in the transformants suggested no other resistance genes were transferred. A similar case was the transformants of A118 with donor strain 189 (Figure 2), where the lack of amplicon suggested no transference of the *mcr-1* occurred with the natural transformation assay as with 189 donor strain.

The transformants from the donor strain Sal25 that were acquired in media under cefotaxime selective pressure and had a viscous phenotype did not acquired the insertion sequence IS*Aba1* (Figure 3).

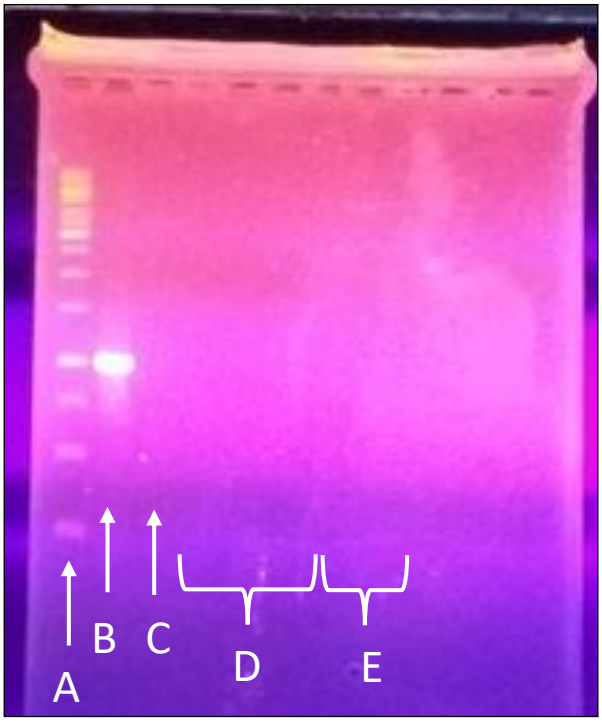

Figure 1 - Agarose gel electrophoresis of the class 1 integron on the selected transformants that resulted from transformation of the recipient *Acinetobacter baumannii* A118 with plasmid DNA from donor strains *Salmonella enterica* serovars Typhimurium Sal25 and 490S. A – DNA ladder 1kb; B – donor strain Sal25 (positive control); C – recipient strain A118 (negative control); D – transformants nº 8, 12 and 16 that had Sal25 as donor; E – transformants nº 8 and 12 that had 490S as donor.

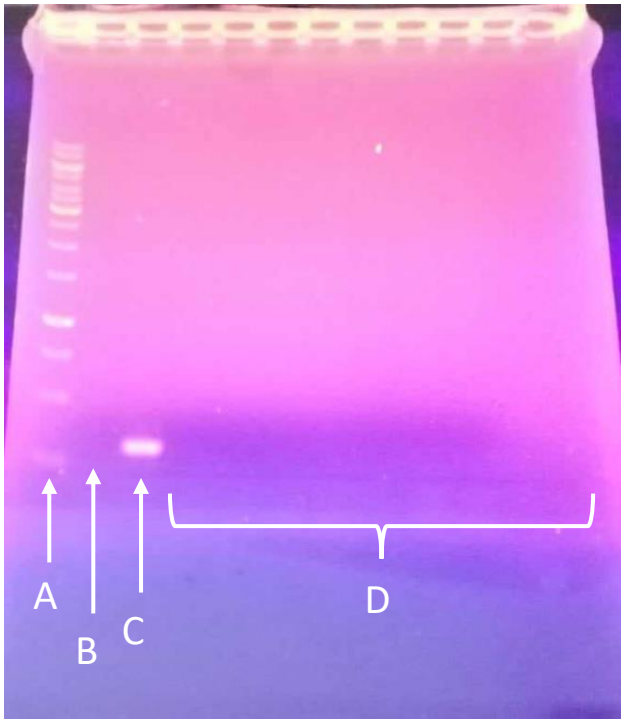

Figure 2 - Agarose gel electrophoresis of the *mcr-1* gene on the selected transformants that resulted from transformation of the recipient *Acinetobacter baumannii* A118 with donor strain *Escherichia coli* 189. A – DNA ladder 1kb; B – recipient strain A118 (negative control); C – donor strain 189 (positive control); D – transformants nº 1, 2, 3, 4, 5, 9, 12, 13 and 14.

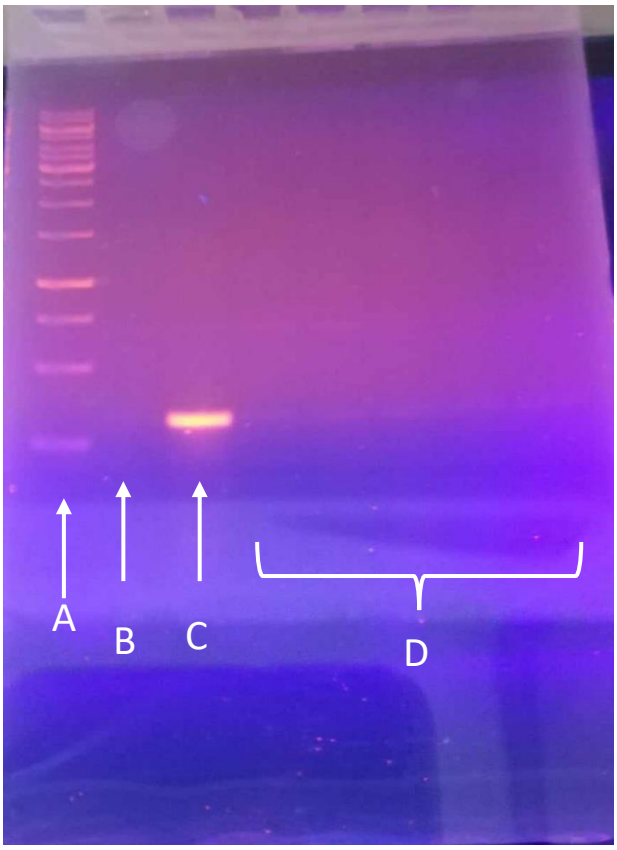

Figure 3 - Agarose gel electrophoresis of the insertion sequence IS*Aba1* on the transformants selected in cefotaxime media that resulted from transformation of the recipient *Acinetobacter baumannii* A118 with whole DNA from donor strain *Salmonella enterica* serovar Typhimurium Sal25. A – DNA ladder 1kb; B – recipient strain A118 (negative control); C – donor Sal25 (positive control); D – transformants nº 1-8.

3.5.2 Characterization of transconjugants

The tested transconjugants that resulted from conjugation with donor strain Sal25 acquired the class 1 integron, as the amplicon is visible in transconjugants 14 and 17 (Figure 4), unlike the tested transconjugants that had 490S as the donor strain, in which no amplicon was observed (Figure4). Further PCRs were performed on the transconjugants that had Sal25 as the donor strain to assess the passage of the $bla_{\text{CTX-M-1}}$ gene, in which there was only one amplicon visible on transconjugant 17 (Figure 5). The passage of the *mcr-1* gene onto the transconjugant 17 with Sal25 as donor was verified by the visible amplicon (Figure 6). Finally the kanamycin resistance marker was verified to be passed onto the recipient strain (Figure 7)

on the transconjugant 17, suggesting the passage of the rest of the genes from donor strain Sal25.

The conjugation of the strain A118K with donor strain 189 (Figure 8) appeared to not have been successful, as no amplicon was possible to be observed in any of the amplified transconjugants.

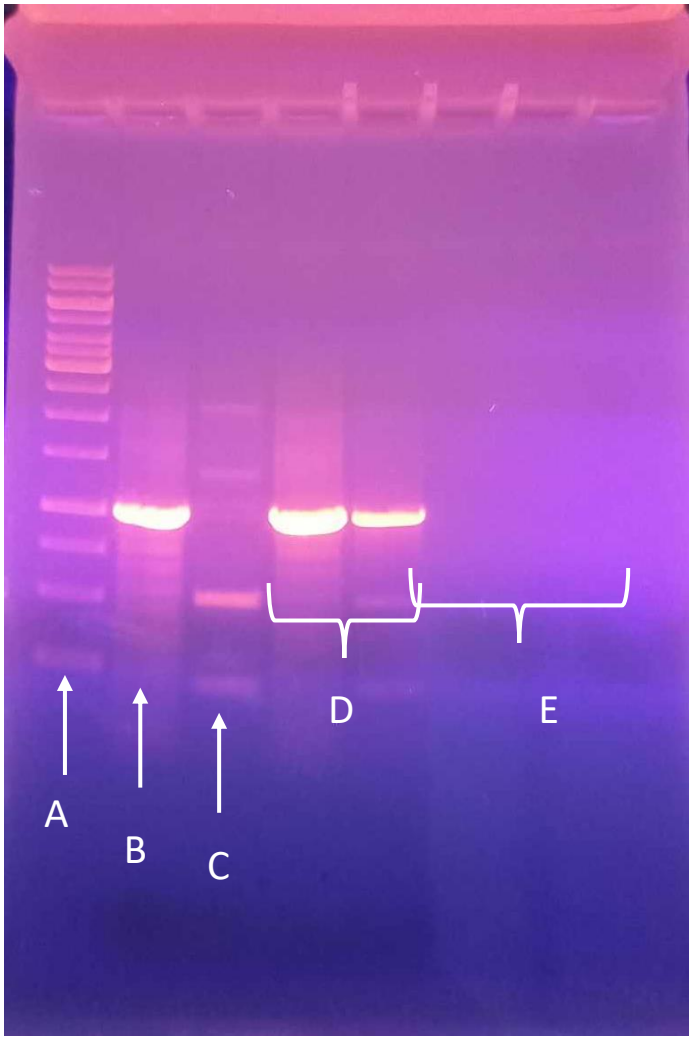

Figure 4 - Agarose gel electrophoresis of the class 1 integron on the selected transconjugants resulting from conjugation of the recipient *Acinetobacter baumannii* A118k with donor strains *Salmonella enterica* serovars Typhimurium Sal25 and 490S. A – DNA ladder 1kb; B – donor Sal25 (positive control); C – recipient strain A118K (negative control); D – transconjugants with donor Sal25 nº 14 and 17; E – transconjugants with donor 490S nº 14, 16 and 17.

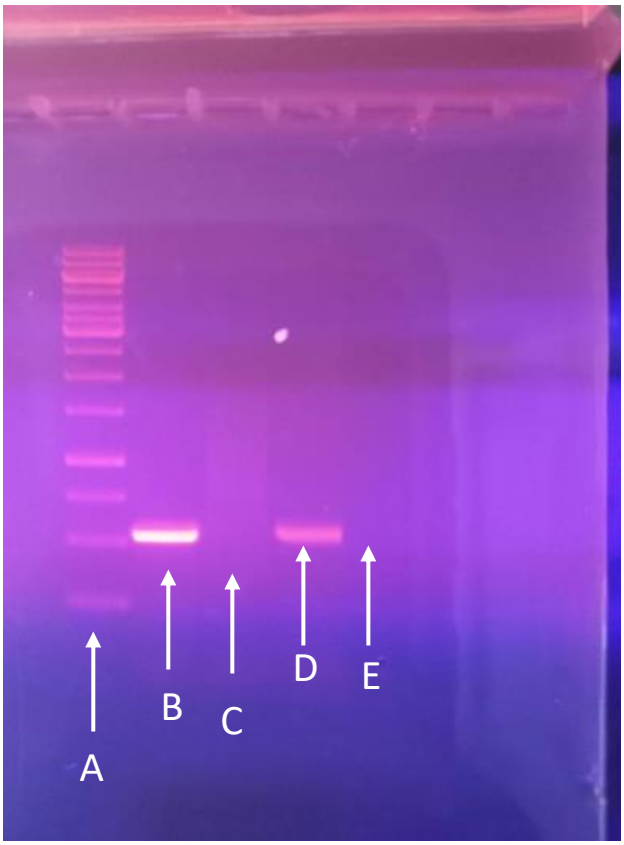

Figure 5 - Agarose gel electrophoresis of the *bla*$_{CTX\text{-}M\text{-}1}$ gene on the selected transconjugants resulting from conjugation of the recipient *Acinetobacter baumannii* A118k with donor strain *Salmonella enterica* serovar Typhimurium Sal25. A – DNA ladder 1kb; B – donor Sal25 (positive control); C – recipient strain A118K (negative control); D – transconjugant nº 17; E – transconjugant nº 14.

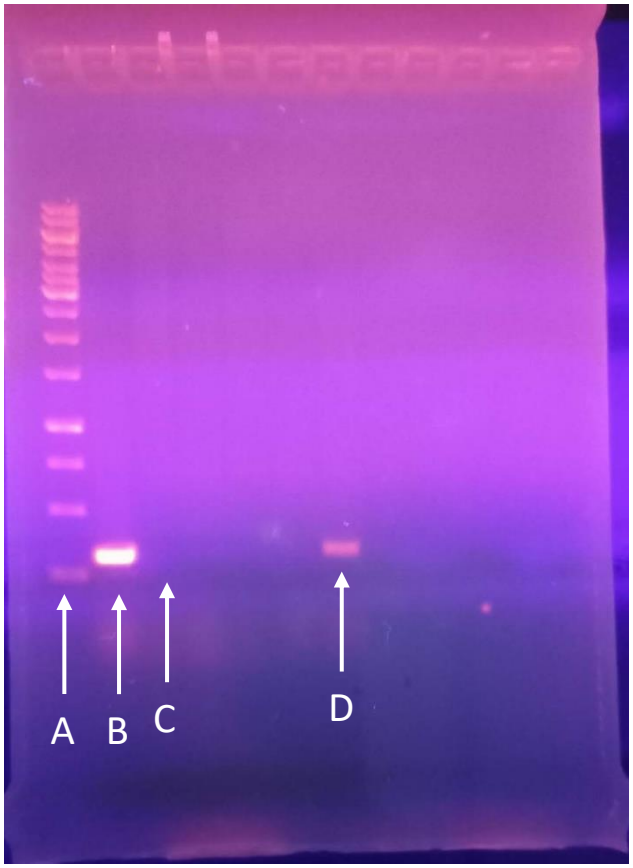

Figure 6 - Agarose gel electrophoresis of the *mcr-1* gene on the selected transconjugant resulting from conjugation of the recipient *Acinetobacter baumannii* A118k with donor strain *Salmonella enterica* serovar Typhimurium Sal25. A – DNA ladder 1kb; B – donor Sal25 (positive control); C – recipient strain A118K (negative control); D – transconjugant nº 17.

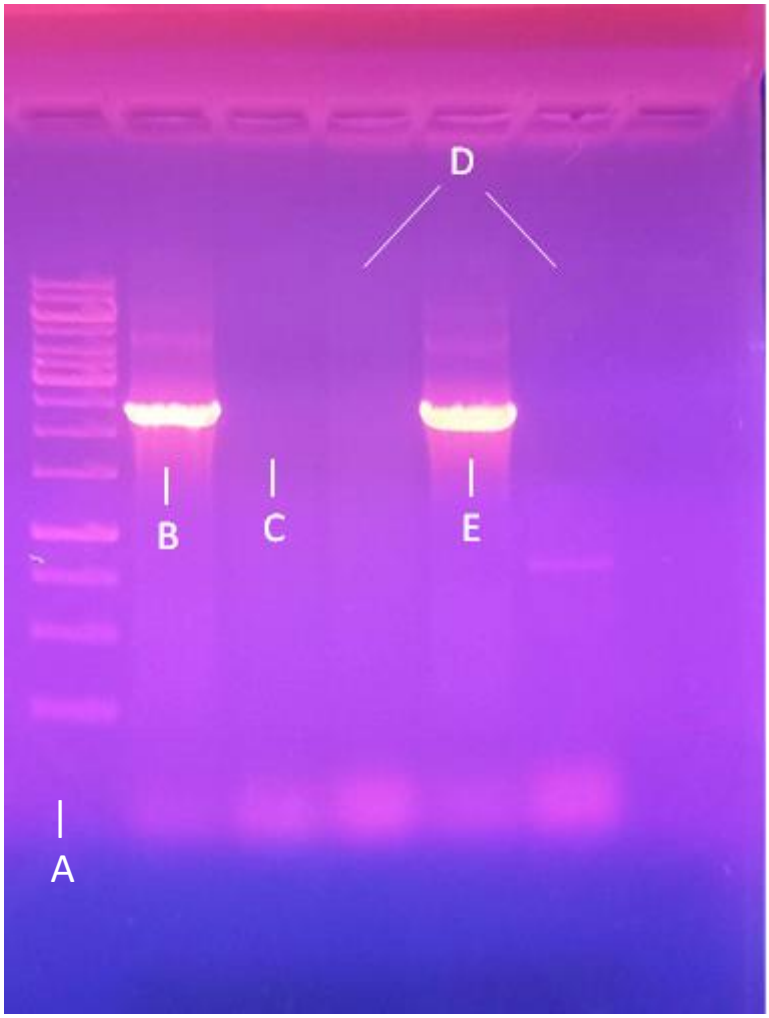

Figure 7 - Agarose gel electrophoresis of the kanamycin resistance marker on the selected transconjugant resulting from conjugation of the recipient *Acinetobacter baumannii* A118k with donor strains *Salmonella enterica* serovar Typhimurium Sal25. A – DNA ladder 1kb; B – recipient strain A118K (positive control); C – donor Sal25 (negative control); D - transconjugants with donor Sal25 nº 14 and 16; E – transconjugant with donor Sal25 nº 17.

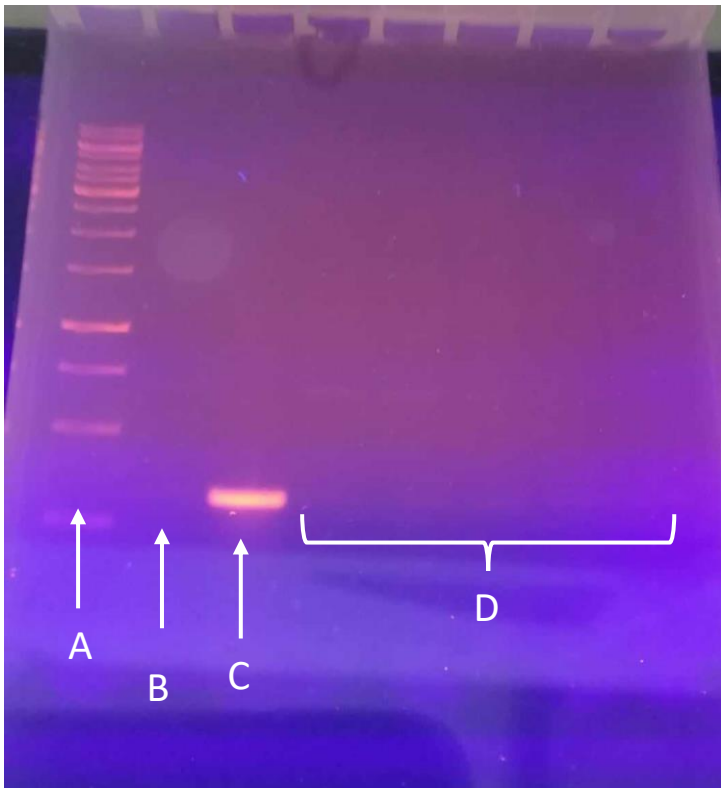

Figure 8 - Agarose gel electrophoresis of the *mcr-1* gene on the selected transconjugants resulting from conjugation of the recipient *Acinetobacter baumannii* A118k with donor strain *Escherichia coli*. Strain 189. A – DNA ladder 1kb; B – recipient strain A118K (negative control); C – donor strain 189 (positive control); D – transconjugants 1, 2, 3, 6 and 7.

Chapter 4 | Discussion

A. baumannii A118 is a naturally competent bacterium that can access foreign DNA through natural transformation. *A. baumannii* A118k is isogenic of A118 with the difference of the kanamycin resistance marker inserted into the chromosome. Both isolates were shown to acquired foreign DNA by natural transformation or conjugation, respectively, in this study.

Natural transformation of *A. baumannii* A118 with homologous DNA occurred at a frequency of $1{,}03*10^{-6}$ where intracellular acquisition relies on homologous recombination (Wilharm et 2013). In this study, DNA from genetically divergent species was used as donor DNA and lower transformation frequencies were obtained, as expected (Domingues *et al*, 2012 Plos Pathog). During transformation of *A. baumannii* A118 with the donor *S. enterica* serovar Typhimurium Sal25 under cefotaxime selective pressure, the transformants produced CFUs with a mucoid phenotype, similar to hydrogel in consistency, suggesting an effect of cefotaxime on transformants' synthesis of the peptidoglycan layer of the cell wall of the bacteria. The transformants that presented this phenotype did not acquire $bla_{\text{CTX-M-1}}$ gene nor the class 1 integron, nor insertion sequences close to the $bla_{\text{OXA-51}}$ or *ampC* intrinsic genes of *A. baumannii*. This result may suggest an altered expression of a different gene that was not targeted for amplification, which may not outright kill the bacteria but affects its synthesis of extracellular matrix. Cefotaxime, as a β-lactam antibiotic, has an effect on the synthesis of the peptidoglycan wall and leads to the synthesis of a new peptidoglycan which is less cross-linked with more pentapeptide side chains. This may lead to the extracellular matrix with a higher water retention capacity with this less-cross-linked polysaccharide chains that have increased moisture retention (Zhang *et al*., 2022b). Amongst other macromolecules, peptidoglycan is a constituent of biofilms, which have a high water retention, being made up of 90% of it in wet weight and has the function of protecting against environmental stress and antimicrobials, which can explain the high water-retention after exposure to cefotaxime (Abebe, 2020; Khan, Altaf & Ahmad, 2017). Alternatively, changes in the capsule of the cells may have occurred. *A. baumannii* nosocomial isolates with a mucoid phenotype were shown to present increased capsule thickness when compared with non-mucoid phenotypes (Hu *et al*., 2020). Antibiotic resistance was found to be unrelated to capsule thickness, as well as no correlation was found with the amount of biofilm produced by the mucoid strains (Hu *et al*., 2020). Formation of

mosaic penicillin-binding proteins genes (Reichmann *et al*., 1997), where parts of the *pbp* genes of the recipient replace sequences of the donor strain, could also explain the altered susceptibility to cefotaxime.

It has also been demonstrated that various genetic changes may occur after natural transformation and be in the origin of susceptibility alterations in transformant cells that are not explained by the acquisition of full genes or the formation of mosaic. In most studies the mosaic genes resulting from recombination events has occurred between same-species or same-genera interaction, however, changes in susceptibility can occur in cases of natural transformation with both related and unrelated donor species, and even when susceptibility changes occur, they may not be explained by major DNA acquisitions. It has been suggested that the alterations of the susceptibility profiles following transformation can be a result of smaller recombination events occurring in structural or regulatory genes not considered as related to resistance, or the transference of unknown traits (Domingues, Nielsen & Da Silva, 2012).

The natural transformation assay conducted with *S. enterica* serovar Typhimurium 490S donor strain was successful in the transmission of resistance, with a transformation frequency of $7{,}45 \times 10^{-8}$ transformants/recipients, within the range of previously obtained results. However, acquisition of integron was not successful in this work, as proven by the observation of the electrophoresis.

The class 1 integron of *S. enterica* serovar Typhimurium 490 has been shown to be transferred in transformation assays (Domingues, Nielsen, *et al*., 2012) to an *Acinetobacter* recipient, *A. baylyi*, suggesting natural transformation with strain 490S as donor and *A. baumannii* A118 should be possible. Following a different transformation protocol using nitrocellulose filters placed on agar surfaces as done by Domingues, Nielsen, *et al*. (Domingues, Nielsen, *et al*., 2012) can be tested in future assays.

Transformation of the recipient strain *Acinetobacter baumannii* strain A118 with the *E. coli* strain 189 was in the order of 10^{-8}, while previous studies have shown transformation of several *A. baumannii* recipient strains with a plasmid isolated from *E. coli* strain DH5α had a frequency ranging from 10^{-6} to 10^{-8} (Wilharm *et al*., 2013), which is consistent with the results obtained in this study.

The possible acquisition of the *mcr-1* gene from *E. coli* strain 189 (Lima *et al*., 2022) was evaluated through the determination of the colistin MIC by broth microdilution; the majority

of the transformants showed an elevated MIC, similar or even higher to the MIC of the donor strain. However, the PCR assays to assess the presence of the *mcr-1* gene were negative. Up to now, only the *mcr-5* gene was demonstrated to be transferred by natural transformation in *Aeromonas hydrophila* (Ma *et al*., 2018). Changes in the MIC of colistin may be explained by changes in the chromosomal genes associated with LPS production in *A. baumannii* (Da Silva & Domingues, 2017).

The substrate used in the natural transformation assays, the purified plasmid DNA, may have not be optimal for gene acquisition for this HGT mechanism, as DNA purification is known to lead to some degradation, and DNA released in the extracellular medium during growth or bacterial interaction renders better results comparatively, but when naked DNA was used, the frequencies were in the order of 10^{-8}, as well as not having statistically significant differences amongst each other (Godeaux *et al*., 2022). Nonetheless, the number of transformant CFUs obtained per µg of DNA were similar to the previous reported for the strain A118 (Ramirez *et al*., 2010).

Most of the transformants acquired through natural transformation of *A. baumannii* A118 with *S. enterica* serovar Typhimurium Sal25 and 490S and *E. coli* 189 donor DNA showed altered antimicrobial susceptibility to the antibiotics utilized for selective pressure, but the complete susceptibility profile of each transformant did not suggest the acquisition of the resistance genes or mobile genetic elements tested. The occurrence of random mutations during selection can be excluded, as there was a lack of growth in the negative controls of natural transformation.

Although disc diffusion is not the reference method to determine susceptibility to colistin, acquisition of the *mcr-1* from *S. enterica* serovar Typhimurium Sal25 was firstly screened by measurement of the growth inhibition diameter; slight differences in the halo could have been the result of this gene acquisition. Nonetheless, the tested transformants did not show significant changes in the growth halos nor acquired the *mcr-1* gene.

Transformation may also be influenced by environmental factors, as was verified with chemical transformation with a plasmidic vector, such as surfactant concentration, which at environmental concentrations can facilitate HGT, specifically by the production of reactive oxygen species that further increase cell membrane permeability (Wang *et al*., 2023).

Natural competence in *A. baumannii* could be increased by changing several parameters that are known to induce natural competence in other naturally competent bacteria, such as high cell density, antibiotic stress, DNA damage, intracellular growth, absence of preferred carbon sources and general starvation (Blokesch, 2016). Some studies where these environmental conditions are manipulated must be conducted to evaluate their impact on the transformation frequency in *A. baumannii*, and where these parameters can be employed to prevent the emergence of novel resistant strains as the result of natural transformation in this pathogen of critical concern.

The difference in the use of DNA from different species could have resulted in the low frequency of success of the HGT around 10^{-8}, which has been shown to vary from 10^{-5} to 10^{-9} per recipient cell in *A. baumannii*, when homologous DNA was used, and is supported by the higher frequency of 10^{-6} when the homologous DNA of *A. baumanni* strain 121.1 was used (Domingues *et al*., 2019; Godeaux *et al*., 2022)

Another important factor for the success of natural transformation could be the populations used for the natural transformation assays, as combinations of different pathogenic *Acinetobacter* strains result in higher efficiency of resistance acquisition (Godeux *et al*., 2022).

The conjugation of *A. baumannii* A118k with the *S. enterica* serovar Typhimurium strains Sal25 and 490S and *E. coli* 189 had similar conjugation frequencies in the order of 10^{-8}.

Conjugation of *S. enterica* serovar Typhimurium MDR strains has been successful in the past with a spontaneously antibiotic resistant *E. coli* strain, *E. coli* K-12 strain MG1655, with varying degrees of plasmid DNA transfer depending on the resistance profile of the donor strains used, where the resistance genes were contained in conjugative plasmids (Gebreyes & Altier, 2002). This was also verified with class 1 integrons embedded into plasmids that were transferred via conjugation from *S. enterica* serovar Typhimurium to recipients of the same species and serovar (Hradecka, Karasova & Rychlik, 2008).

Conjugation frequencies in *A. baumannii* obtained in previous research are overall higher than the ones obtained here, raging between the values of $10^{-1} – 10^{-7}$ (Zhang ***et al***., 2022a; Khongfak ***et al***., 2022). Given the fact that the procedure used differed from the one in this study, where recipient and donor strains were both *A. baumannii* strains incubated in the same proportion (L. Zhang *et al*., 2022; Khongfak ***et al***., 2022), unlike the 10:1 used in this study, the increase of the amount of donor strain may have influenced the outcome.

The frequency of conjugation obtained in the past with an *E. coli* as donor was substantially higher than the ones obtained in these assays, in the range of 10^{-1} to 10^{-3} and 10^{-6} to 10^{-1} transconjugants/recipients, but the recipients were Gram-negative bacteria different from *A. baumannii*, having the results in this experiment be below what was expected (Thoma & Schobert, 2009; Liu *et al*., 2016).

The evaluation of the antimicrobial susceptibility profile of transconjugants suggested different outcomes depending on the donor species. Transconjugants from *S. enterica* serovar Typhimurium 490S did not present changes in the susceptibility profile that suggested acquisition of the class 1 integron. Transconjugants from *S. enterica* serovar Typhimurium Sal25 showed different susceptibility profiles; while some did not suggest the acquisition of resistance genes or mobile genetic elements, two were resistant to spectinomycin and streptomycin, two were resistant to spectinomycin, streptomycin, sulphonamide compounds and cefotaxime (suggestive of acquisition of the class 1 integron-carrying *aadA1* and $bla_{\text{CTX-M-1}}$), and resistance to colistin mediated by the *mcr-1* gene, as confirmed in the electrophoresis, and one was resistant to spectinomycin, streptomycin and sulphonamide compounds (suggestive of acquisition of the class 1 integron-carrying *aadA1*), in addition to the kanamycin resistance of the recipient cell. Finally, transconjugants of the *E. coli* 189 had increased colistin MICs, which could be compatible with the *mcr-1* gene acquisition.

S. enterica serovar Typhimurium 490S was not previously tested in conjugation assays. Therefore, the absence of transfer of the 490S' plasmid may be due to a non-conjugative nature of the plasmid. The *E. coli* 189 strain was previously shown to transfer the *mcr-1* and $bla_{\text{CTX-M-1}}$ genes by conjugation to *E. coli* J53 strain, where were successfully transferred (Lima *et al*., 2022). However, in this study, the *mcr*-1-embedded plasmid from E*. coli* 189 was not transferred to *A. baumannii* A118k, as demonstrated by the absence of amplification of the *mcr-1* gene in PCR. The used protocol of conjugation can be further optimized in order to increase the conjugation rate as well as the successful transfer of the resistance genes with *E. coli* as the donor strain, as there is a small-time window for conjugation when it happens under growing conditions after the mixing of the donor and recipient strains (Headd & Bradford, 2020). In the past, conjugation frequencies with *E. coli* as donor strain resulted in a higher frequency of the results observed in the range of 10^{-7} to 10^{-2} cells per recipient, but obtained with another *E. coli* bacterium as recipient, not consistent with the results obtained, which were in the order of 10^{-8} with *A. baumannii* as recipient (Lima *et al*., 2022).

In the conjugation with *S. enterica* serovar Typhimurium Sal25, transfer of plasmid-mediated resistance genes was verified in one transconjugant. The transconjugant number 17 had the kanamycin resistance marker of the recipient strain and acquired the resistance genes $bla_{CTX\text{-}M\text{-}1}$ and *mcr-1* and the class 1 integron carrying the *aadaA1* gene cassette. This result proves that interspecies conjugation is possible with *Acinetobacter baumannii* A118k as recipient and *Salmonella enterica* serovar Typhimurium Sal25 as donor, despite this may be a rare event.

It is of note that, despite the acquisition if the *mcr-1* gene by transconjugant nº 17 the inhibition growth diameter obtained with the colistin disc did not show a change when compared with the recipient or other transconjugants that did not acquire the gene, highlighting why this method is not recommended to evaluate the susceptibility profile to colistin.

Conjugation has been possible in the past to horizontally transfer the *mcr-1* gene, though both recipient and donor strains were *E. coli* SPH45 and C600, respectively (Liu *et al*., 2016).

Although the transfer of resistance genes in plasmids through conjugation is relatively common among *Enterobacteriaceae* (Gebreyes & Altier, 2002; Hradecka, Karasova & Rychlik, 2008), transfer by this method to *A. baumannii* seems rare. Different environmental conditions can also be changed in future assays, such as temperature, which can have a positive or negative effect on the result of conjugation (Hradecka, Karasova & Rychlik, 2008).

The transformation and conjugation frequencies for the recipients A118 and A118k were all in the order of 10^{-8} values, regardless of the donor strain used or if the DNA used in the transformation was genomic or plasmidic. When the transformation and conjugation frequencies were compared on a T-test of independent samples, as each assay used genomic material from different donor strains for the natural transformation assays, and the same donor strains for the conjugation assays were used, no significant differences were detected. Even though the values obtained were different from transformation to conjugation when the same donor strain was used, all the *P* values were above the limit of 0,05, meaning all the null hypothesis of inexistent significant differences were accepted. Overall, natural transformation has been considered the main driver of HGT of antibiotic resistance genes in *A. baumannii* (Godeaux *et al*., 2022), and has been verified in the case of last-line carbapenem antibiotic resistance, but the results of this thesis do not support this idea. Nonetheless, although these results suggest there is no significant differences amongst different HGT mechanisms using the same donors, a higher number of repeated assays and more recipient and donor strains

should be used to attain a wider variety of data for better assessment of HGT mechanisms in *A. baumannii* and how they impact the acquisition of resistance.

Even with protocols matching the natural transformation and conjugation occurrence in bacteria, these genes may not be able to be passed at all with the methodology and the strains used, as disparity between species can lead to different results with different HGT methods and protocols (Zhou, Beltrán & Brito, 2021). The change of environmental conditions during the processes of HGT may lead to different results, so performing alterations in the methodologies should be tested in future studies.

Despite the acquisition of transformants and transconjugants, the detection of acquired resistance genes was very scarce. Whole genome sequencing, which can predict antimicrobial phenotypes it is still not sufficient to take over conventional antimicrobial susceptibility testing (Kumburu *et al*., 2021). Nonetheless, this method can potentially further clarify the genetic changes of transformants and transconjugants with already identified antimicrobial susceptibility alterations.

The observed low transformation and conjugation frequencies may also not be a clear representation of the frequency at which HGT occurred with the given conditions, since HGT is the result of the integration of new DNA into the host's genome and the chromosomal regions may be disrupted, and fitness could be lowered in specific conditions. The synthesis of new DNA could have had a potential cost in bacterial replication, as the available energy and building blocks directed towards the extra synthesis could represent a setback, and certain critical housekeeping processes could be affected with the sequestering of the cellular machinery by the extra genomic regions, so HGT may not have been observed (Baltrus, 2013). Hence registered resistance without the presence of the targeted genes may be considered unsuccessful HGT, as resistance may have been indeed induced by the DNA that entered the recipient cells, but the negative results of the electrophoresis proved most of the genes were not maintained after their internalization and momentary expression, thus cannot be referred to as HGT.

In conclusion, the tested donor strains against the single recipient strain resulted in a very narrow proof of interspecies transfer of resistance by the studied HGT, requiring more assays with a wider variety of recipient and donor strains, as well as changing parameters on the protocols depending on the strains used to improve the obtained frequencies. This would lead

to a better understanding of the emergence of resistance in pathogenic strains both inside and outside of the healthcare environment and to study ways to avoid its increase in the future.

References:

Abdi, S. N., Ghotaslou, R., Ganbarov, K., Mobed, A., Tanomand, A., Yousefi, M., Asgharzadeh, M. and Kafil, H. S. (2020) '*Acinetobacter baumannii* Efflux Pumps and Antibiotic Resistance', *Infection and Drug Resistance,* 13, pp. 423-434.

Abebe, G. M. (2020) 'The Role of Bacterial Biofilm in Antibiotic Resistance and Food Contamination', *International Journal of Microbiology,* 2020, pp. 1705814.

Adams-Haduch, J. M., Paterson, D. L., Sidjabat, H. E., Pasculle, A. W., Potoski, B. A., Muto, C. A., Harrison, L. H. and Doi, Y. (2008) 'Genetic Basis of Multidrug Resistance in *Acinetobacter baumannii* Clinical Isolates at a Tertiary Medical Center in Pennsylvania', *Antimicrobial Agents and Chemotherapy,* 52(11), pp. 3837-3843.

Ahmadian, L., Haghshenas, M. R., Mirzaei, B., Norouzi Bazgir, Z. and Goli, H. R. (2020) 'Distribution and Molecular Characterization of Resistance Gene Cassettes Containing Class 1 Integrons in Multi-Drug Resistant (MDR) Clinical Isolates of *Pseudomonas aeruginosa*', *Infection and Drug Resistance,* 13, pp. 2773-2781.

Al-Kadmy, I. M. S., Ali, A. N. M., Salman, I. M. A. and Khazaal, S. S. (2018) 'Molecular characterization of *Acinetobacter baumannii* isolated from Iraqi hospital environment', *New Microbes and New Infections,* 21, pp. 51-57.

Al-Kadmy, I. M. S., Ibrahim, S. A., Al-Saryi, N., Aziz, S. N., Besinis, A. and Hetta, H. F. (2020) 'Prevalence of Genes Involved in Colistin Resistance in *Acinetobacter baumannii*: First Report from Iraq', *Microbial Drug Resistance,* 26(6), pp. 616-622.

AlAmri, A. M., AlQurayan, A. M., Sebastian, T. and AlNimr, A. M. (2020) 'Molecular Surveillance of Multidrug-Resistant *Acinetobacter baumannii*', *Current Microbiology,* 77(3), pp. 335-342.

Almutairi, M. M. (2022) 'Synergistic activities of colistin combined with other antimicrobial agents against colistin-resistant *Acinetobacter baumannii* clinical isolates', *PLoS One,* 17(7), pp. e0270908.

Aly, M., Tayeb, H. T., Al Johani, S. M., Alyamani, E. J., Aldughaishem, F., Alabdulkarim, I. and Balkhy, H. H. (2014) 'Genetic diversity of *OXA-51-like* genes among multidrug-resistant *Acinetobacter baumannii* in Riyadh, Saudi Arabia', *European Journal of Clinical Microbiology & Infectious Diseases,* 33(7), pp. 1223-1228.

Andrade, F. F., Silva, D., Rodrigues, A. and Pina-Vaz, C. (2020) 'Colistin Update on Its Mechanism of Action and Resistance, Present and Future Challenges', *Microorganisms,* 8(11).

Anthony, K. B., Fishman, N. O., Linkin, D. R., Gasink, L. B., Edelstein, P. H. and Lautenbach, E. (2008) 'Clinical and Microbiological Outcomes of Serious Infections with Multidrug-Resistant Gram-Negative Organisms Treated with Tigecycline', *Clinical Infectious Diseases,* 46(4), pp. 567-570.

Arnold, B. J., Huang, I. T. and Hanage, W. P. (2022) 'Horizontal gene transfer and adaptive evolution in bacteria', *Nature Reviews Microbiology,* 20(4), pp. 206-218.

Aruna, N., Archana, K., Reena, J. and Swaraj, B. (2015) 'Colistin neurotoxicity: revisited', *BMJ Case Reports,* 2015, pp. bcr2015210787.

Avedissian, S. N., Liu, J., Rhodes, N. J., Lee, A., Pais, G. M., Hauser, A. R. and Scheetz, M. H. (2019) 'A Review of the Clinical Pharmacokinetics of Polymyxin B', *Antibiotics,* 8(1), pp. 31.

Baltrus, D. A. (2013) 'Exploring the costs of horizontal gene transfer', *Trends in Ecology & Evolution,* 28(8), pp. 489-495.

Baltrus, D. A., Guillemin, K. and Phillips, P. C. (2008) 'Natural Transformation Increases the Rate of Adaptation in the Human Pathogen *Helicobacter pylori*', *Evolution,* 62(1), pp. 39-49.

Ban, K. A., Minei, J. P., Laronga, C., Harbrecht, B. G., Jensen, E. H., Fry, D. E., Itani, K. M. F., Dellinger, P. E., Ko, C. Y. and Duane, T. M. (2017) 'American College of Surgeons and Surgical Infection Society: Surgical Site Infection Guidelines, 2016 Update', *Journal of the American College of Surgeons,* 224(1), pp. 59-74.

Baumann, P. (1968) 'Isolation of *Acinetobacter* from Soil and Water', *Journal of Bacteriology,* 96(1), pp. 39-42.

Baumann, P., Doudoroff, M. and Stanier, R. Y. (1968) 'A Study of the *Moraxella* Group II. Oxidative-negative Species (Genus *Acinetobacter*)', *Journal of Bacteriology,* 95(5), pp. 1520-1541.

Beijerinck, M. (1911) 'Pigmenten als oxydatieproducten gevormd door bacterien', *Versl Koninklijke Akad Wetensch Amsterdam,* 19, pp. 1092-103.

Bergogne-Bérézin, E. and Towner, K. J. (1996) '*Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features', *Clinical Microbiology Reviews,* 9(2), pp. 148-65.

Berlau, J., Aucken, H. M., Houang, E. and Pitt, T. L. (1999) 'Isolation of *Acinetobacter* spp including *A. baumannii* from vegetables: implications for hospital-acquired infections', *Journal of Hospital Infection,* 42(3), pp. 201-204.

Bhattacharya, M., Parakh, A. and Narang, M. (2009) 'Tigecycline', *Journal of Postgraduate Medicine,* 55(1), pp. 65-8.

Biemer, J. J. (1973) 'Antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method', *Annals of clinical laboratory science,* 3(2), pp. 135-40.

Birnboim, H. C. and Doly, J. (1979) 'A rapid alkaline extraction procedure for screening recombinant plasmid DNA', *Nucleic Acids Research,* 7(6), pp. 1513-1523.

Blokesch, M. (2016) 'Natural competence for transformation', *Current Biology,* 26(21), pp. R1126-R1130.

Bogaerts, P., Naas, T., El Garch, F., Cuzon, G., Deplano, A., Delaire, T., Huang, T., Lissoir, B., Nordmann, P. and Glupczynski, Y. (2010) 'GES Extended-Spectrum Beta-Lactamases in *Acinetobacter baumannii* Isolates in Belgium', *Antimicrobial Agents and Chemotherapy,* 54(11), pp. 4872-4878.

Burmeister, A. R. (2015) 'Horizontal Gene Transfer', *Evolution, Medicine, and Public Health,* 2015(1), pp. 193-194.

Cabezón, E., Ripoll-Rozada, J., Peña, A., de la Cruz, F. and Arechaga, I. (2014) 'Towards an integrated model of bacterial conjugation', *FEMS Microbiology Reviews,* 39(1), pp. 81-95.

Cai, Y., Chai, D., Wang, R., Liang, B. and Bai, N. (2012) 'Colistin resistance of *Acinetobacter baumannii*: clinical reports, mechanisms and antimicrobial strategies', *Journal of Antimicrobial Chemotherapy,* 67(7), pp. 1607-1615.

Casal, M., Rodríguez, F., Johnson, B., Garduno, E., Tubau, F., de Lejarazu, R. O., Tenorio, A., Giménez, M. J., Bartolomé, R., Garcia-Rey, C., Aguilar, L. and García-Escribano, N. (2009) 'Influence of testing

methodology on the tigecycline activity profile against presumably tigecycline-non-susceptible *Acinetobacter* spp', *Journal of Antimicrobial Chemotherapy,* 64(1), pp. 69-72.

Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., Colomb-Cotinat, M., Kretzschmar, M. E., Devleesschauwer, B., Cecchini, M., Ouakrim, D. A., Oliveira, T. C., Struelens, M. J., Suetens, C., Monnet, D. L., Strauss, R., Mertens, K., Struyf, T., Catry, B., Latour, K., Ivanov, I. N., Dobreva, E. G., Tambic Andraševic, A., Soprek, S., Budimir, A., Paphitou, N., Žemlicková, H., Schytte Olsen, S., Wolff Sönksen, U., Märtin, P., Ivanova, M., Lyytikäinen, O., Jalava, J., Coignard, B., Eckmanns, T., Abu Sin, M., Haller, S., Daikos, G. L., Gikas, A., Tsiodras, S., Kontopidou, F., Tóth, Á., Hajdu, Á., Guólaugsson, Ó., Kristinsson, K. G., Murchan, S., Burns, K., Pezzotti, P., Gagliotti, C., Dumpis, U., Liuimiene, A., Perrin, M., Borg, M. A., de Greeff, S. C., Monen, J. C. M., Koek, M. B. G., Elstrøm, P., Zabicka, D., Deptula, A., Hryniewicz, W., Caniça, M., Nogueira, P. J., Fernandes, P. A., Manageiro, V., Popescu, G. A., Serban, R. I., Schréterová, E., Litvová, S., Štefkovicová, M., Kolman, J., Klavs, I., Korošec, A., Aracil, B., Asensio, A., Pérez-Vázquez, M., Billström, H., Larsson, S., Reilly, J. S., Johnson, A. and Hopkins, S. (2019) 'Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis', *The Lancet Infectious Diseases,* 19(1), pp. 56-66.

Castanheira, M., Costello, S. E., Woosley, L. N., Deshpande, L. M., Davies, T. A. and Jones, R. N. (2014) 'Evaluation of Clonality and Carbapenem Resistance Mechanisms among *Acinetobacter baumannii-Acinetobacter calcoaceticus* Complex and Enterobacteriaceae Isolates Collected in European and Mediterranean Countries and Detection of Two Novel Beta-Lactamases, GES-22 and VIM-35', *Antimicrobial Agents and Chemotherapy,* 58(12), pp. 7358-7366.

Catchpole, C. R., Andrews, J. M., Brenwald, N. and Wise, R. (1997) 'A reassessment of the in-vitro activity of colistin sulphomethate sodium', *Journal of Antimicrobial Chemotherapy,* 39(2), pp. 255-260.

CDC, C. f. D. C. a. P. (2004) '*Acinetobacter baumannii* infections among patients at military medical facilities treating injured U.S. service members, 2002-2004', *MMWR Morb Mortal Wkly Rep,* 53(45), pp. 1063-6.

Cerezales, M., Xanthopoulou, K., Wille, J., Krut, O., Seifert, H., Gallego, L. and Higgins, P. G. (2020) 'Mobile Genetic Elements Harboring Antibiotic Resistance Determinants in *Acinetobacter baumannii* Isolates From Bolivia', *Frontiers in Microbiology,* 11.

Chapartegui-González, I., Lázaro-Díez, M., Redondo-Salvo, S., Navas, J. and Ramos-Vivas, J. (2021) 'Antimicrobial Resistance Determinants in Genomes and Plasmids from *Acinetobacter baumannii* Clinical Isolates', *Antibiotics,* 10(7), pp. 753.

Chatterjee, S., Mondal, A., Mitra, S. and Basu, S. (2017) '*Acinetobacter baumannii* transfers the bla_{NDM-1} gene via outer membrane vesicles', *The Journal of antimicrobial chemotherapy,* 72(8), pp. 2201-2207.

Chen, Y., Zhou, Z., Jiang, Y. and Yu, Y. (2011) 'Emergence of *NDM-1*-producing *Acinetobacter baumannii* in China', *Journal of Antimicrobial Chemotherapy,* 66(6), pp. 1255-1259.

Chereau, F., Opatowski, L., Tourdjman, M. and Vong, S. (2017) 'Risk assessment for antibiotic resistance in South East Asia', *Bmj,* 358, pp. j3393.

Chew, K. L., La, M., Lin, R. T. P. and Teo, J. W. P. (2017) 'Colistin and Polymyxin B Susceptibility Testing for Carbapenem-Resistant and *mcr*-Positive Enterobacteriaceae: Comparison of Sensititre, MicroScan, Vitek 2, and Etest with Broth Microdilution', *Journal of Clinical Microbiology,* 55(9), pp. 2609-2616.

Chim, H., Tan, B. H. and Song, C. (2007) 'Five-year review of infections in a burn intensive care unit: High incidence of *Acinetobacter baumannii* in a tropical climate', *Burns,* 33(8), pp. 1008-1014.

Cho, H., Sondak, T. and Kim, K. (2023) 'Characterization of Increased Extracellular Vesicle-Mediated Tigecycline Resistance in *Acinetobacter baumannii*', *Pharmaceutics,* 15(4), pp. 1251.

Christie, P. J. (2001) 'Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines', *Molecular microbiology,* 40(2), pp. 294-305.

Chusri, S., Chongsuvivatwong, V., Silpapojakul, K., Singkhamanan, K., Hortiwakul, T., Charernmak, B. and Doi, Y. (2019) 'Clinical characteristics and outcomes of community and hospital-acquired *Acinetobacter baumannii* bacteremia', *Journal of Microbiology, Immunology and Infection,* 52(5), pp. 796-806.

Codjoe, F. S. and Donkor, E. S. (2018) 'Carbapenem Resistance: A Review', *Medical Sciences,* 6(1), pp. 1.

Corbella, X., Ariza, J., Ardanuy, C., Vuelta, M., Tubau, F., Sora, M., Pujol, M. and Gudiol, F. (1998) 'Efficacy of sulbactam alone and in combination with ampicillin in nosocomial infections caused by multiresistant *Acinetobacter baumannii*', *Journal of Antimicrobial Chemotherapy,* 42(6), pp. 793-802.

Coyne, S., Courvalin, P. and Périchon, B. (2011) 'Efflux-Mediated Antibiotic Resistance in *Acinetobacter* spp', *Antimicrobial Agents and Chemotherapy,* 55(3), pp. 947-953.

Da Silva, G. J. and Domingues, S. (2016) 'Insights on the Horizontal Gene Transfer of Carbapenemase Determinants in the Opportunistic Pathogen *Acinetobacter baumannii*', *Microorganisms,* 4(3), pp. 29.

Da Silva, G. J. and Domingues, S. (2017) 'Interplay between Colistin Resistance, Virulence and Fitness in *Acinetobacter baumannii*', *Antibiotics,* 6(4), pp. 28.

Datta, N. and Kontomichalou, P. (1965) 'Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae', *Nature,* 208(5007), pp. 239-41.

Davies, O. L. and Bennett, S. (2017) *WHO publishes list of bacteria for which new antibiotics are urgently needed*. WHO Newsletters.

De Boever, P., Mergeay, M., Ilyin, V., Forget-Hanus, D., Van der Auwera, G. and Mahillon, J. (2007) 'Conjugation-mediated plasmid exchange between bacteria grown under space flight conditions', *Microgravity Science and Technology,* 19(5), pp. 138-144.

De Francesco, M. A., Ravizzola, G., Peroni, L., Bonfanti, C. and Manca, N. (2013) 'Prevalence of multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in an Italian hospital', *Journal of Infection and Public Health,* 6(3), pp. 179-185.

De Oliveira, D. M. P., Forde, B. M., Kidd, T. J., Harris, P. N. A., Schembri, M. A., Beatson, S. A., Paterson, D. L. and Walker, M. J. (2020) 'Antimicrobial Resistance in ESKAPE Pathogens', *Clinical Microbiology Reviews,* 33(3), pp. e00181-19.

Dell'Annunziata, F., Folliero, V., Giugliano, R., De Filippis, A., Santarcangelo, C., Izzo, V., Daglia, M., Galdiero, M., Arciola, C. R. and Franci, G. (2021) 'Gene Transfer Potential of Outer Membrane Vesicles of Gram-Negative Bacteria', *International Journal of Molecular Sciences,* 22(11), pp. 5985.

Deng, Y., Bao, X., Ji, L., Chen, L., Liu, J., Miao, J., Chen, D., Bian, H., Li, Y. and Yu, G. (2015) 'Resistance integrons: class 1, 2 and 3 integrons', *Annals of Clinical Microbiology and Antimicrobials,* 14(1), pp. 45.

Di Venanzio, G., Flores-Mireles, A. L., Calix, J. J., Haurat, M. F., Scott, N. E., Palmer, L. D., Potter, R. F., Hibbing, M. E., Friedman, L., Wang, B., Dantas, G., Skaar, E. P., Hultgren, S. J. and Feldman, M. F. (2019) 'Urinary tract colonization is enhanced by a plasmid that regulates uropathogenic *Acinetobacter baumannii* chromosomal genes', *Nature Communications,* 10(1), pp. 2763.

Dijkshoorn, L., Aucken, H., Gerner-Smidt, P., Janssen, P., Kaufmann, M. E., Garaizar, J., Ursing, J. and Pitt, T. L. (1996) 'Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods', *Journal of Clinical Microbiology,* 34(6), pp. 1519-1525.

Domingues, S., Harms, K., Fricke, W. F., Johnsen, P. J., Da Silva, G. J. and Nielsen, K. M. (2012) 'Natural transformation facilitates transfer of transposons, integrons and gene cassettes between bacterial species', *PLoS Pathog,* 8(8), pp. e1002837.

Domingues, S., Lima, T., Saavedra, M. J. and Da Silva, G. J. (2023) 'An Overview of Cefiderocol's Therapeutic Potential and Underlying Resistance Mechanisms', *Life,* 13(7), pp. 1427.

Domingues, S., Nielsen, K. M. and Da Silva, G. J. (2012) 'Various pathways leading to the acquisition of antibiotic resistance by natural transformation', *Mobile Genetic Elements,* 2(6), pp. 257-260.

Domingues, S., Rosário, N., Cândido, Â., Neto, D., Nielsen, K. M. and Da Silva, G. J. (2019) 'Competence for Natural Transformation Is Common among Clinical Strains of Resistant *Acinetobacter* spp', *Microorganisms,* 7(2), pp. 30.

Dong, J., Liu, C., Wang, P., Li, L. and Zou, Q. (2022) 'The type VI secretion system in *Acinetobacter baumannii* clinical isolates and its roles in antimicrobial resistance acquisition', *Microbial Pathogenesis,* 169, pp. 105668.

Durante-Mangoni, E., Utili, R. and Zarrilli, R. (2014) 'Combination therapy in severe *Acinetobacter baumannii* infections: an update on the evidence to date', *Future Microbiol,* 9(6), pp. 773-89.

Edelstein, M., Pimkin, M., Palagin, I., Edelstein, I. and Stratchounski, L. (2003) 'Prevalence and molecular epidemiology of *CTX-M* extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals', *Antimicrob Agents Chemother,* 47(12), pp. 3724-32.

El-Kazzaz, W., Metwally, L., Yahia, R., Al-Harbi, N., El-Taher, A. and Hetta, H. F. (2020) 'Antibiogram, Prevalence of *OXA* Carbapenemase Encoding Genes, and RAPD-Genotyping of Multidrug-Resistant *Acinetobacter baumannii* Incriminated in Hidden Community-Acquired Infections', *Antibiotics*, 9(9). DOI: 10.3390/antibiotics9090603.

Eliopoulos, G. M., Maragakis, L. L. and Perl, T. M. (2008) '*Acinetobacter baumannii*: Epidemiology, Antimicrobial Resistance, and Treatment Options', *Clinical Infectious Diseases,* 46(8), pp. 1254-1263.

Escudero*, J. A., Loot*, C., Nivina, A. and Mazel, D. (2015) 'The Integron: Adaptation On Demand', *Microbiology Spectrum,* 3(2), pp. 3.2.10.

Etowski, D. C. and Beckwith, D. G. (1981) 'Performance of a Commercial Microdilution Minimal Inhibitory Concentration Procedure for Aminoglycoside Susceptibility Testing of *Pseudomonas aeruginosa*', *American Journal of Clinical Pathology,* 75(6), pp. 830-833.

Evans, B. A., Hamouda, A., Towner, K. J. and Amyes, S. G. B. (2008) '*OXA-51-like* β-lactamases and their association with particular epidemic lineages of *Acinetobacter baumannii*', *Clinical Microbiology and Infection,* 14(3), pp. 268-275.

Eveillard, M., Kempf, M., Belmonte, O., Pailhoriès, H. and Joly-Guillou, M. (2013) 'Reservoirs of *Acinetobacter baumannii* outside the hospital and potential involvement in emerging human community-acquired infections', *International Journal of Infectious Diseases,* 17(10), pp. e802-e805.

Falagas, M. E., Rafailidis, P. I., Ioannidou, E., Alexiou, V. G., Matthaiou, D. K., Karageorgopoulos, D. E., Kapaskelis, A., Nikita, D. and Michalopoulos, A. (2010) 'Colistin therapy for microbiologically documented multidrug-resistant Gram-negative bacterial infections: a retrospective cohort study of 258 patients', *Int J Antimicrob Agents,* 35(2), pp. 194-9.

Figueiredo, R., Card, R. M., Nunez, J., Pomba, C., Mendonça, N., Anjum, M. F. and Da Silva, G. J. (2016) 'Detection of an *mcr-1*-encoding plasmid mediating colistin resistance in *Salmonella enterica* from retail meat in Portugal', *Journal of Antimicrobial Chemotherapy,* 71(8), pp. 2338-2340.

Figueiredo, R., Card, R. M., Nunez-Garcia, J., Mendonça, N., da Silva, G. J. and Anjum, M. F. (2019) 'Multidrug-Resistant *Salmonella enterica* Isolated from Food Animal and Foodstuff May Also Be Less Susceptible to Heavy Metals', *Foodborne Pathog Dis,* 16(3), pp. 166-172.

Antimicrobial susceptibility of Salmonella enterica serovars from retail meat: first report CTX-M β-lactamase in Portugal [Poster].

Figueiredo, R., Henriques, A., Sereno, R., Mendonça, N. and da Silva, G. J. (2015) 'Antimicrobial resistance and extended-spectrum β-lactamases of *Salmonella enterica* serotypes isolated from livestock and processed food in Portugal: an update', *Foodborne Pathog Dis,* 12(2), pp. 110-7.

Fondi, M., Bacci, G., Brilli, M., Papaleo, M. C., Mengoni, A., Vaneechoutte, M., Dijkshoorn, L. and Fani, R. (2010) 'Exploring the evolutionary dynamics of plasmids: the *Acinetobacter* pan-plasmidome', *BMC Evolutionary Biology,* 10(1), pp. 59.

Fournier, P. E., Vallenet, D., Barbe, V., Audic, S., Ogata, H., Poirel, L., Richet, H., Robert, C., Mangenot, S., Abergel, C., Nordmann, P., Weissenbach, J., Raoult, D. and Claverie, J. M. (2006) 'Comparative genomics of multidrug resistance in *Acinetobacter baumannii*', *PLoS Genetics,* 2(1), pp. e7.

Fragkou, P. C., Poulakou, G., Blizou, A., Blizou, M., Rapti, V., Karageorgopoulos, D. E., Koulenti, D., Papadopoulos, A., Matthaiou, D. K. and Tsiodras, S. (2019) 'The Role of Minocycline in the Treatment of Nosocomial Infections Caused by Multidrug, Extensively Drug and Pandrug Resistant *Acinetobacter baumannii*: A Systematic Review of Clinical Evidence', *Microorganisms,* 7(6), pp. 159.

Fu, Y., Jiang, J., Zhou, H., Jiang, Y., Yu, Y. and Zhou, J. (2014) 'Characterization of a novel plasmid type and various genetic contexts of bla_{OXA-58} in *Acinetobacter* spp. from multiple cities in China', *PLoS One,* 9(1), pp. e84680.

Fulsundar, S., Domingues, S. and Nielsen, K. M. (2019) 'Vesicle-Mediated Gene Transfer in *Acinetobacter baumannii*', *Methods Mol Biol,* 1946, pp. 87-94.

Fulsundar, S., Harms, K., Flaten, G. E., Johnsen, P. J., Chopade, B. A. and Nielsen, K. M. (2014) 'Gene transfer potential of outer membrane vesicles of *Acinetobacter baylyi* and effects of stress on vesiculation', *Applied and Environmental Microbiology,* 80(11), pp. 3469-83.

Gajdács, M., Urbán, E., Stájer, A. and Baráth, Z. (2021) 'Antimicrobial Resistance in the Context of the Sustainable Development Goals: A Brief Review', *European Journal of Investigation in Health, Psychology and Education,* 11(1), pp. 71-82.

Gaudin, M., Krupovic, M., Marguet, E., Gauliard, E., Cvirkaite-Krupovic, V., Le Cam, E., Oberto, J. and Forterre, P. (2014) 'Extracellular membrane vesicles harbouring viral genomes', *Environmental Microbiology,* 16(4), pp. 1167-75.

Gebreyes, W. A. and Altier, C. (2002) 'Molecular Characterization of Multidrug-Resistant *Salmonella enterica* subsp. *enterica* Serovar Typhimurium Isolates from Swine', *Journal of Clinical Microbiology,* 40(8), pp. 2813-2822.

Gerner-Smidt, P., Tjernberg, I. and Ursing, J. (1991) 'Reliability of phenotypic tests for identification of *x* species', *Journal of Clinical Microbiology,* 29(2), pp. 277-282.

Giraldi, G., Montesano, M., Napoli, C., Frati, P., La Russa, R., Santurro, A., Scopetti, M. and Orsi, G. B. (2019) 'Healthcare-Associated Infections Due to Multidrug-Resistant Organisms: a Surveillance Study on Extra Hospital Stay and Direct Costs', *Current Pharmaceutical Biotechnology,* 20(8), pp. 643-652.

Girija, S. A., Priyadharsini, J. V. and Paramasivam, A. (2019) 'Prevalence of carbapenem-hydrolyzing OXA-type β-lactamases among *Acinetobacter baumannii* in patients with severe urinary tract infection', *Acta Microbiologica et Immunologica Hungarica,* 67(1), pp. 49-55.

Godeux, A.-S., Svedholm, E., Barreto, S., Potron, A., Venner, S., Charpentier, X. and Laaberki, M.-H. (2022) 'Interbacterial Transfer of Carbapenem Resistance and Large Antibiotic Resistance Islands by Natural Transformation in Pathogenic *Acinetobacter*', *mBio,* 13(1), pp. e02631-21.

Goessweiner-Mohr, N., Arends, K., Keller, W. and Grohmann, E. (2014) 'Conjugation in Gram-Positive Bacteria', *Microbiology Spectrum,* 2(4), pp. Plas-0004-2013.

Goldstein, F. W., Labigne-Roussel, A., Gerbaud, G., Carlier, C., Collatz, E. and Courvalin, P. (1983) 'Transferable plasmid-mediated antibiotic resistance in *Acinetobacter*', *Plasmid,* 10(2), pp. 138-147.

Griffith, F. (1928) 'The Significance of Pneumococcal Types', *Journal of Hygiene,* 27(2), pp. 113-159.

Gu, B., Tong, M., Zhao, W., Liu, G., Ning, M., Pan, S. and Zhao, W. (2007) 'Prevalence and Characterization of Class I Integrons among *Pseudomonas aeruginosa* and *Acinetobacter baumannii* Isolates from Patients in Nanjing, China', *Journal of Clinical Microbiology,* 45(1), pp. 241-243.

Hall, R. M. and Collis, C. M. (1995) 'Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination', *Molecular Microbiology,* 15(4), pp. 593-600.

Hamidian, M. and Nigro, S. J. (2019) 'Emergence, molecular mechanisms and global spread of carbapenem-resistant *Acinetobacter baumannii*', *Microbial Genomics,* 5(10).

Harding, C. M., Hennon, S. W. and Feldman, M. F. (2018) 'Uncovering the mechanisms of *Acinetobacter baumannii* virulence', *Nature Reviews Microbiology,* 16(2), pp. 91-102.

Harris, S.-R., Feil, E. J., Holden, M. T. G., Quail, M. A., Nickerson, E. K., Chantratita, N., Gardete, S., Tavares, A., Day, N., Lindsay, J. A., Edgeworth, J. D., de Lencastre, H., Parkhill, J., Peacock, S. J. and Bentley, S. D. (2010) 'Evolution of MRSA During Hospital Transmission and Intercontinental Spread', *Science,* 327(5964), pp. 469-474.

Hawley, J. S., Murray, C. K. and Jorgensen, J. H. (2008) 'Colistin Heteroresistance in *Acinetobacter* and Its Association with Previous Colistin Therapy', *Antimicrobial Agents and Chemotherapy,* 52(1), pp. 351-352.

Hayashi, Y., Roberts, J. A., Paterson, D. L. and Lipman, J. (2010) 'Pharmacokinetic evaluation of piperacillin-tazobactam', *Expert Opinion on Drug Metabolism & Toxicology,* 6(8), pp. 1017-31.

Headd, B. and Bradford, S. A. (2020) 'The Conjugation Window in an *Escherichia coli* K-12 Strain with an IncFII Plasmid', *Applied and Environmental Microbiology,* 86(17), pp. e00948-20.

Holloway, K. P., Rouphael, N. G., Wells, J. B., King, M. D. and Blumberg, H. M. (2006) 'Polymyxin B and Doxycycline Use in Patients with Multidrug-Resistant *Acinetobacter baumannii* Infections in the Intensive Care Unit', *Annals of Pharmacotherapy,* 40(11), pp. 1939-1945.

Holton, J. (1982) 'A report of a further hospital outbreak caused by a multi-resistant *Acinetobacter anitratus*', *Journal of Hospital Infection,* 3(3), pp. 305-309.

Hooper, D. C. (2000) 'Mechanisms of Action and Resistance of Older and Newer Fluoroquinolones', *Clinical Infectious Diseases,* 31(Supplement_2), pp. S24-S28.

Hope, R., Warner, M., Mushtaq, S., Ward, M. E., Parsons, T. and Livermore, D. M. (2005) 'Effect of medium type, age and aeration on the MICs of tigecycline and classical tetracyclines', *Journal of Antimicrobial Chemotherapy,* 56(6), pp. 1042-1046.

Howard, A., O'Donoghue, M., Feeney, A. and Sleator, R. D. (2012) '*Acinetobacter baumannii*: an emerging opportunistic pathogen', *Virulence,* 3(3), pp. 243-250.

Hradecka, H., Karasova, D. and Rychlik, I. (2008) 'Characterization of *Salmonella enterica* serovar Typhimurium conjugative plasmids transferring resistance to antibiotics and their interaction with the virulence plasmid', *Journal of Antimicrobial Chemotherapy,* 62(5), pp. 938-941.

Hu, L., Shi, Y., Xu, Q., Zhang, L., He, J., Jiang, Y., Liu, L., Leptihn, S., Yu, Y., Hua, X. and Zhou, Z. (2020) 'Capsule Thickness, Not Biofilm Formation, Gives Rise to Mucoid *Acinetobacter baumannii* Phenotypes That are More Prevalent in Long-Term Infections: A Study of Clinical Isolates from a Hospital in China.', *Infect Drug Resist.,* 13, pp. 99-109.

Hu, W. S., Yao, S. M., Fung, C. P., Hsieh, Y. P., Liu, C. P. and Lin, J. F. (2007) 'An *OXA-66/OXA-51-like* carbapenemase and possibly an efflux pump are associated with resistance to imipenem in *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 51(11), pp. 3844-52.

Hujer, K. M., Hujer, A. M., Hulten, E. A., Bajaksouzian, S., Adams, J. M., Donskey, C. J., Ecker, D. J., Massire, C., Eshoo, M. W., Sampath, R., Thomson, J. M., Rather, P. N., Craft, D. W., Fishbain, J. T., Ewell, A. J., Jacobs, M. R., Paterson, D. L. and Bonomo, R. A. (2006) 'Analysis of Antibiotic Resistance Genes in Multidrug-Resistant *Acinetobacter* spp. Isolates from Military and Civilian Patients Treated at the Walter Reed Army Medical Center', *Antimicrobial Agents and Chemotherapy,* 50(12), pp. 4114-4123.

Humphrey, S., Fillol-Salom, A., Quiles-Puchalt, N., Ibarra-Chávez, R., Haag, A. F., Chen, J. and Penadés, J. R. (2021) 'Bacterial chromosomal mobility via lateral transduction exceeds that of classical mobile genetic elements', *Nature Communications,* 12(1), pp. 6509.

Hussein, M., Allobawi, R., Levou, I., Blaskovich, M. A. T., Rao, G. G., Li, J. and Velkov, T. (2022) 'Mechanisms Underlying Synergistic Killing of Polymyxin B in Combination with Cannabidiol against *Acinetobacter baumannii*: A Metabolomic Study', *Pharmaceutics,* 14(4), pp. 786.

Hussein, N. H., Al-Kadmy, I. M. S., Taha, B. M. and Hussein, J. D. (2021) 'Mobilized colistin resistance (*mcr*) genes from 1 to 10: a comprehensive review', *Molecular Biology Reports,* 48(3), pp. 2897-2907.

Héritier, C., Dubouix, A., Poirel, L., Marty, N. and Nordmann, P. (2005) 'A nosocomial outbreak of *Acinetobacter baumannii* isolates expressing the carbapenem-hydrolysing oxacillinase *OXA-58*', *Journal of Antimicrobial Chemotherapy,* 55(1), pp. 115-118.

Héritier, C., Poirel, L. and Nordmann, P. (2006) 'Cephalosporinase over-expression resulting from insertion of IS*Aba1* in *Acinetobacter baumannii*', *Clinical Microbiology and Infection,* 12(2), pp. 123-130.

Iswarya Jaisankar, A., Smiline Girija, A. S., Gunasekaran, S. and Vijayashree Priyadharsini, J. (2020) 'Molecular characterisation of csgA gene among ESBL strains of *A. baumannii* and targeting with essential oil compounds from *Azadirachta indica*', *Journal of King Saud University - Science,* 32(8), pp. 3380-3387.

Jiménez-Guerra, G., Heras-Cañas, V., Gutiérrez-Soto, M., Del Pilar Aznarte-Padial, M., Expósito-Ruiz, M., Navarro-Marí, J. M. and Gutiérrez-Fernández, J. (2018) 'Urinary tract infection by *Acinetobacter baumannii* and *Pseudomonas aeruginosa*: evolution of antimicrobial resistance and therapeutic alternatives', *Journal of Medical Microbiology,* 67(6), pp. 790-797.

Jin, M., Liu, L., Wang, D., Yang, D., Liu, W., Yin, J., Yang, Z., Wang, H., Qiu, Z., Shen, Z., Shi, D., Li, H., Guo, J. and Li, J. (2020) 'Chlorine disinfection promotes the exchange of antibiotic resistance genes across bacterial genera by natural transformation', *The ISME Journal,* 14(7), pp. 1847-1856.

Jouybari, M. A., Ahanjan, M., Mirzaei, B. and Goli, H. R. (2021) 'Role of aminoglycoside-modifying enzymes and 16S rRNA methylase (*ArmA*) in resistance of *Acinetobacter baumannii* clinical isolates against aminoglycosides', *Revista da Sociedade Brasileira de Medicina Tropical,* 54, pp. e05992020.

Juni, E. (1972) 'Interspecies Transformation of *Acinetobacter*: Genetic Evidence for a Ubiquitous Genus', *Journal of Bacteriology,* 112(2), pp. 917-931.

Kallel, H., Hergafi, L., Bahloul, M., Hakim, A., Dammak, H., Chelly, H., Hamida, C. B., Chaari, A., Rekik, N. and Bouaziz, M. (2007) 'Safety and efficacy of colistin compared with imipenem in the treatmentof ventilator-associated pneumonia: a matched case–control study', *Intensive Care Medicine,* 33(7), pp. 1162-1167.

Kang, H. Y., Jeong, Y. S., Oh, J. Y., Tae, S. H., Choi, C. H., Moon, D. C., Lee, W. K., Lee, Y. C., Seol, S. Y., Cho, D. T. and Lee, J. C. (2005) 'Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea', *Journal of Antimicrobial Chemotherapy,* 55(5), pp. 639-644.

Karakonstantis, S., Kritsotakis, E. I. and Gikas, A. (2020) 'Treatment options for *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* co-resistant to carbapenems, aminoglycosides, polymyxins and tigecycline: an approach based on the mechanisms of resistance to carbapenems', *Infection,* 48(6), pp. 835-851.

Khaledi, A., Esmaeili, D., Jamehdar, S. A., Esmaeili, S., Neshani, A. and Bahador, A. (2016) 'Expression of MFS efflux pumps among multidrug resistant *Acinetobacter baumannii* clinical isolates', *Der Pharmacia Lettre,* 8(2), pp. 262-267.

Khan, M. S. A., Altaf, M. M. and Ahmad, I. (2017) 'Chemical nature of biofilm matrix and its significance', *Biofilms in plant and soil health*, pp. 151-177.

Khongfak, S., Thummeepak, R., Leungtongkam, U., Tasanapak, K., Thanwisai, A. and Sitthisak, S. (2022) 'Insights into mobile genetic elements and the role of conjugative plasmid in transferring aminoglycoside resistance in extensively drug-resistant *Acinetobacter baumannii* AB329', *PeerJ,* 10, pp. e13718.

Kidsley, A. K., Abraham, S., Bell, J. M., O'Dea, M., Laird, T. J., Jordan, D., Mitchell, P., McDevitt, C. A. and Trott, D. J. (2018) 'Antimicrobial Susceptibility of *Escherichia coli* and *Salmonella* spp. Isolates From Healthy Pigs in Australia: Results of a Pilot National Survey', *Front Microbiol,* 9, pp. 1207.

Ko, K. S., Suh, J. Y., Kwon, K. T., Jung, S., Park, K., Kang, C. I., Chung, D. R., Peck, K. R. and Song, J. (2007) 'High rates of resistance to colistin and polymyxin B in subgroups of *Acinetobacter baumannii* isolates from Korea', *Journal of Antimicrobial Chemotherapy,* 60(5), pp. 1163-1167.

Krahn, T., Wibberg, D., Maus, I., Winkler, A., Bontron, S., Sczyrba, A., Nordmann, P., Pühler, A., Poirel, L. and Schlüter, A. (2016) 'Intraspecies Transfer of the Chromosomal *Acinetobacter baumannii bla*$_{NDM-1}$ Carbapenemase Gene', *Antimicrob Agents Chemother,* 60(5), pp. 3032-40.

Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., Balakrishnan, R., Chaudhary, U., Doumith, M., Giske, C. G., Irfan, S., Krishnan, P., Kumar, A. V., Maharjan, S., Mushtaq, S., Noorie, T., Paterson, D. L., Pearson, A., Perry, C., Pike, R., Rao, B., Ray, U., Sarma, J. B., Sharma, M., Sheridan, E., Thirunarayan, M. A., Turton, J., Upadhyay, S., Warner, M., Welfare, W., Livermore, D. M. and Woodford, N. (2010) 'Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study', *The Lancet Infectious Diseases,* 10(9), pp. 597-602.

Kumburu, H. H., Sonda, T., van Zwetselaar, M., Leekitcharoenphon, P., Lukjancenko, O., Mmbaga, B. T., Alifrangis, M., Lund, O., Aarestrup, F. M. and Kibiki, G. S. (2019) 'Using WGS to identify antibiotic resistance genes and predict antimicrobial resistance phenotypes in MDR *Acinetobacter baumannii* in Tanzania', *Journal of Antimicrobial Chemotherapy,* 74(6), pp. 1484-1493.

Kurihara, M. N. L., Sales, R. O., Silva, K. E. D., Maciel, W. G. and Simionatto, S. (2020) 'Multidrug-resistant *Acinetobacter baumannii* outbreaks: a global problem in healthcare settings', *Revista da Sociedade Brasileira de Medicina Tropical,* 53, pp. e20200248.

Labbate, M., Case, R. J. and Stokes, H. W. (2009) 'The integron/gene cassette system: an active player in bacterial adaptation', *Methods in Molecular Biology,* 532, pp. 103-25.

Lam, M. M. C., Koong, J., Holt, K. E., Hall, R. M. and Hamidian, M. (2023) 'Detection and Typing of Plasmids in *Acinetobacter baumannii* Using *rep* Genes Encoding Replication Initiation Proteins', *Microbiology Spectrum,* 11(1), pp. e02478-22.

Le, C., Pimentel, C., Tuttobene, M. R., Subils, T., Nishimura, B., Traglia, G. M., Perez, F., Papp-Wallace, K. M., Bonomo, R. A., Tolmasky, M. E. and Ramirez, M. S. (2021) 'Interplay between Meropenem and Human Serum Albumin on Expression of Carbapenem Resistance Genes and Natural Competence in *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 65(10), pp. e01019-21.

Lederberg, J. and Tatum, E. L. (1946) 'Gene Recombination in *Escherichia coli*', *Nature,* 158(4016), pp. 558-558.

Lerminiaux, N. A. and Cameron, A. D. S. (2019) 'Horizontal transfer of antibiotic resistance genes in clinical environments', *Canadian Journal of Microbiology,* 65(1), pp. 34-44.

Leungtongkam, U., Thummeepak, R., Tasanapak, K. and Sitthisak, S. (2018) 'Acquisition and transfer of antibiotic resistance genes in association with conjugative plasmid or class 1 integrons of *Acinetobacter baumannii*', *PLoS One,* 13(12), pp. e0208468.

Levin, A. S., Barone, A. A., Penço, J., Santos, M. V., Marinho, I. S., Arruda, E. A. G., Manrique, E. I. and Costa, S. F. (1999) 'Intravenous Colistin as Therapy for Nosocomial Infections Caused by Multidrug-Resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*', *Clinical Infectious Diseases,* 28(5), pp. 1008-1011.

Levin, A. S., Levy, C. E., Manrique, A. E. I., Medeiros, E. A. S. and Costa, S. F. (2003) 'Severe nosocomial infections with imipenem-resistant *Acinetobacter baumannii* treated with ampicillin/sulbactam', *International Journal of Antimicrobial Agents,* 21(1), pp. 58-62.

Li, J., Rayner, C. R., Nation, R. L., Owen, R. J., Spelman, D., Tan, K. E. and Liolios, L. (2006) 'Heteroresistance to Colistin in Multidrug-Resistant *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 50(9), pp. 2946-2950.

Liakopoulos, A., Mevius, D. and Ceccarelli, D. (2016) 'A Review of SHV Extended-Spectrum β-Lactamases: Neglected Yet Ubiquitous', *Frontiers in Microbiology,* 7, pp. 1374.

Liao, Y., Kuo, S., Chiang, M., Lee, Y., Sung, W., Chen, Y., Chen, T. and Fung, C. (2015) '*Acinetobacter baumannii* Extracellular *OXA-58* Is Primarily and Selectively Released via Outer Membrane Vesicles after Sec-Dependent Periplasmic Translocation', *Antimicrobial Agents and Chemotherapy,* 59(12), pp. 7346-7354.

Lima, T., Loureiro, D., Henriques, A., Ramos, F., Pomba, C., Domingues, S. and da Silva, G. J. (2022) 'Occurrence and Biological Cost of *mcr-1*-Carrying Plasmids Co-harbouring Beta-Lactamase Resistance Genes in Zoonotic Pathogens from Intensive Animal Production', *Antibiotics,* 11(10), pp. 1356.

Lin, M., Lin, Y., Yeh, H. and Lan, C. (2014) 'Role of the BaeSR two-component system in the regulation of *Acinetobacter baumannii adeAB* genes and its correlation with tigecycline susceptibility', *BMC Microbiology,* 14(1), pp. 119.

Ling, T. K., Xiong, J., Yu, Y., Lee, C. C., Ye, H. and Hawkey, P. M. (2006) 'Multicenter antimicrobial susceptibility survey of gram-negative bacteria isolated from patients with community-acquired infections in the People's Republic of China', *Antimicrob Agents Chemother,* 50(1), pp. 374-8.

Liu, Y., Wang, Y., Walsh, T. R., Yi, L., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J. and Shen, J. (2016) 'Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study', *The Lancet Infectious Diseases,* 16(2), pp. 161-168.

Lorenz, M. G. and Wackernagel, W. (1994) 'Bacterial gene transfer by natural genetic transformation in the environment', *Microbiological Reviews,* 58(3), pp. 563-602.

Lévesque, C., Piché, L., Larose, C. and Roy, P. H. (1995) 'PCR mapping of integrons reveals several novel combinations of resistance genes', *Antimicrobial Agents and Chemotherapy,* 39(1), pp. 185-91.

López-Leal, G., Santamaria, R. I., Cevallos, M. Á., Gonzalex, V. and Castillo-Ramírez, S. (2020) 'Letter to the Editor: Prophages Encode Antibiotic Resistance Genes in *Acinetobacter baumannii*', *Microbial Drug Resistance,* 26(10), pp. 1275-1277.

Ma, S., Sun, C., Hulth, A., Li, J., Nilsson, L. E., Zhou, Y., Börjesson, S., Bi, Z., Bi, Z., Sun, Q. and Wang, Y. (2018) 'Mobile colistin resistance gene *mcr-5* in porcine *Aeromonas hydrophila*', *Journal of Antimicrobial Chemotherapy,* 73(7), pp. 1777-1780.

MacWilliams, M. P. and Liao, M. (2006) 'Luria broth (LB) and Luria agar (LA) media and their uses protocol'.

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T. and Monnet, D. L. (2012) 'Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance', *Clinical Microbiology and Infection,* 18(3), pp. 268-281.

Mak, J. K., Kim, M., Pham, J., Tapsall, J. and White, P. A. (2008) 'Antibiotic resistance determinants in nosocomial strains of multidrug-resistant *Acinetobacter baumannii*', *Journal of Antimicrobial Chemotherapy,* 63(1), pp. 47-54.

Mamishi, S., Mahmoudi, S., Naserzadeh, N., Hosseinpour Sadeghi, R., Haghi Ashtiani, M. T., Bahador, A., Abdosalehi, M. R., Rahmani, M. and Pourakbari, B. (2019) 'Antibiotic resistance and genotyping of gram-negative bacteria causing hospital-acquired infection in patients referred to Children's Medical Center', *Infection and Drug Resistance,* 12, pp. 3377-3384.

Martí, S., Fernández-Cuenca, F., Pascual, A., Ribera, A., Rodríguez-Baño, J., Bou, G., Miguel Cisneros, J., Pachón, J., Martínez-Martínez, L. and Vila, J. (2006) 'Prevalence of the *tetA* and *tetB* genes as mechanisms of resistance to tetracycline and minocycline in *Acinetobacter baumannii* clinical isolates', *Enfermedades infecciosas y microbiologia clinica,,* 24(2), pp. 77-80.

Matic, I., Rayssiguier, C. and Radman, M. (1995) 'Interspecies gene exchange in bacteria: The role of SOS and mismatch repair systems in evolution of species', *Cell,* 80(3), pp. 507-515.

Mazel, D. (2006) 'Integrons: agents of bacterial evolution', *Nature Reviews Microbiology,* 4(8), pp. 608-620.

McDermott, J. H., Wolf, J., Hoshitsuki, K., Huddart, R., Caudle, K. E., Whirl-Carrillo, M., Steyger, P. S., Smith, R. J. H., Cody, N., Rodriguez-Antona, C., Klein, T. E. and Newman, W. G. (2022) 'Clinical Pharmacogenetics Implementation Consortium Guideline for the Use of Aminoglycosides Based on MT-RNR1 Genotype', *Clinical Pharmacology and Therapeutics,* 111(2), pp. 366-372.

Medvedeva, E. S., Baranova, N. B., Mouzykantov, A. A., Grigorieva, T. Y., Davydova, M. N., Trushin, M. V., Chernova, O. A. and Chernov, V. M. (2014) 'Adaptation of mycoplasmas to antimicrobial agents: *Acholeplasma laidlawii* extracellular vesicles mediate the export of ciprofloxacin and a mutant gene related to the antibiotic target', *ScientificWorldJournal,* 2014, pp. 150615.

Mestrovic, T., Robles Aguilar, G., Swetschinski, L. R., Ikuta, K. S., Gray, A. P., Davis Weaver, N., Han, C., Wool, E. E., Gershberg Hayoon, A., Hay, S. I., Dolecek, C., Sartorius, B., Murray, C. J. L., Addo, I. Y., Ahinkorah, B. O., Ahmed, A., Aldeyab, M. A., Allel, K., Ancuceanu, R., Anyasodor, A. E., Ausloos, M., Barra, F., Bhagavathula, A. S., Bhandari, D., Bhaskar, S., Cruz-Martins, N., Dastiridou, A., Dokova, K., Dubljanin, E., Durojaiye, O. C., Fagbamigbe, A. F., Ferrero, S., Gaal, P. A., Gupta, V. B., Gupta, V. K., Gupta, V. K., Herteliu, C., Hussain, S., Ilic, I. M., Ilic, M. D., Jamshidi, E., Joo, T., Karch, A., Kisa, A., Kisa, S., Kostyanev, T., Kyu, H. H., Lám, J., Lopes, G., Mathioudakis, A. G., Mentis, A. A., Michalek, I. M., Moni, M. A., Moore, C. E., Mulita, F., Negoi, I., Negoi, R. I., Palicz, T., Pana, A., Perdigão, J., Petcu, I., Rabiee, N., Rawaf, D. L., Rawaf, S., Shakhmardanov, M. Z., Sheikh, A., Silva, L. M. L. R., Skryabin, V. Y., Skryabina, A. A., Socea, B., Stergachis, A., Stoeva, T. Z., Sumi, C. D., Thiyagarajan, A., Tovani-Palone, M. R., Yesiltepe, M., Zaman, S. B. and Naghavi, M. (2022) 'The burden of bacterial antimicrobial

resistance in the WHO European region in 2019: a cross-country systematic analysis', *The Lancet Public Health,* 7(11), pp. e897-e913.

Michalopoulos, A. and Falagas, M. E. (2010) 'Treatment of *Acinetobacter* infections', *Expert Opin Pharmacother,* 11(5), pp. 779-88.

Michalopoulos, A. S., Tsiodras, S., Rellos, K., Mentzelopoulos, S. and Falagas, M. E. (2005) 'Colistin treatment in patients with ICU-acquired infections caused by multiresistant Gram-negative bacteria: the renaissance of an old antibiotic', *Clinical Microbiology and Infection,* 11(2), pp. 115-121.

Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D. F., Vinogradov, E., Seemann, T., Henry, R., Crane, B., St. Michael, F., D. Cox, A., Adler, B., Nation, R. L., Li, J. and Boyce, J. D. (2010) 'Colistin Resistance in *Acinetobacter baumannii* Is Mediated by Complete Loss of Lipopolysaccharide Production', *Antimicrobial Agents and Chemotherapy,* 54(12), pp. 4971-4977.

Mohammadi, M., Khayat, H., Sayehmiri, K., Soroush, S., Sayehmiri, F., Delfani, S., Bogdanovic, L. and Taherikalani, M. (2017) 'Synergistic Effect of Colistin and Rifampin Against Multidrug Resistant *Acinetobacter baumannii*: A Systematic Review and Meta-Analysis', *The Open Microbiology Journal,* 11, pp. 63-71.

Moosavian, M., Ahmadi, K., Shoja, S., Mardaneh, J., Shahi, F. and Afzali, M. (2020) 'Antimicrobial resistance patterns and their encoding genes among clinical isolates of *Acinetobacter baumannii* in Ahvaz, Southwest Iran', *MethodsX,* 7, pp. 101031.

Naas, T., Bogaerts, P., Bauraing, C., Degheldre, Y., Glupczynski, Y. and Nordmann, P. (2006) 'Emergence of PER and VEB extended-spectrum β-lactamases in *Acinetobacter baumannii* in Belgium', *Journal of Antimicrobial Chemotherapy,* 58(1), pp. 178-182.

Necati Hakyemez, I., Kucukbayrak, A., Tas, T., Burcu Yikilgan, A., Akkaya, A., Yasayacak, A. and Akdeniz, H. (2013) 'Nosocomial *Acinetobacter baumannii* Infections and Changing Antibiotic Resistance', *Pakistan Journal of Medical Sciences,* 29(5), pp. 1245-8.

Nield, B. S., Holmes, A. J., Gillings, M. R., Recchia, G. D., Mabbutt, B. C., Nevalainen, K. M. H. and Stokes, H. W. (2001) 'Recovery of new integron classes from environmental DNA', *FEMS Microbiology Letters,* 195(1), pp. 59-65.

Novović, K. and Jovčić, B. (2023) 'Colistin Resistance in *Acinetobacter baumannii*: Molecular Mechanisms and Epidemiology', *Antibiotics,* 12(3), pp. 516.

Nowak, P. and Paluchowska, P. (2016) '*Acinetobacter baumannii*: biology and drug resistance — role of carbapenemases', *Folia Histochemica et Cytobiologica,* 54(2), pp. 61-74.

Nurtop, E., Bilman, F. B., Menekse, S., Azap, O. K., Gönen, M., Ergonul, O. and Can, F. (2019) 'Promoters of Colistin Resistance in Acinetobacter baumannii Infections', *Microbial Drug Resistance,* 25(7), pp. 997-1002.

O'Neill, J. 2014. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. *Rev. Antimicrob. Resist.*

Oliveira, M. S., Prado, G. V. B., Costa, S. F., Grinbaum, R. S. and Levin, A. S. (2008) 'Ampicillin/sulbactam compared with polymyxins for the treatment of infections caused by carbapenem-resistant *Acinetobacter* spp', *Journal of Antimicrobial Chemotherapy,* 61(6), pp. 1369-1375.

Organization, W. H. (2014) *Antimicrobial resistance: global report on surveillance.* World Health Organization.

Pachón-Ibáñez, M. E., Fernández-Cuenca, F., Docobo-Pérez, F., Pachón, J. and Pascual, Á. (2006) 'Prevention of rifampicin resistance in *Acinetobacter baumannii* in an experimental pneumonia

murine model, using rifampicin associated with imipenem or sulbactam', *Journal of Antimicrobial Chemotherapy,* 58(3), pp. 689-692.

Park, S., Lee, K. M., Yoo, Y. S., Yoo, J. S., Yoo, J. I., Kim, H. S., Lee, Y. S. and Chung, G. T. (2011) 'Alterations of *gyrA*, *gyrB*, and *parC* and Activity of Efflux Pump in Fluoroquinolone-resistant *Acinetobacter baumannii*', *Osong Public Health and Research Perspectives,* 2(3), pp. 164-170.

Partridge, S. R., Kwong, S. M., Firth, N. and Jensen, S. O. (2018) 'Mobile Genetic Elements Associated with Antimicrobial Resistance', *Clinical Microbiology Reviews,* 31(4), pp. e00088-17.

Partridge, S. R., Tsafnat, G., Coiera, E. and Iredell, J. R. (2009) 'Gene cassettes and cassette arrays in mobile resistance integrons', *FEMS Microbiology Reviews,* 33(4), pp. 757-784.

Peleg, A. Y., Adams, J. and Paterson, D. L. (2007) 'Tigecycline Efflux as a Mechanism for Nonsusceptibility in *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 51(6), pp. 2065-2069.

Peleg, A. Y., de Breij, A., Adams, M. D., Cerqueira, G. M., Mocali, S., Galardini, M., Nibbering, P. H., Earl, A. M., Ward, D. V., Paterson, D. L., Seifert, H. and Dijkshoorn, L. (2012) 'The success of *Acinetobacter* species; genetic, metabolic and virulence attributes', *PLoS One,* 7(10), pp. e46984.

Peleg, A. Y., Potoski, B. A., Rea, R., Adams, J., Sethi, J., Capitano, B., Husain, S., Kwak, E. J., Bhat, S. V. and Paterson, D. L. (2006) '*Acinetobacter baumannii* bloodstream infection while receiving tigecycline: a cautionary report', *Journal of Antimicrobial Chemotherapy,* 59(1), pp. 128-131.

Penwell, W. F., Shapiro, A. B., Giacobbe, R. A., Gu, R., Gao, N., Thresher, J., McLaughlin, R. E., Huband, M. D., DeJonge, B. L. M., Ehmann, D. E. and Miller, A. A. (2015) 'Molecular Mechanisms of Sulbactam Antibacterial Activity and Resistance Determinants in *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 59(3), pp. 1680-1689.

Petca, A., Negoita, S., Petca, R., Borislavschi, A., Calo, I. G., Popescu, V. G. and Sinescu, R. D. (2021) 'Bacterial pathogens isolated from surgical site infections and their antibiotic susceptibility', *Farmacia,* 69, pp. 741-748.

Petersen, P. J. and Bradford, P. A. (2005) 'Effect of Medium Age and Supplementation with the Biocatalytic Oxygen-Reducing Reagent Oxyrase on In Vitro Activities of Tigecycline against Recent Clinical Isolates', *Antimicrobial Agents and Chemotherapy,* 49(9), pp. 3910-3918.

Pfeifer, Y., Wilharm, G., Zander, E., Wichelhaus, T. A., Göttig, S., Hunfeld, K., Seifert, H., Witte, W. and Higgins, P. G. (2011) 'Molecular characterization of bla_{NDM-1} in an *Acinetobacter baumannii* strain isolated in Germany in 2007', *Journal of Antimicrobial Chemotherapy,* 66(9), pp. 1998-2001.

Phe, K., Lee, Y., McDaneld, P. M., Prasad, N., Yin, T., Figueroa, D. A., Musick, W. L., Cottreau, J. M., Hu, M. and Tam, V. H. (2014) 'In vitro assessment and multicenter cohort study of comparative nephrotoxicity rates associated with colistimethate versus polymyxin B therapy', *Antimicrobial Agents and Chemotherapy,* 58(5), pp. 2740-6.

Pormohammad, A., Mehdinejadiani, K., Gholizadeh, P., Nasiri, M. J., Mohtavinejad, N., Dadashi, M., Karimaei, S., Safari, H. and Azimi, T. (2020) 'Global prevalence of colistin resistance in clinical isolates of *Acinetobacter baumannii*: A systematic review and meta-analysis', *Microbial Pathogenesis,* 139, pp. 103887.

Pormohammad, A., Pouriran, R., Azimi, H. and Goudarzi, M. (2019) 'Prevalence of integron classes in Gram-negative clinical isolated bacteria in Iran: a systematic review and meta-analysis', *Iranian Journal of Basic Medical Sciences,* 22(2), pp. 118-127.

Pérez-Cruz, C., Carrión, O., Delgado, L., Martinez, G., López-Iglesias, C. and Mercade, E. (2013) 'New type of outer membrane vesicle produced by the Gram-negative bacterium *Shewanella vesiculosa* M7T: implications for DNA content', *Applied and Environmental Microbiololgy,* 79(6), pp. 1874-81.

Pérez-Varela, M., Corral, J., Aranda, J. and Barbé, J. (2018) 'Functional Characterization of AbaQ, a Novel Efflux Pump Mediating Quinolone Resistance in *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 62(9), pp. 10.1128/aac.00906-18.

Qureshi, Z. A., Hittle, L. E., O'Hara, J. A., Rivera, J. I., Syed, A., Shields, R. K., Pasculle, A. W., Ernst, R. K. and Doi, Y. (2015) 'Colistin-Resistant *Acinetobacter baumannii*: Beyond Carbapenem Resistance', *Clinical Infectious Diseases,* 60(9), pp. 1295-1303.

Ramirez, M. S., Bonomo, R. A. and Tolmasky, M. E. (2020) 'Carbapenemases: Transforming *Acinetobacter baumannii* into a Yet More Dangerous Menace', *Biomolecules,* 10(5), pp. 720.

Ramirez, M. S., Don, M., Merkier, A. K., Bistué, A. J. S., Zorreguieta, A., Centrón, D. and Tolmasky, M. E. (2010) 'Naturally Competent *Acinetobacter baumannii* Clinical Isolate as a Convenient Model for Genetic Studies', *Journal of Clinical Microbiology,* 48(4), pp. 1488-1490.

Rebelo, A. R., Bortolaia, V., Kjeldgaard, J. S., Pedersen, S. K., Leekitcharoenphon, P., Hansen, I. M., Guerra, B., Malorny, B., Borowiak, M., Hammerl, J. A., Battisti, A., Franco, A., Alba, P., Perrin-Guyomard, A., Granier, S. A., De Frutos Escobar, C., Malhotra-Kumar, S., Villa, L., Carattoli, A. and Hendriksen, R. S. (2018) 'Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for surveillance purposes', *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin,* 23(6).

Recchia, G. D. and Hall, R. M. (1995) 'Gene cassettes: a new class of mobile element', *Microbiology,* 141(12), pp. 3015-3027.

Reichmann, P., König, A., Linares, J., Alcaide, F., Tenover, F. C., McDougal, L., Swidsinski, S. and Hakenbeck, R. (1997) 'A Global Gene Pool for High-Level Cephalosporin Resistance in Commensal *Streptococcus* Species and *Streptococcus pneumoniae*', *The Journal of Infectious Diseases,* 176(4), pp. 1001-1012.

Reid, G. E., Grim, S. A., Aldeza, C. A., Janda, W. M. and Clark, N. M. (2007) 'Rapid development of *Acinetobacter baumannii* resistance to tigecycline', *Pharmacotherapy,* 27(8), pp. 1198-201.

Rumbo, C., Fernández-Moreira, E., Merino, M., Poza, M., Mendez, J. A., Soares, N. C., Mosquera, A., Chaves, F. and Bou, G. (2011) 'Horizontal transfer of the *OXA-24* carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 55(7), pp. 3084-90.

Safari, M., Mozaffari Nejad, A. S., Bahador, A., Jafari, R. and Alikhani, M. Y. (2015) 'Prevalence of ESBL and MBL encoding genes in *Acinetobacter baumannii* strains isolated from patients of intensive care units (ICU)', *Saudi Journal of Biological Sciences,* 22(4), pp. 424-429.

Salgado-Camargo, A. D., Castro-Jaimes, S., Gutierrez-Rios, R. M., Lozano, L. F., Altamirano-Pacheco, L., Silva-Sanchez, J., Pérez-Oseguera, Á., Volkow, P., Castillo-Ramírez, S. and Cevallos, M. A. (2020) 'Structure and Evolution of *Acinetobacter baumannii* Plasmids', *Front Microbiol,* 11, pp. 1283.

San Millan, A. (2018) 'Evolution of Plasmid-Mediated Antibiotic Resistance in the Clinical Context', *Trends in Microbiology,* 26(12), pp. 978-985.

Schafer, J. J., Goff, D. A., Stevenson, K. B. and Mangino, J. E. (2007) 'Early experience with tigecycline for ventilator-associated pneumonia and bacteremia caused by multidrug-resistant *Acinetobacter baumannii*', *Pharmacotherapy,* 27(7), pp. 980-7.

Schneider, C. L. (2021) 'Bacteriophage-Mediated Horizontal Gene Transfer: Transduction', in Harper, D.R., Abedon, S.T., Burrowes, B.H. and McConville, M.L. (eds.) *Bacteriophages: Biology, Technology, Therapy*. Cham: Springer International Publishing, pp. 151-192.

Schumacher, M. A. and Brennan, R. G. (2002) 'Structural mechanisms of multidrug recognition and regulation by bacterial multidrug transcription factors', *Molecular Microbiology,* 45(4), pp. 885-93.

Schäfer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A. H. and Pühler, A. (1990) 'High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria', *Journal of Bacteriology,* 172(3), pp. 1663-1666.

Seifert, H., Müller, C., Stefanik, D., Higgins, P. G., Miller, A. and Kresken, M. (2020) 'In vitro activity of sulbactam/durlobactam against global isolates of carbapenem-resistant *Acinetobacter baumannii*', *Journal of Antimicrobial Chemotherapy,* 75(9), pp. 2616-2621.

Seitz, P. and Blokesch, M. (2013) 'Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria', *FEMS Microbiology Reviews,* 37(3), pp. 336-363.

Sharma, A., Sharma, R., Bhattacharyya, T., Bhando, T. and Pathania, R. (2016) 'Fosfomycin resistance in *Acinetobacter baumannii* is mediated by efflux through a major facilitator superfamily (MFS) transporter—AbaF', *Journal of Antimicrobial Chemotherapy,* 72(1), pp. 68-74.

Shaw, K. J., Rather, P. N., Hare, R. S. and Miller, G. H. (1993) 'Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes', *Microbiological Reviews,* 57(1), pp. 138-163.

Sherertz, R. J. and Sullivan, M. L. (1985) 'An Outbreak of Infections with *Acinetobacter calcoaceticus* in Burn Patients: Contamination of Patients' Mattresses', *The Journal of Infectious Diseases,* 151(2), pp. 252-258.

Sobouti, B., Mirshekar, M., Fallah, S., Tabaei, A., Fallah Mehrabadi, J. and Darbandi, A. (2020) 'Pan drug-resistant *Acinetobacter baumannii* causing nosocomial infections among burnt children', *Med J Islam Repub Iran,* 34, pp. 24.

Sopirala, M. M., Mangino, J. E., Gebreyes, W. A., Biller, B., Bannerman, T., Balada-Llasat, J. and Pancholi, P. (2010) 'Synergy Testing by Etest, Microdilution Checkerboard, and Time-Kill Methods for Pan-Drug-Resistant *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 54(11), pp. 4678-4683.

Stokes, H. W. and Hall, R. M. (1989) 'A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons', *Molecular Microbiology,* 3(12), pp. 1669-83.

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., Pulcini, C., Kahlmeter, G., Kluytmans, J. and Carmeli, Y. (2018) 'Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis', *The Lancet infectious diseases,* 18(3), pp. 318-327.

Temocin, F., Erdinc, F. S., Tulek, N., Demirelli, M., Ertem, G., Kinikli, S. and Koksal, E. (2015) 'Synergistic effects of sulbactam in multi-drug-resistant *Acinetobacter baumannii*', *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology],* 46(4), pp. 1119-24.

Tennstedt, T., Szczepanowski, R., Braun, S., Pühler, A. and Schlüter, A. (2003) 'Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant', *FEMS Microbiology Ecology,* 45(3), pp. 239-252.

Thoma, S. and Schobert, M. (2009) 'An improved *Escherichia coli* donor strain for diparental mating', *FEMS Microbiology Letters,* 294(2), pp. 127-132.

Thomas, C. M. and Nielsen, K. M. (2005) 'Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria', *Nature Reviews Microbiology,* 3(9), pp. 711-721.

Thomas, V. M., Brown, R. M., Ashcraft, D. S. and Pankey, G. A. (2019) 'Synergistic effect between nisin and polymyxin B against pandrug-resistant and extensively drug-resistant *Acinetobacter baumannii*', *International Journal of Antimicrobial Agents,* 53(5), pp. 663-668.

Towner, K. J., Evans, B., Villa, L., Levi, K., Hamouda, A., Amyes, S. G. B. and Carattoli, A. (2011) 'Distribution of Intrinsic Plasmid Replicase Genes and Their Association with Carbapenem-Hydrolyzing Class D Beta-Lactamase Genes in European Clinical Isolates of *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 55(5), pp. 2154-2159.

Trebosc, V., Gartenmann, S., Tötzl, M., Lucchini, V., Schellhorn, B., Pieren, M., Lociuro, S., Gitzinger, M., Tigges, M., Bumann, D. and Kemmer, C. (2019) 'Dissecting Colistin Resistance Mechanisms in Extensively Drug-Resistant *Acinetobacter baumannii* Clinical Isolates', *mBio,* 10(4).

Tripodi, M., Durante-Mangoni, E., Fortunato, R., Utili, R. and Zarrilli, R. (2007) 'Comparative activities of colistin, rifampicin, imipenem and sulbactam/ampicillin alone or in combination against epidemic multidrug-resistant *Acinetobacter baumannii* isolates producing *OXA-58* carbapenemases', *International Journal of Antimicrobial Agents,* 30(6), pp. 537-540.

Turton, J. F., Woodford, N., Glover, J., Yarde, S., Kaufmann, M. E. and Pitt, T. L. (2006) 'Identification of *Acinetobacter baumannii* by Detection of the $bla_{\text{OXA-51-like}}$ Carbapenemase Gene Intrinsic to This Species', *Journal of Clinical Microbiology,* 44(8), pp. 2974-2976.

Velimirov, B. and Ranftler, C. (2018) 'Unexpected aspects in the dynamics of horizontal gene transfer of prokaryotes: the impact of outer membrane vesicles', *Wiener medizinische Wochenschrift (1946),* 168(11-12), pp. 307-313.

Vijayashree Priyadharsini, J., Smiline Girija, A. S. and Paramasivam, A. (2018) 'An insight into the emergence of *Acinetobacter baumannii* as an oro-dental pathogen and its drug resistance gene profile – An in silico approach', *Heliyon,* 4(12), pp. e01051.

Vila, J., Ruiz, J., Goñi, P., Marcos, A. and Jimenez de Anta, T. (1995) 'Mutation in the *gyrA* gene of quinolone-resistant clinical isolates of *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 39(5), pp. 1201-1203.

Virolle, C., Goldlust, K., Djermoun, S., Bigot, S. and Lesterlin, C. (2020) 'Plasmid Transfer by Conjugation in Gram-Negative Bacteria: From the Cellular to the Community Level', *Genes,* 11(11), pp. 1239.

Vu, H. and Nikaido, H. (1985) 'Role of beta-lactam hydrolysis in the mechanism of resistance of a beta-lactamase-constitutive *Enterobacter cloacae* strain to expanded-spectrum beta-lactams', *Antimicrobial Agents and Chemotherapy,* 27(3), pp. 393-398.

Wachino, J., Jin, W., Kimura, K. and Arakawa, Y. (2019) 'Intercellular Transfer of Chromosomal Antimicrobial Resistance Genes between *Acinetobacter baumannii* Strains Mediated by Prophages', *Antimicrobial Agents and Chemotherapy,* 63(8), pp. 10.1128/aac.00334-19.

Wang, H., Wang, J., Yu, P., Ge, P., Jiang, Y., Xu, R., Chen, R. and Liu, X. (2017) 'Identification of antibiotic resistance genes in the multidrug-resistant *Acinetobacter baumannii* strain, MDR-SHH02, using whole-genome sequencing', *International Journal of Molecular Medicine,* 39(2), pp. 364-372.

Wang, X., Du, G., Shi, H., Qiao, Z., Zhang, D. and Pan, X. (2023) 'Environmental concentrations of surfactants as a trigger for climax of horizonal gene transfer of antibiotic resistance', *Heliyon,* 9(6).

Weber, B. S., Ly, P. M., Irwin, J. N., Pukatzki, S. and Feldman, M. F. (2015) 'A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*', *Proceedings of the National Academy of Sciences,* 112(30), pp. 9442-9447.

Weernink, A., Severin, W. P. J., Tjernberg, I. and Dijkshoorn, L. (1995) 'Pillows, an unexpected source of *Acinetobacter*', *Journal of Hospital Infection,* 29(3), pp. 189-199.

Weinstein, R. A. (1991) 'Epidemiology and control of nosocomial infections in adult intensive care units', *The American Journal of Medicine,* 91(3, Supplement 2), pp. S179-S184.

Weinstein, R. A., Gaynes, R., Edwards, J. R. and System, N. N. I. S. (2005) 'Overview of Nosocomial Infections Caused by Gram-Negative Bacilli', *Clinical Infectious Diseases,* 41(6), pp. 848-854.

Wendt, C., Dietze, B., Dietz, E. and Rüden, H. (1997) 'Survival of *Acinetobacter baumannii* on dry surfaces', *Journal of Clinical Microbiology,* 35(6), pp. 1394-1397.

Wilharm, G., Piesker, J., Laue, M. and Skiebe, E. (2013) 'DNA Uptake by the Nosocomial Pathogen *Acinetobacter baumannii* Occurs during Movement along Wet Surfaces', *Journal of Bacteriology,* 195(18), pp. 4146-4153.

Wood, G. C., Jonap, B. L., Maish, G. O., Magnotti, L. J., Swanson, J. M., Boucher, B. A., Croce, M. A. and Fabian, T. C. (2018) 'Treatment of *Achromobacter* Ventilator-Associated Pneumonia in Critically Ill Trauma Patients', *Annals of Pharmacotherapy,* 52(2), pp. 120-125.

Xie, R., Zhang, X. D., Zhao, Q., Peng, B. and Zheng, J. (2018) 'Analysis of global prevalence of antibiotic resistance in *Acinetobacter baumannii* infections disclosed a faster increase in OECD countries', *Emerging Microbes & Infections,* 7(1), pp. 1-10.

Xu, Z., Li, L., Shirtliff, M. E., Alam, M. J., Yamasaki, S. and Shi, L. (2009) 'Occurrence and Characteristics of Class 1 and 2 Integrons in *Pseudomonas aeruginosa* Isolates from Patients in Southern China', *Journal of Clinical Microbiology,* 47(1), pp. 230-234.

Yamamoto, S., Okujo, N. and Sakakibara, Y. (1994) 'Isolation and structure elucidation of acinetobactin., a novel siderophore from Acinetobacter baumannii', *Archives of Microbiology,* 162(4), pp. 249-254.

Yang, H., Hu, L., Liu, Y., Ye, Y. and Li, J. (2016a) 'Detection of the plasmid-mediated quinolone resistance determinants in clinical isolates of *Acinetobacter baumannii* in China', *J Chemother,* 28(5), pp. 443-5.

Yang, Y., Lee, Y., Tseng, K., Huang, W., Chuang, M., Kuo, S., Lauderdale, T. Y. and Chen, T. (2016b) '*In Vivo* and *In Vitro* Efficacy of Minocycline-Based Combination Therapy for Minocycline-Resistant *Acinetobacter baumannii*', *Antimicrobial Agents and Chemotherapy,* 60(7), pp. 4047-4054.

Zavascki, A. P., Goldani, L. Z., Li, J. and Nation, R. L. (2007) 'Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review', *Journal of Antimicrobial Chemotherapy,* 60(6), pp. 1206-1215.

Zhanel, G. G., Lawson, C. D., Adam, H., Schweizer, F., Zelenitsky, S., Lagacé-Wiens, P. R. S., Denisuik, A., Rubinstein, E., Gin, A. S., Hoban, D. J., Lynch, J. P. and Karlowsky, J. A. (2013) 'Ceftazidime-Avibactam: a Novel Cephalosporin/β-lactamase Inhibitor Combination', *Drugs,* 73(2), pp. 159-177.

Zhang, L., Fu, Y., Xu, Q., Yang, Y., He, J., Leptihn, S., Loh, B., Moran, R. A., van Schaik, W., Toleman, M. A., Chen, Q., Liu, L., Yu, Y. and Hua, X. (2022a) 'Co-evolutionary adaptations of *Acinetobacter baumannii* and a clinical carbapenemase-encoding plasmid during carbapenem exposure', *Evol Appl,* 15(7), pp. 1045-1061.

Zhang, T., Guo, Q., Xin, Y. and Liu, Y. (2022b) 'Comprehensive review in moisture retention mechanism of polysaccharides from algae, plants, bacteria and fungus', *Arabian Journal of Chemistry,* 15(10), pp. 104163.

Zhao, Y., Hu, K., Zhang, J., Guo, Y., Fan, X., Wang, Y., Mensah, S. D. and Zhang, X. (2019) 'Outbreak of carbapenem-resistant *Acinetobacter baumannii* carrying the carbapenemase OXA-23 in ICU of the eastern Heilongjiang Province, China', *BMC Infectious Diseases,* 19(1), pp. 452.

Zhou, H., Beltrán, J. F. and Brito, I. L. (2021) 'Functions predict horizontal gene transfer and the emergence of antibiotic resistance', *Science Advances,* 7(43), pp. eabj5056.

Zhu, L. J., Chen, X. Y. and Hou, P. F. (2019) 'Mutation of *CarO* participates in drug resistance in imipenem-resistant *Acinetobacter baumannii*', *Journal of Clinical Laboratory Analysis,* 33(8), pp. e22976.

Čiginskienė, A., Dambrauskienė, A., Rello, J. and Adukauskienė, D. (2019) 'Ventilator-Associated Pneumonia due to Drug-Resistant *Acinetobacter baumannii*: Risk Factors and Mortality Relation with Resistance Profiles, and Independent Predictors of In-Hospital Mortality', *Medicina*, 55(2). DOI: 10.3390/medicina55020049.