Characterisation of the resistome and virulome of *Pseudomonas aeruginosa* isolates from patients with ventilator-associated pneumonia

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Project submitted in partial fulfilment of MSc Degree in Molecular Biology and Biomedicine

October, 2020

Abstract

Introduction/objective: Ventilator-associated pneumonia (VAP) is the hospital-acquired infection with the greatest impact on patient outcomes and health care costs. Endogenous colonization by aerobic gram-negative bacteria such as *Pseudomonas aeruginosa* play a pivotal role in the pathogenesis of VAP. The aim of this work was the characterisation of the resistome and virulome of a collection of *P. aeruginosa* isolates from patients with VAP collected during a multicentre study.

Material and methods: We analysed 38 isolates (1 per patient) of *P. aeruginosa* from patients with VAP. Minimum inhibitory concentrations (MICs) of 16 different antibiotics were determined by broth microdilution according to the Clinical and Laboratory Standard Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints. The complete genomes of all the isolates were sequenced using the MiSeq platform (Illumina) to determine their clonal relationship, as well as the presence of antibiotic resistance and virulence related genes.

Results: According to antibiotic resistance, 10.5% (n=4) of the isolates were multi-susceptible and 23.7% (n=9) moderately resistant, while 21.1% (n=8) were multidrug-resistant and 44.7% (n=17) were extensively resistant. Forty-five genes related with antibiotic resistance were detected: $bla_{\text{GES-7}}$ and bla_{VIM} (1,2 and 20) were observed on extensively resistant isolates. According to the results obtained from the Virulence finder database (VFDB), the virulome of the whole population was made up of 294 genes, among which the most significant were the effectors (*exo*-) from the type III secretion system.

Index

1.-Introduction

1.1-Ventilator-Associated Pneumonia

Pneumonia is defined as an inflammatory condition of the lungs, caused mostly by bacteria or virus. Affected people usually report cough, chest pain, fever, and difficulty for breathing.

Ventilator-Associated Pneumonia (VAP) is defined as a pneumonia that appears 48h+ after the initiation of mechanical ventilation or endotracheal intubation (Davis; 2006). It is the most acquired infection in the intensive-care-unit with an incidence sometimes greater than 50% (Koenig & Truwit; 2006). The wall of the tubes inserted into the patient body allow for a great development of different pathogens, mostly bacteria, that will continue their way up to the lungs of the individual, causing this infection.

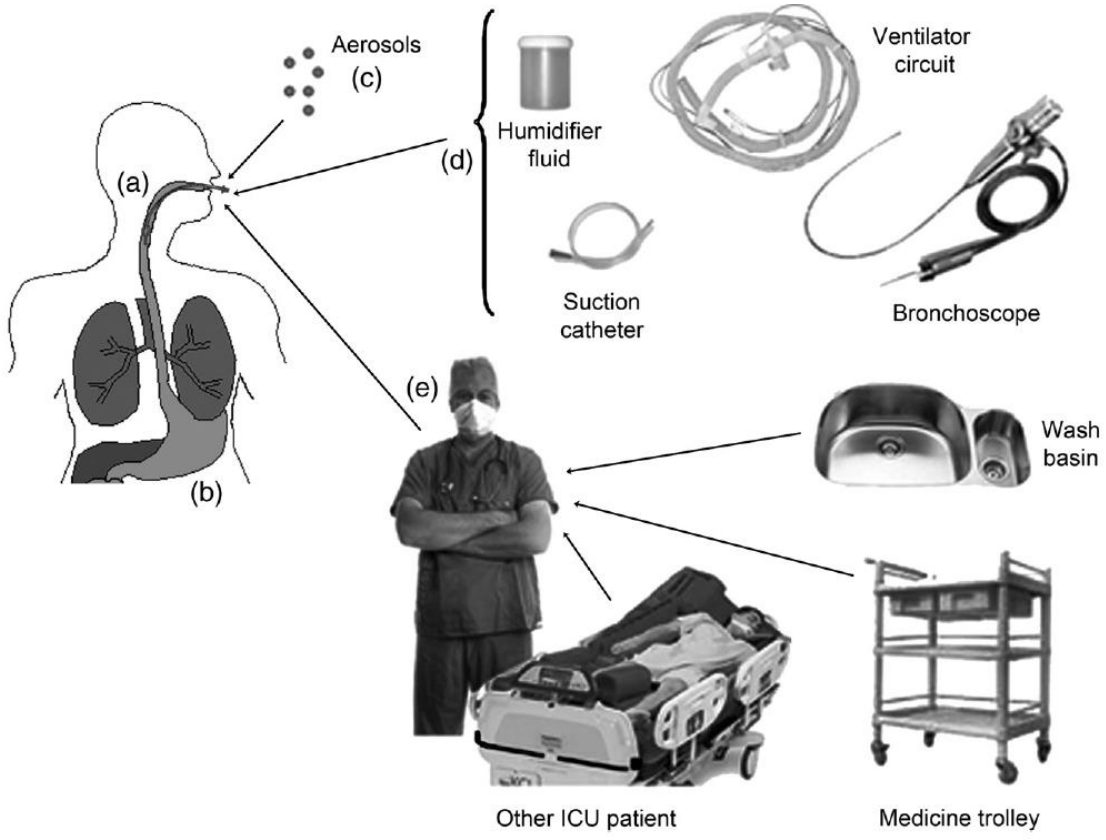

Figure 1: Source of VAP. pathogens which lead to the infection. (a) & (b) represent endogenous sources (oropharyngeal and gastric colonization), (c), (d) & (e) exogenous ones (contaminated air, instruments, or other medical materials). (Joseph et al; 2010)

There are many risk factors related to this condition, being burnt patients the most problematic one, as they tend to get infected more consistently and usually have worse prognosis. Age, immunosuppression, organ failure, and post-traumatic situations should also be considered.

Table 1: Complete list of risk factors on VAP. Divided on host or intervention factors. (Joseph et al; 2010)

Host factors	Intervention factors
Oro-pharyngeal colonization	Emergency intubation
Gastric colonization	Re-intubation
Thermal injury (Burns)	Tracheostomy
Post-traumatic	Bronchoscopy
Post-surgical	Nasogastric tube
Impaired consciousness	Duration of hospital stay/ICU stay
Immunosuppression	Multiple central venous line insertions
Organ failure	Sedatives
Sinusitis	Stress ulcer prophylaxis
Severity of underlying illness	Prior antibiotics/no antibiotic prophylaxis
Old age (≥60 years)	Immunosuppressives (Corticosteroids)
Presence of comorbidities	Supine head position

MV = mechanical ventilation; ICU = intensive care unit.

The most common pathogens causing VAP are *Pseudomonas* species, *Acinetobacter* species, and Gram-negative bacilli expressing Extended Spectrum β-lactamases (ESBL) and AmpC β-lactamases. Due to these characteristics these bacteria are known as multridug-resistant (MDR) pathogens (Trouillet et al; 1998). Prior hospitalization or antibiotic treatment within the past 90 days predisposes to colonization and infection by MDR pathogens. Usually VAP is caused by more than 1 microorganism, between 30 and 70% of the cases are polymicrobial (Torres & Carlet; 2001).

In order to understand how to better treat this kind of infections, we must know first who we are fighting against. The complex resistance mechanisms and virulence factors seen on *Pseudomonas aeruginosa* will be explained briefly in this work.

1.2-*P. aeruginosa*

Pseudomonas aeruginosa is a Gram-negative rod-shaped gamma-proteobacterium. It can be found in many ecological niches due to its metabolic versatility. It is capable of colonizing different living beings such as plants, animals, and humans. It is associated with a wide range of acute and chronic infections such as cystic fibrosis (CF), VAP, urinary tract infections, otitis, burn injuries and bacteremia.

P. aeruginosa is one of the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa,* and *Enterobacter* species), which confirms its impact on hospital infections and how capable it is to "escape" the activity of a wide range of antibiotics. This species has also been included in the World Health Organization (WHO) list of pathogens under the "critical" category for which new antibiotics research is urgently required. The United States Center for Disease Control and prevention (CDC) flagged *P. aeruginosa* as a serious threat.

Figure 2: P. aeruginosa is considered one of the most important bacterial pathogens affecting human populations according to a big number of organizations like WHO or CDC. (Botelho et al; 2019)

Metabolic versatility, a huge number of virulence factors, the formation of biofilm, and its antibiotic resistance (AR) are the pillars of *P. aeruginosa* pathogenicity. All of them will be explored in this work.

1.3-Antibiotic resistance on *P. aeruginosa*

Antibiotics can be both a really powerful weapon against pathogens and the last ingredient needed to create a pan-resistant bacteria depending on the proper application. Its inappropriate use has promoted the appearance of AR in most bacteria. The WHO made a report with data from more than 100 countries showing that AR is present everywhere (WHO; 2015). It also lists AR as one of the top risks the world will face in the near future. Around 700.000 people die every year because of AR infections worldwide (O´Neill; 2016). According to the latest European Centre for Disease Control (ECDC) AR report, 12.9% of *P. aeruginosa* isolates have resistance to three or more antibiotic groups (ECDC; 2017). Eight families of antibiotics are generally used for treatment of infections provoked by *P. aeruginosa*:

Table 2: Different antibiotic families, their action mechanism and resistance mechanisms developed by P. aeruginosa to counteract them.

Antibiotic	Action mechanism	Resistance mechanism
Monobactam **AZT**	Inhibition of mucopeptide synthesis in the bacterial cell wall blocking peptidoglycan crosslinking	Inactivating β-lactamases
Carbapenem **IMP, MER, DOR**	Inhibition of mucopeptide synthesis in the bacterial cell wall blocking peptidoglycan crosslinking	Inactivating β-lactamases Alteration of permeability of the membrane (mutations of OprD) Efflux pumps
Penicillin **PIP, TZP**	Inhibition of mucopeptide synthesis in the bacterial cell wall blocking peptidoglycan crosslinking	Inactivating β-lactamases
Cephalosporines **FEP, CAZ**	Inhibition of mucopeptide synthesis in the bacterial cell wall blocking peptidoglycan crosslinking	Inactivating β-lactamases
Aminoglycosides **GEN, TOB, AMK, NET**	Protein synthesis inhibitor losing the functional integrity of the bacterial cell membrane	Aminoglycoside modifying enzymes (AMEs)
Fluoroquinolones **LEV, CIP**	Interaction with DNA gyrase	Mutations in DNA gyrase subunit A
Polymyxins **COL**	Disruption of the bacterial cell membrane by surfactant reaction	LPS modifications, efflux pumps, capsules, overexpression of OprH
Phosphoric acid **FOS**	Inhibition of bacterial cell wall biogenesis by inactivating MurA enzyme	Mutations that inactivate the glycerophosphate transporter Glyoxalase enzymes, FosC enzymes

Abbreviations: AZT = Aztreonam; IMP = Imipenem; MER = Meropenem; DOR = Doripenem; PIP = Piperacillin; TZP = Piperacillin - Tazobactam; FEP = Cefepime; CAZ = Ceftazidime; GEN = Gentamicin; TOB = Tobramycin; AMK = Amikacin; NET = Netilmicin; LEV = Levofloxacin; CIP = Ciprofloxacin; COL = Colistin; FOS = Fosfomycin.

Pseudomonas aeruginosa shows resistance to a wide number of antibiotics, including aminoglycosides, quinolones, and β-lactams (Hancock and Speert; 2000). In order to classify the different mechanisms that *P. aeruginosa* has developed to fight antibiotic attacks we will use three different categories:

Intrinsic antibiotic resistance

The intrinsic antibiotic resistance of a bacterial species can be defined as the inherent ability to block partially or completely the efficacy of a given antibiotic by structural or functional characteristics (Blair et al; 2015). *P. aeruginosa* has three mechanisms which provide great resistance to most antibiotics intrinsically:

- **Outer membrane permeability:** Most antibiotics need to penetrate the cell membrane in order to work (Aminoglycoside by binding to 30S subunits (Mingeot-Leclercq et al; 1999), quinolones by inhibiting DNA gyrase and topoisomerase (Aldred et al; 2014), β-lactams by blocking cell wall biosynthesis (Poole; 2004), Polymyxins by binding to lipopolysaccharides (LPS) of the outer membrane (Zavascki et al; 2007)). *P. aeruginosa* outer membrane is an asymmetric bilayer of phospholipid and LPS, with embedded β-barrel protein channels called porins (Delcour; 2009). Porins can be divided into four classes, depending on the substrates they work with. Regarding the topic of this work the most important ones are efflux porins, which are key components of efflux pumps, in P. aeruginosa, efflux porins include the following main proteins: OprM, OprN, and OprJ (Hancock & Brinkman; 2002). Also, *P. aeruginosa* membrane has an extremely low permeability, around 100-fold lower than E. coli K12 (Yoshimura et al; 1982). OprF protein is the main porin of *P. aeruginosa* responsible for non-specific uptake of ions and saccharides and it has a low efficiency on antibiotic uptake, this is caused by the fact that this protein has 2 conformational states, being the closed one the most common (about 95%) allowing only for a 5% of open channels (Bellido et al; 1992).

- **Efflux systems:** Out of the five families of efflux pumps; Resistance-Nodulation-Division (RND) plays a key role in *P. aeruginosa* antibiotic resistance and thus is the one we are going to focus on in this work. They are made of 3 components: Cytoplasmic membrane transporters, periplasmic linker proteins and outer membrane porin channel proteins. The cytoplasmic and periplasmic components of this pumps are named multidrug efflux (Mex) along with a letter, and the porin is named Opr along with a letter as we have already seen above. Out of the 12 pumps among this family, 4 are capable of pumping out the different drugs (Dreier & Ruggerone; 2015). Overexpression of this

RND family efflux pumps has been found in some clinical strains of *P. aeruginosa*, contributing to the development of multidrug-resistance (Cabot et al; 2011).

Table 3: Major RND multridug efflux pumps of P. aeruginosa and their substrates. (El Zowalaty et al; 2015)

System	Regulatory gene	Cytoplasmic membrane transporter protein	Membrane fusion protein	Outer membrane efflux protein	Mutation causing upregulation	Substrates
MexAB-OprM	*mexR*	MexB	MexA	OprM	nalB (affects *mexR*) and *nalC* (lies outside *mexR*)	BL, FQ, CM, TC, NV, TP, SM, ML, EB, AC, CV, SDS, AH, HL, CL, TL, IR, TS
MexCD-OprJ	*nfxB*	MexD	MexC	OprJ	*nfxB*	BL, FQ, CM, TC, NV, TP, ML, CV EB, AC, SDS, AH, CL, TS
MexEF-OprN	*mexT*	MexF	MexE	OprN	*nfxC*	FQ, CM, TP, AH, TS
MexXY-OprM	*mexZ*	MexY	MexX	OprM	ParRS[§]	FQ, AG, TC, ER

AC: Acriflavine; AG: Aminoglycosides; AH: Aromatic hydrocarbons; BL: β-lactams; CL: Cerulenin; CM: Chloramphenicol; CV: Crystal violet; EB: Ethidium bromide; ER: Erythromycin; FQ: Fluoroquinolones; HL: Homoserine lactones; IR: Irgasan; ML: Macrolides; NV: Novobiocin; SDS: Sodium dodecyl sulphate; SM: Sulphonamides; TC: Tetracycline; TL: Thiolactomycin; TO: Toluene; TP: Trimethoprim; TS: Triclosan.

- **Antibiotic-inactivating enzymes:** One of the major mechanisms of intrinsic resistance seen in bacteria. Most antibiotics have chemical bonds susceptible to hydrolysis by enzymes commonly found on *P. aeruginosa* like β-lactamases (Wolter & Lister; 2013). ampC is the gene encoding this enzyme, it will break the amide bond of the β-lactam leading to its inactivation. β-lactamases can be classified into four groups A, B, C, and D based on their amino acid sequences. Some strains of *P. aeruginosa* have been found to produce ESBLs conferring great resistance to most β-lactam antibiotics, including penicillins, cephalosporins and aztreonam (Paterson & Bonomo; 2015). Antibiotic-inactivating enzymes are probably the most important method of resistance seen on *P. aeruginosa* and will be examined in this work.

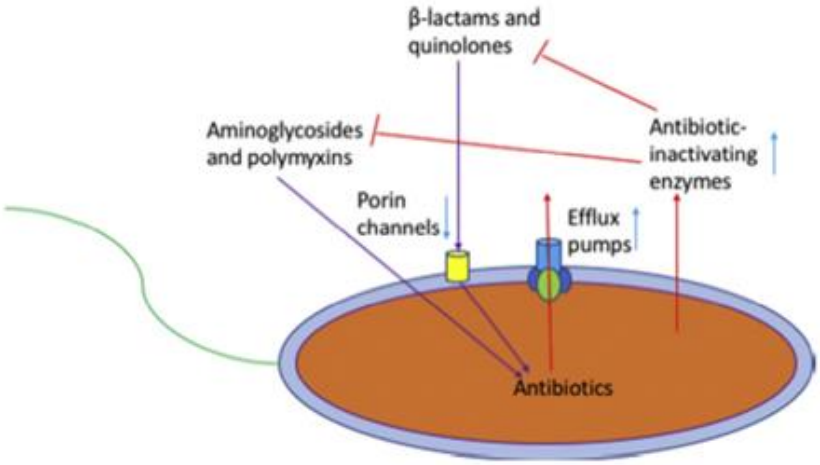

Figure 3: Intrinsic resistance mechanisms on P. aeruginosa. Restricted outer membrane permeability, pump efflux systems, and inactivating enzymes work all together to avoid being affected by the drugs. (Pang et al; 2019)

Acquired antibiotic resistance

There are two mechanisms by which *P. aeruginosa* can gain this kind of resistance: Mutational changes and horizontal gene transfer. Both methods contribute to the already high intrinsic antibiotic resistance of this bacteria increasing the difficulty eradicating the microorganism and allowing for more persistent infections (Henrichfreise et al; 2007):

- **Resistance by mutation:** Mutational changes allow for reduced antibiotic penetration, modification of targets and overexpression of efflux pumps or inactivating enzymes. For example, mutations on OprD allow *P. aeruginosa* to resist extremely well against carbapenems by modifying its binding site on the porins (Fang et al; 2014) also the overexpression of MexAB-OprM enhances the resistance against β-lactams and fluoroquinolones by increasing the production of RND efflux pumps (Tian et al; 2016). Detailed mechanism on *Figure 4*.

- **Acquisition of resistance genes:** These genes can be carried on plasmids, transposons, integrons, prophages and the chromosomes. They can be exchanged with other species of bacteria and not only *P. aeruginosa* (Breidenstein et al; 2011). The most interesting example is probably metallo-beta-lactamases (MBLs) a group of enzymes (class B β-lactamases) that hydrolyze most β-lactam-based antibiotics. These MBLs, which are not originally found on *P. aeruginosa* have been detected in integrons as well as plasmids of this species (Cavalcanti; 2015). Mechanisms by which this process takes place can be seen on *Figure 5*.

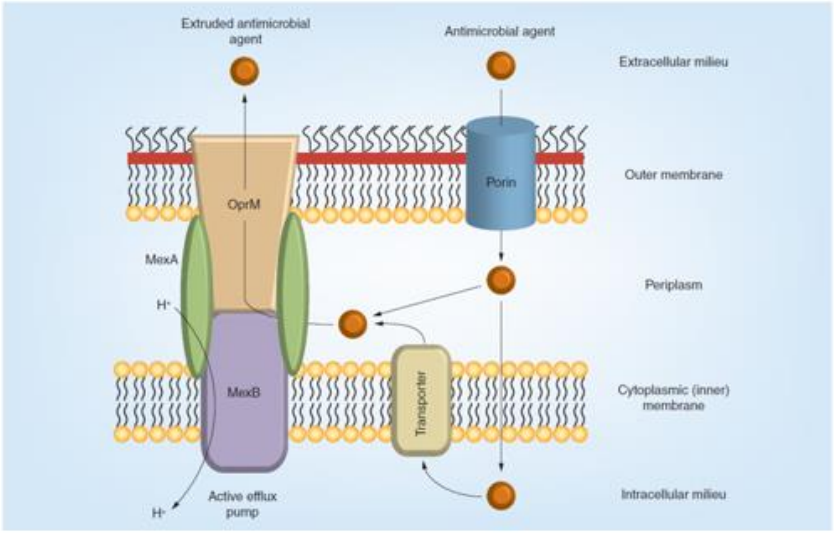

Figure 4: Scheme of the RDN family efflux pump seen on P. aeruginosa. Antibiotics are sent off the cell with the energy of the proton-motive force through OprM channel protein. (El Zowalaty et al; 2015)

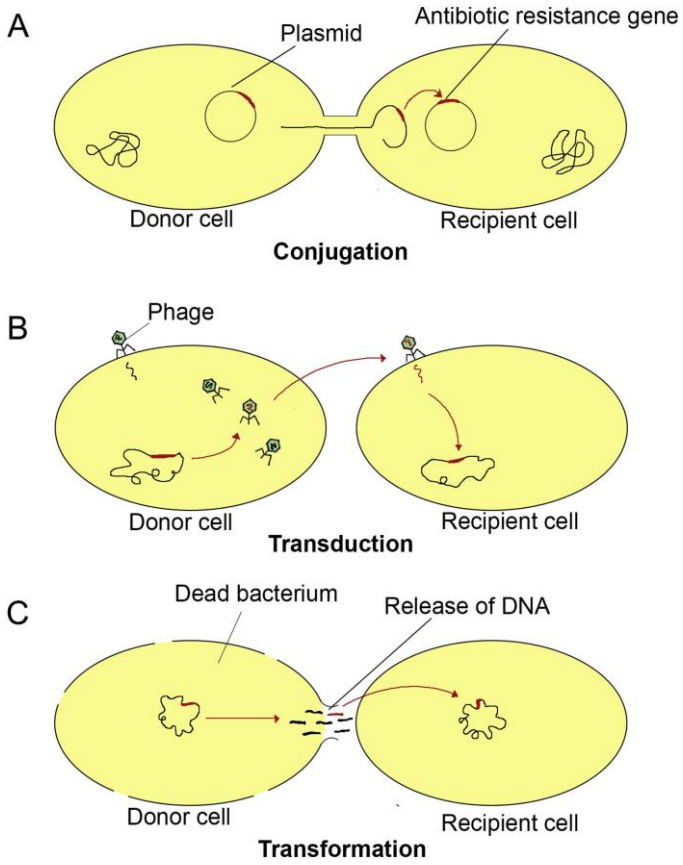

Figure 5: Mechanisms of horizontal gene transfer between bacteria. A) Conjugation involves direct contact between two individuals for DNA transfer. B) Transduction requires a bacteriophage to transfer the DNA. C) Transformation requires the release of DNA fragments from the donor cell that are later picked up from the medium by the recipient cell. (Pang et al; 2019)

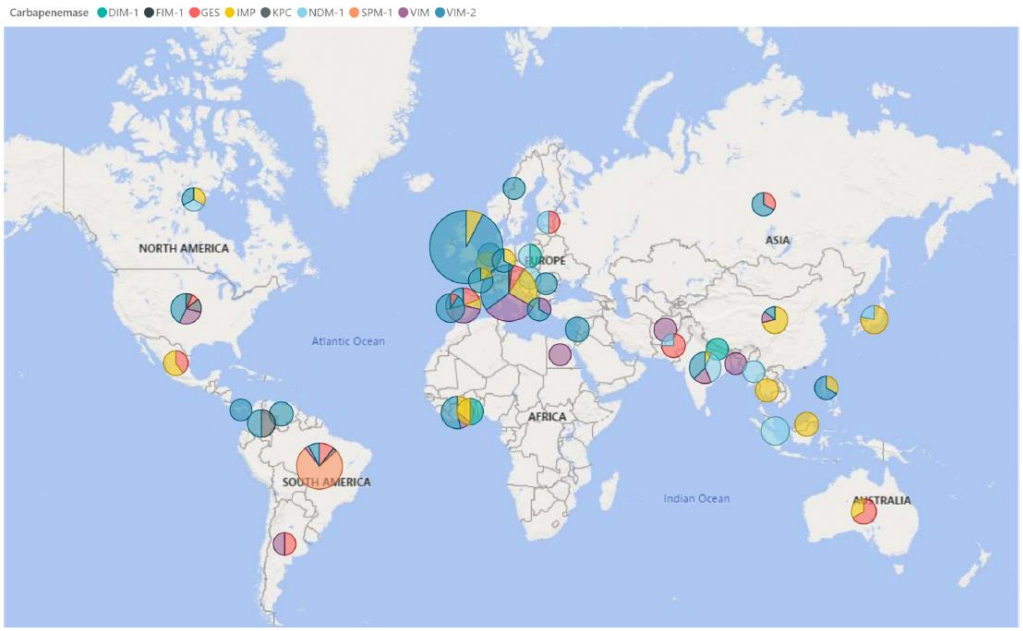

Figure 6: Different carbapenemases found on P. aeruginosa isolates from NCBI database. Each colour represents a family. In Spain we can find 4 different families: VIM, DIM-1 GES, and IMP (from more common to scarcer). (Botelho et al; 2019)

Adaptive antibiotic resistance

It is defined as the ability to resist an antibiotic attack by the alteration of gene and/or protein expression due to an environmental stimulus. It must be reversible in order to be considered as an adaptive response (Sandoval-Motta & Aldana; 2016). In *P. aeruginosa*, 2 strategies have been widely characterized:

- **Biofilm-mediated resistance:** A biofilm is an aggregate of microorganisms (mostly bacteria) on top of a living or non-living surface. Biofilm is made of a matrix where cells are embedded, mostly made of extracellular polymeric substances (EPS) which includes exopolysaccharides, proteins, DNA fragments and other metabolites (Donlan; 2002). Biofilm protects bacteria from antibiotics by preventing its penetration and slowing down the metabolism or inducing stress responses of the cells inside of it. In *P. aeruginosa*, biofilm formation is multifactorial. Quorum sensing (Bacterial cells communicate through metabolites and react to cell density altering gene expression) as well as exopolysaccharides and cdi-GMP coordinate this process. Once the cells take part in the biofilm structure, they begin to change their physical and physiological shape (Drenkard; 2003). Alginate production gets upregulated to create the matrix and flagellum gets downregulated as the cells will not move anymore. This makes it harder for the immune system to detect *P. aeruginosa (*Jyot et al; 2007), (*Figure 7).*

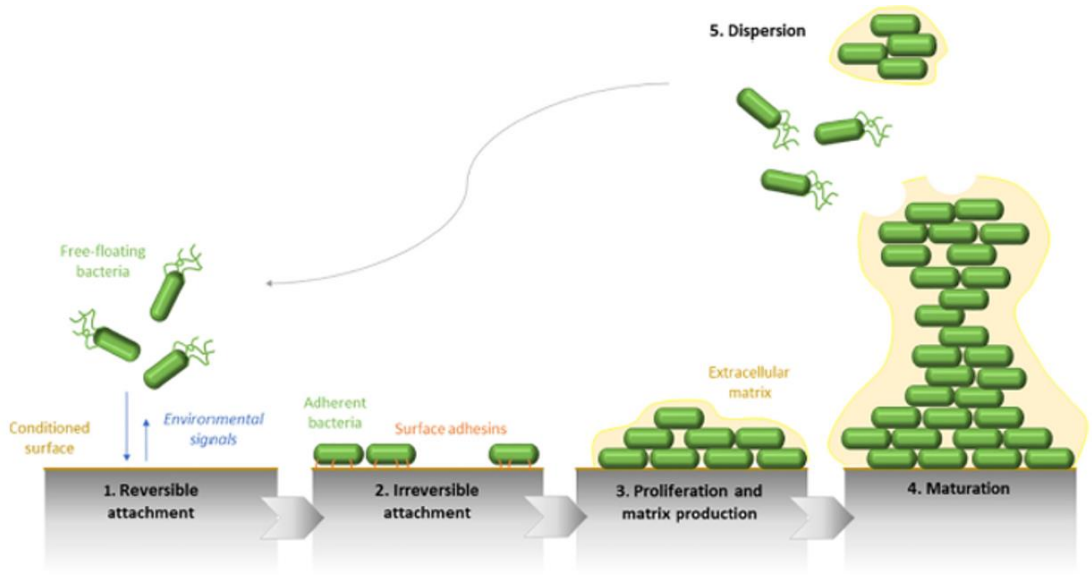

Figure 7: Scheme of the basic P. aeruginosa biofilm formation process. Motile cells attach themselves to a surface in a reversible way, after that they get attached completely through adhesins to form a monolayer. These cells will produce the matrix and multiply creating complex 3D structures known as mature biofilm. Finally, some cells leave the biofilm to create a new one where good conditions are met. (Olivares et al; 2020)

- **Persister cells in antibiotic resistance:** These cells are phenotypical variants without any genetical antibiotic resistance but with high tolerance due to its almost inexistent metabolism. Around 1% of the *P. aeruginosa* individuals inside a biofilm are persister cells (Wood et al; 2013). While most of the cells can be killed with antibiotics, this special kind can resist the attack by shutting down the synthesis of the antibiotic targets and entering dormancy (Van den Bergh et al; 2017). Persister cells are not able to grow under presence of antibiotics but will resume its growth once the treatment is over, that is why it is believed that they are responsible for chronic infections (Maisonneuve & Gerdes; 2014). They should not be confused with cells that are resistant due to mutations (*Figure 8*).

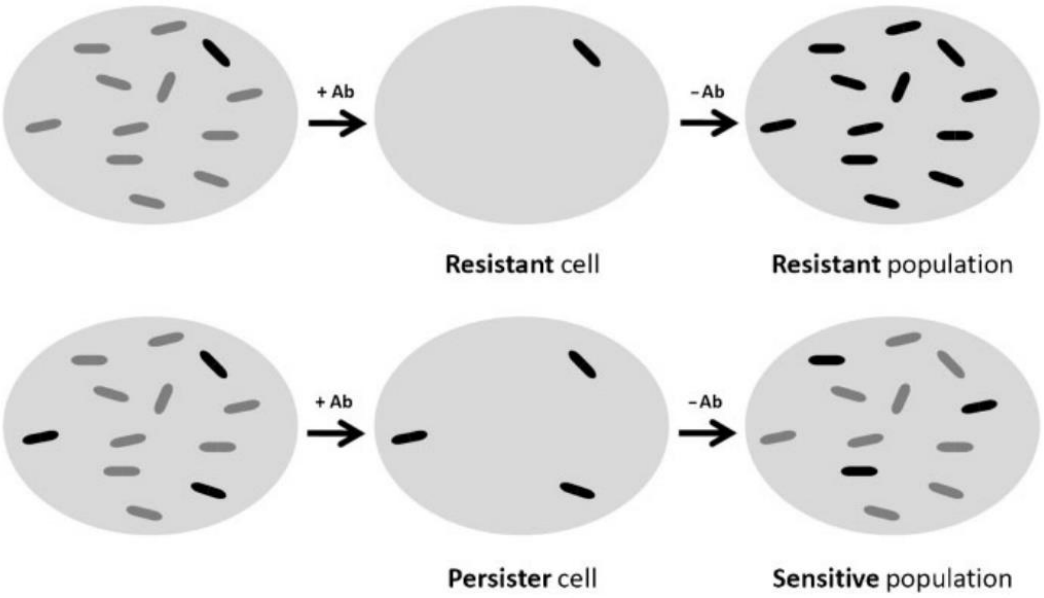

Figure 8: Comparison between resistant and persister cells. Top panel shows a bacterial population with resistant individual reacting to an antibiotic, only resistant cells will survive and thus replenish the population later on. Lower panel shows a bacterial population with persister cells, once the treatment has finished, they will multiply and colonize the environment again, but with bacteria that are not resistant to the antibiotic previously applied. (Fauvert et al; 2011)

1.4-Virulence factors on *P. aeruginosa*

Several virulence factors can be found in different bacteria, these will facilitate adhesion and/or disrupt host cell signalling pathways. *P. aeruginosa* is known of having a wide number of them: LPS, Flagellum, Type IV Pili, Type I, II, III, V and VI Secretion Systems, Proteases, Alginate, Quorum Sensing and Biofilm formation being the most known (Rocha et al; 2019). The most important ones will be resumed in the following pages:

- **LPS:** Consists of 3 parts: Lipid A, which is the hydrophobic portion that anchors the molecule in the outer membrane. The core, which is made mostly of hexoses and hexosamines and due to its negative charge provides membrane stability, and lastly, the O antigen, which is the long polysaccharide ranging from one to hundreds of sugars (Huszczynski et al; 2020). It is synthesized separately from the Lipid A-core and then attached to it. LPS is an inducer of the complement system thus producing different cascades of inflammation on the host (Schreiber et al; 1993). There are some strains that lack O antigen, which makes them less aggressive against the host as O antigen protects bacteria from phagocytosis and antibodies reaching the surface of the cell (Engels et al; 1985).

- **Type I Secretion System:** T1SS displays the typical ATP binding cassete-exporter secretion complex, capable of exporting proteins out of the cell. Little is known about the importance of this system on the virulence of *P. aeruginosa*, but an alkaline protease (AprA) uses this system to reach the environment (Guzzo et al; 1991).

- **Type II Secretion System:** T2SS secretes a wide variety of toxins: LasA and LasB proteases, phospholipase H, lipolytic enzimes and the most important one in which we will focus: Exotoxin A. It is a protein of 638 amino acids coming from PE gene and divides in several structural and functional domains. Once it is secreted from the bacteria, Exotoxin A will bind to KDEL receptors of the Golgi apparatus of the host (Hessler & Kreitman; 1997), later, following two different pathways, the toxin will reach its objective: Endoplasmic Reticulum (ER). After reaching ER, the toxin activates a protein degradation route to be freed at the cytosol (Ogata et al; 1992). In the cytosol, the toxin binds to the ribosomes activating its enzymatic activity, producing the ADP-Ribosylation of eukaryotic elongation factor-2 (eEF-2) thus blocking protein synthesis and inducing apoptosis.

- **Type III Secretion System:** T3SS is made of thirty-six genes encoded in five operons clustered together in the P. aeruginosa chromosome. Another six different genes encode for the effector proteins and their chaperones. Only 4 effectors have been characterised on P. aeruginosa: ExoS, ExoU, ExoT, and ExoY. Nearly all strains have either the *exoS* or the *exoU* gene and both the *exoT* and the *exoY*. They will be briefly described now based on information from *Table 4*.

Table 4: Characteristics of Pseudomonas aeruginosa T3SS efector proteins. (Hauser; 2009)

Effector protein	Size (kDa)	Chaperone	Substrates	Cofactor for activation
ExoS	48	SpcS	GAP activity: Rho, Rac and CDC42; ADPRT activity: ezrin, radixin, moesin, vimentin, cyclophilin A, IgG_3, apolipoprotein A1, RAS, RAC1, CDC42, RAB1, 3, 5, 7, 8 and 11, RALA, RAP1 and RAP2	14-3-3 proteins
ExoT	49	SpcS	GAP activity: Rho, Rac and CDC42; ADPRT activity: CRKI, CRKII and phosphoglycerate kinase	14-3-3 proteins
ExoU	74	SpcU	Phospholipids, lysophospholipids and neutral lipids	SOD1
ExoY	42	Unknown	ATP	Unknown

ADPRT, ADP ribosyl transferase; CDC42, cell division cycle 42; CRK, CT10 regulator of kinase; GAP, GTPase-activating protein; Ig, immunoglobulin; SOD1, Cu^{2+}, Zn^{2+}-superoxide dismutase; T3SS, type III secretion system.

1. **ExoS:** It is a bifunctional toxin that has both GTPase-activating protein (GAP) and ADP ribosyl trans-ferase (ADPRT) activity. The GAP domain targets Rho, Rac and cell division cycle 42 (CDC42) which are small GTPases related to the organization of the actin cytoskeleton in the host cell. ExoS inactivates all of them leading to the disruption of the cytoskeleton (Pederson et al; 1999) It has been suggested that this has a role in avoidance of phagocytosis. ADPRT domain has a binding site for a eukaryotic cofactor needed for ADPRT activity. This protects *P. aeruginosa* from attacking itself with such a potent weapon. Once active, ExoS has several adverse effects: Cell death, actin cytoskeleton disruption, and inhibition of DNA synthesis (Rocha et al; 2003).

2. **ExoT:** It shares 76% amino acid identity with ExoS. It has the same GAP and ADPRT activity as ExoS. The enzymatic activities of this enzyme have been linked with delays in wound healing, which can allow *P. aeruginosa* to exploit breaches in mucosal barriers more strongly (Garrity-Ryan et al; 2004).

3. **ExoU:** It is a potent phospholipase, capable of causing fast cell death. Like ExoS or ExoT it requires an eukaryotic cofactor in order to work. It has been found that it binds to Superoxide dismutase (SOD1), but its enzymatic activity is not needed for

ExoU to work (Sato et al; 2003). Cell death caused by this effector is produced by the breaking off the membrane which is consistent with necrosis (Finck-Barbançon et al; 1997).

4. **ExoY:** It is considered as an adenylyl cyclase. It binds to ATP and also requires a host cell factor for full enzymatic activity. If injected on mammalian cells an elevation on cAMP is observed (Yahr et al; 1998). This effect disrupts the actin cytoskeleton while increasing endothelial permeability. This is yet to be observed on real situations and the importance of ExoY in infection remains unclear.

- **Type V Secretion System:** T5SS works in a very similar way to T2SS, it is widespread on Gram negative bacteria and it can secret a wide number of proteins. Recently PlpD enzyme was described. This enzyme has great phospholipase activity allowing for the recognition of many phosphatydilinositols, key components of eukaryotic cell membrane thus allowing for an easier colonization (da Mata Madeira et al; 2016).

- **Type VI Secretion System:** The latest secretion system described. Works like a needle, inyecting toxins inside the cells with whom it makes contact. It is believed that it helps during competition with other bacteria in the environment. It allows of a better internalization on epithelial cells. Its structure is very similar to the dynamic contractile tail of a phage (*Figure 9*). Different effectors can be found: Tse1 and 3 which acts by degrading peptidoglycan and thus dissolving other cells, Tse2 which targets bacterial cytoplasm inhibiting growth and PldA/B, lipases that degrade phosphatidylethanolamine and phosphatidylinositol respectively, In order to avoid being attacked by its own effectors, *P. aeruginosa* has antitoxins for each of this proteins (Chen et al; 2015).

- **Biofilm:** It has already been briefly summarized under the Resistance methods of P. aeruginosa. Biofilm allows the survival of the cells inside of it because of its extracellular matrix, that does not let antibiotics pass through. Also, the conditions found inside the biofilm makes the perfect environment for the arise of persister cells, even more resistant to drugs due to its almost zero metabolism. *P. aeruginosa* is notorious for causing pneumonia on CF patients, biofilms settle in the mucus and induce inflammation by recruiting polymorphonuclear leukocytes (Murray et al; 2007). The chronic inflammatory response causes tissue damage and leads to lung failure (Tolker-Nielsen; 2014). It also allows for infections in the ears, prostate gland, wounds, and works together with T3SS and other virulence factors to avoid being attacked by the host immune system.

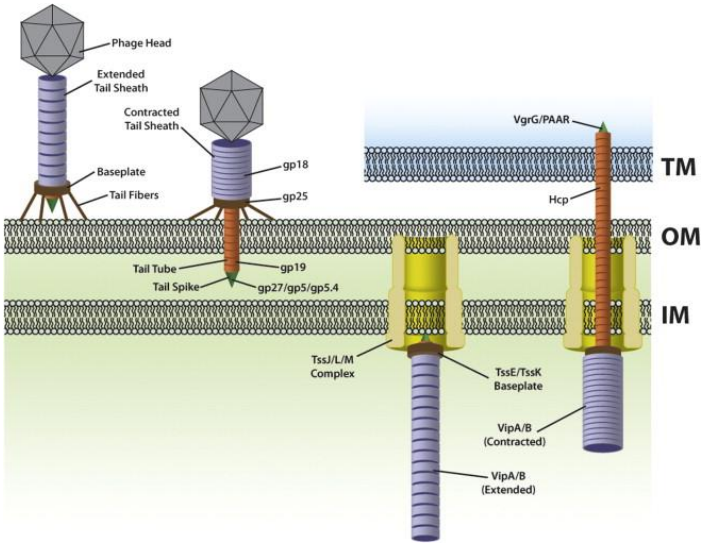

Figure 9: Similarities between phage tails and T6SS complex. Most of the structural proteins found on this secretion system are believed to come from the bacteriophages. While phages contract to inject DNA, T6SS does it to inject certain toxins. (Ho et al; 2014)

2.-Aims of the study

The main goal of this study was to characterize the resistome and virulome of *P. aeruginosa* clinical isolates from patients with Ventilator Associated Pneumonia (VAP).

Specific aims:

1. To study the in vitro activity of antimicrobials of clinical interest using a standardized method against an established collection of *P. aeruginosa* strains isolated from patients with VAP.

2. To determine the clonal relationship between the different clinical isolates of *P. aeruginosa* by multilocus sequence typing.

3. To Sequence the complete genomes of the *P. aeruginosa* isolates from patients with VAP in order to identify the presence of acquired antibiotic resistant genes such a as carbapenemase-coding genes, as well as the distribution of different virulence associated genes.

3.-Materials and Methods

3.1-Bacterial Isolates, Medium and Culture Conditions

Out of a 200+ collection of *P. aeruginosa* non duplicated isolates from patients with VAP collected from different hospitals across Spain, 38 were selected for analysis. Bacteria were routinely grown on Mueller-Hinton agar at 37ºC (typical human body temperature), which is suitable for *P. aeruginosa* growth and standardized for most testing procedures. Once an isolate was received, it was grown in a Mueller-Hinton agar petri dish to confirm its isolation and stored at -80ºC in glycerol until it was needed.

3.2-Antibiotic Susceptibility Testing

Minimum Inhibitory Concentration (MIC) of aztreonam (AZT), ceftazidime (CAZ), cefepime (FEP), imipenem (IMP), meropenem (MER), doripenem (DOR), piperacillin (PIP), piperacillin – tazobactam (TZP), gentamicin (GEN), tobramycin (TOB), amikacin (AK), levofloxacin (LEV), ciprofloxacin (CIP), colistin (COL), polymyxin B (PB) and fosfomycin (FOS) were determined by the broth microdilution method according with CLSI guidelines (CLSI, 2018), *Table 2*. European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints were followed when interpreting results (EUCAST; 2020).

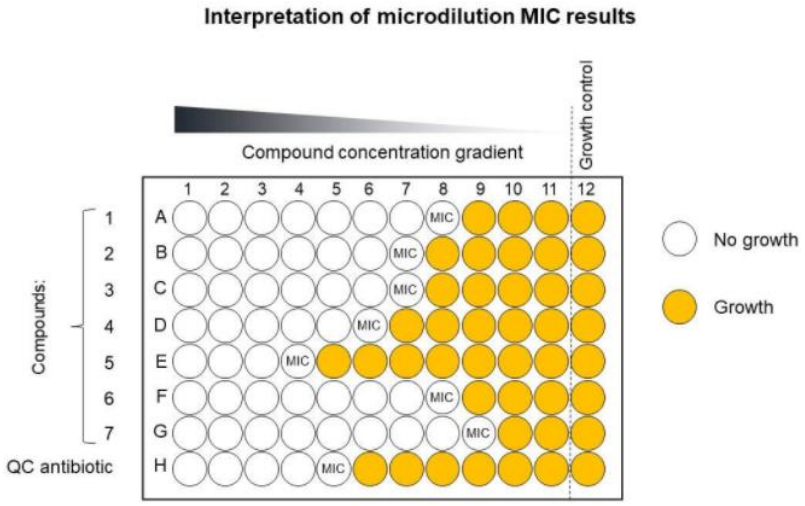

Figure 10: Example of a microdilution essay result. Bacterial growth is shown in yellow colour and the absence of it is white. There is a gradient on the concentration of the antibiotic, halving it on each well from left to right. Each line represents a different drug. The MIC will be found on the well that shows zero growth but is followed by one with bacterial growth. (Emery Pharma)

3.3-Quantification of Biofilm Production by Crystal Violet Staining

Biofilm formation was evaluated by means of the crystal violet staining assay as described before (Merritt et al; 2005). Bacterial isolates were grown overnight in Mueller-Hinton broth (MHB) at 37 °C with shaking. Optical density (OD) of bacterial cultures was measured at 620nm using a plate reader (Infinite® 200 PRO, Tecan) and adjusted with MHB to an OD= 0.1.

Biofilms were developed in 96 well plates. One hundred microliters of the adjusted cultures were placed in each well and statically incubated overnight at 37°C. The following day, planktonic bacteria were removed, and wells were washed three times with milliQ water. Plates were air dried for approximately 20 minutes and then 100µL of a 0.7% crystal violet solution (w/v) were added to the wells. After 15 minutes wells were rinsed three times with milliQ water to remove excess stain. One hundred microliters of a 33% acetic acid solution (v/v) was added to the wells and plates were incubated at room temperature until crystal violet-stained biofilms were completely solubilised. The amount of dye (proportional to the density of adherent cells) was determined at 620nm. Results were corrected for background staining by subtracting the value for crystal violet bound to uninoculated MHB control wells. Normalized biofilms were calculated by dividing the total biofilm value (expressed as the OD_{620}) by the bacterial growth for each isolate (expressed in CFUs). The biofilm assay was performed three times, with duplicates in each assay.

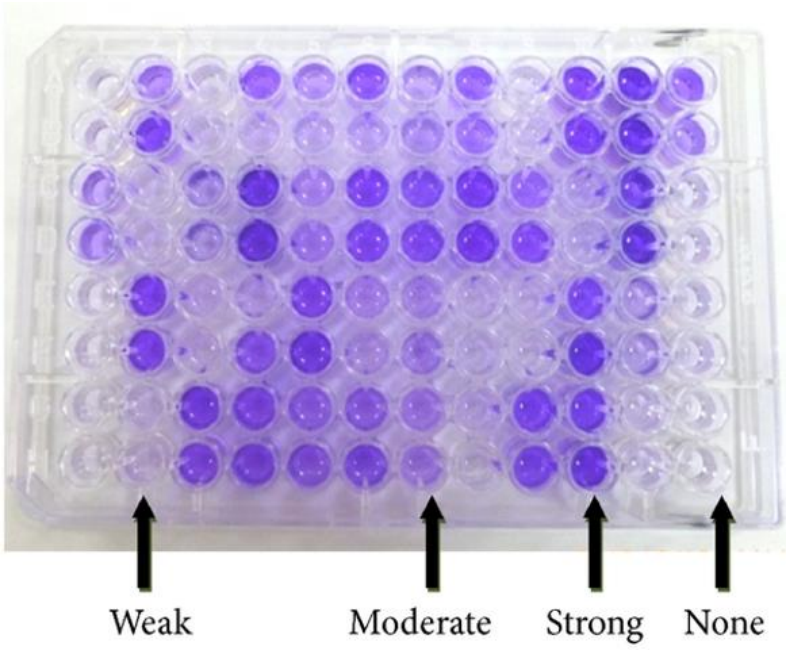

Figure 11: Example of a crystal violet biofilm quantification. The darker the well, the bigger the amount of biofilm produced. (Darwish & Asfour; 2013)

3.4-Development of Biofilm in Chambers for Confocal Microscopy

To evaluate the shape and viability of the biofilms produced by the different isolates, 13ml tubes (Sarstedt, Nümbrecht, Germany) with 3ml of inoculated MHB were grown overnight at 37ºC with shaking. The following day, the different isolates were adjusted at the same OD_{620}. 200µl of culture were placed into each chamber (Ibidi, Martinsried, Germany). Chambers were then placed in the incubator with a certain angle (around 45º), this will allow the biofilm to grow in the middle of the chamber, making it easier to find on the microscope. After overnight growth at 37ºC, supernatant was removed, and the chambers were rinsed twice with 300µl milliQ water. Live/dead staining gets prepared then, by mixing 1.5µl of propidium iodide and 1.5µl of SYTO9 (ThermoFisher) in 2ml of milliQ water. After 15-30 minutes of staining, the chambers were observed through the confocal microscope.

3.5-Bacterial Adhesion and Internalization Assays

A549 human lung epithelial cells ATCC® CCL-185™ (American Type Culture Collection, Manassas, VA) were employed to carry out in parallel adhesion and internalization (invasion) experiments. A549 cells were cultured in DMEM/F12 supplemented with 10% foetal bovine serum (FBS, ThermoFisher Scientific) at 37°C in an incubator with 5% CO2. Cell suspensions were seeded in 24-well standard polystyrene cell culture plates (Sarstedt, Nümbrecht, Germany) at $4x10^5$ cells per well, and grown to confluence in the same medium mention above. Cells were infected with approximately $4x10^6$ CFU/ml of each *P. aeruginosa* isolate, suspended in DMEM/F12 medium to obtain a multiplicity of infection (MOI) of approximately 10, relative to the number of cells initially seeded, and incubated for 1 hour at 37°C with 5% CO2. Strain PAO1 was used as control at the same concentration. After the incubation period the cells were gently washed three times with sterile PBS (1X) to remove non-adherent bacteria. Bacterial suspension were recovered and then vortexed for 1 minutes, and 10-fold serial dilutions of each sample were carried out followed by plating onto Mueller Hinton agar (MHA) to determine the number of CFU/ml which adhered to A549 cells.

For internalization assays, infected cells were extensively washed with sterile PBS after 1 hour of incubation at 37°C and further incubated for 45 minutes in DMEM/F12 medium supplemented with gentamicin (100 µg/ml) (colistin was used for gentamicin-resistant isolates) in order to eliminate extracellular bacteria. After incubation, infected monolayers were washed three times with PBS to remove dead bacteria and then lysed with a solution of 1% Triton X-100 (Sigma-

Aldrich) in PBS for 5 minutes at room temperature. The resultant cell lysates were serially diluted and plated onto Mueller Hinton agar plates to quantify viable intracellular bacteria. For quantification of extracellular bacteria (adherence assay), we subtracted the number of intracellular bacteria (determined by CFUs/ml) obtained after killing extracellular bacteria with the corresponding antibiotics (invasion assay) from the total bacteria recovered in the absence of antibiotics. Internalization is calculated as the ratio between the CFUs after the lysis of the A549 cells and CFUs in the culture supernatant.

3.6-Immunology Assays for Confocal Microscopy

This experiment was conducted in parallel with adhesion and internalization assays. For this experiment, A549 human lung epithelial cells ATCC® CCL-185™ (American Type Culture Collection, Manassas, VA) were employed. A549 cells were cultured in DMEM/F12 supplemented with 10% foetal bovine serum (FBS, ThermoFisher Scientific) at 37°C in an incubator with 5% CO2. Cell suspensions were seeded in 24-well standard polystyrene cell culture plates (Sarstedt, Nümbrecht, Germany) at $4x10^5$ cells per well, and grown to confluence in the same medium mention above. Cells were infected with approximately $4x10^6$ CFU/ml of each *P. aeruginosa* isolate, suspended in DMEM/F12 medium to obtain a multiplicity of infection (MOI) of approximately 10, relative to the number of cells initially seeded, and incubated for 1 hour at 37°C with 5% CO2. After 1h, wells were washed with milliQ water and 3.2% paraformaldehyde is applied to fix the sample and stored in the freezer until next step is taken. The following day, infected monolayers were lysed with a solution of 1% Triton X-100 (Sigma-Aldrich) in PBS for 15 minutes at room temperature. After that, unspecific epitopes were blocked with 1% BSA in PBS for another 15 minutes. Specific anti-Pseudomonas rabbit polyclonal antibodies 1:1000 on PBS were then applied for 15 minutes again, following this step the sample is rinsed carefully with PBS twice and secondary antibody (Alexa goat anti-rabbit) 1:1000 is applied for another 15 minutes. Once this step was done and after the sample was cleaned again with PBS twice, A488 phalloidin 1:200 is applied for 30 minutes. Finally, the sample is mounted with a glue FluoroShield™ (Sigma) that already contains DAPI and it can be carried to the microscope.

3.7-DNA Sequencing and Analysis

Total DNA from the *P. aeruginosa* isolates was purified by using the DNeasy Blood & Tissue Kit (Qiagen) and sequenced on a MiSeq device using reagents kit v3 for 2×300 paired-end libraries (Illumina) as previously described.

Raw reads from the sequencing platform were directly analyzed by using the in-house bioinformatics pipeline TORMES®, using *P. aeruginosa* PAO1 as reference strain. The options used in this study included quality control and filtering of the reads by using Trimmomatic, Prinseq and Kraken. Genome assembly was performed with SPAdes and Quast and genome annotation with Prokka. Multi-locus sequence typing (MLST). Search of antibiotic resistance genes and plasmid replicons was done using BLAST and ABRicate against ResFinder and PlasmidFinder databases, respectively, while point mutation investigation in genes known to confer antibiotic resistance was performed with PointFinder. Pangenome was created with Roary and FastTree. Tormes analysis lasted ~20 hours in a 128 GB RAM 32 cores computer. In silico serotyping was performed using the *Pseudomonas aeruginosa* serotyper (PAst) program.

4.-Results

4.1-Antibiotic susceptibility of clinical isolates

According to EUCAST breakpoints, MIC range for the isolates were 4 - 128µg/ml for AZT, 1 - 128+µg/ml for FEP, 0.5 - 128+µg/ml for CAZ, 2 - 256+µg/ml for PIP, 2 - 256+µg/ml for TZP, 0.5 – 64+µg/ml for IMP, 0.06 – 64+µg/ml for MER, 0.06 – 64+µg/ml for DOR, 0.25 - 64µg/ml for AMK 0.25 - 64µg/ml for GEN, 0.06 – 64+µg/ml for TOB, 0.5 – 256+µg/ml for NET, 0.03 – 32+µg/ml for CIP, 0.125 – 32+µg/ml for LEV, 0.125 – 32+µg/ml for COL, and 4 – 256+µg/ml for FOS.

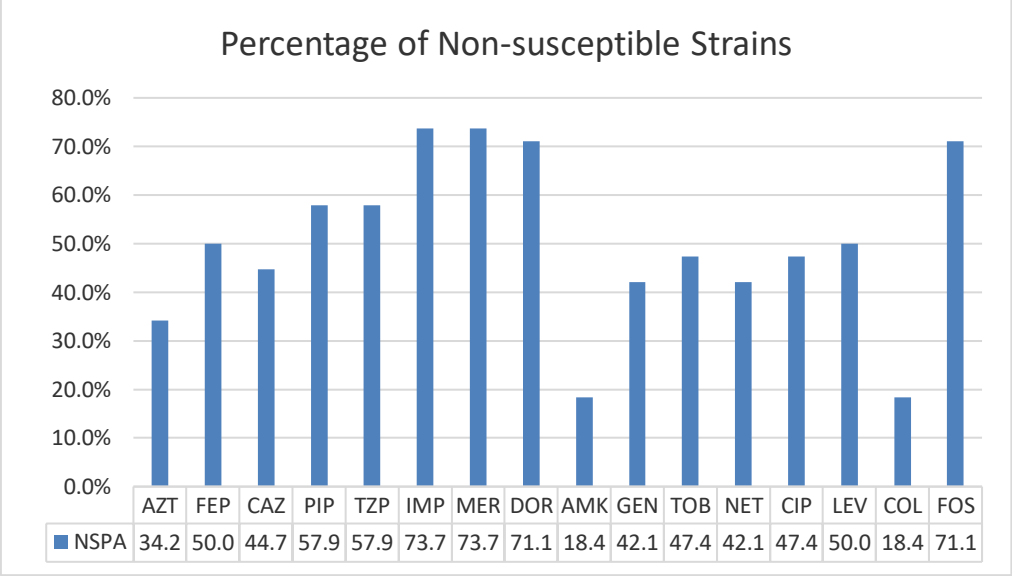

Figure 12: % of the 38 isolates non-susceptible to treatment with the different antibiotics. According to EUCAST, non-susceptible strains are the ones with intermediate or fully resistant profiles against each drug (I + R).

As we can see in *Figure 12*, 34.2% of the isolates were non-susceptible *Pseudomonas aeruginosa* (NSPA) for AZT. In cephalosporines, 50% were NSPA for FEP and 44.7% for CAZ. For antibiotics inactivating B-lactamases, highest values come from PIP and TZP with 57.9% of NSPA isolates. Isolates showed more resistance among carbapenems to IMP and MER with 73.7% of NSPA isolates followed by DOR. For aminoglycosides, isolates were NSPA in more than 40% of the cases, excluding amikacin. Our isolates presented NSPA rates around 50% for fluoroquinolones and 70% for FOS. Finally, antibiotics that seemed to be the most efficient were AMK and COL with 81.6% of susceptible isolates.

After evaluating the susceptibility profiles of the different isolates, the collection of 38 was grouped according to their resistance profiles. Extremely-drug-resistant (XDR) isolates were

those NSPA to at least 6 antibiotic categories, multi-drug-resistant (MDR) isolates were NSPA to 3 – 5 antibiotic categories, moderately resistant (modR) isolates were the ones NSPA to 1 or 2 antibiotic categories, and finally, multi-susceptible (multiS) isolates were those susceptible to all the antibiotic categories.

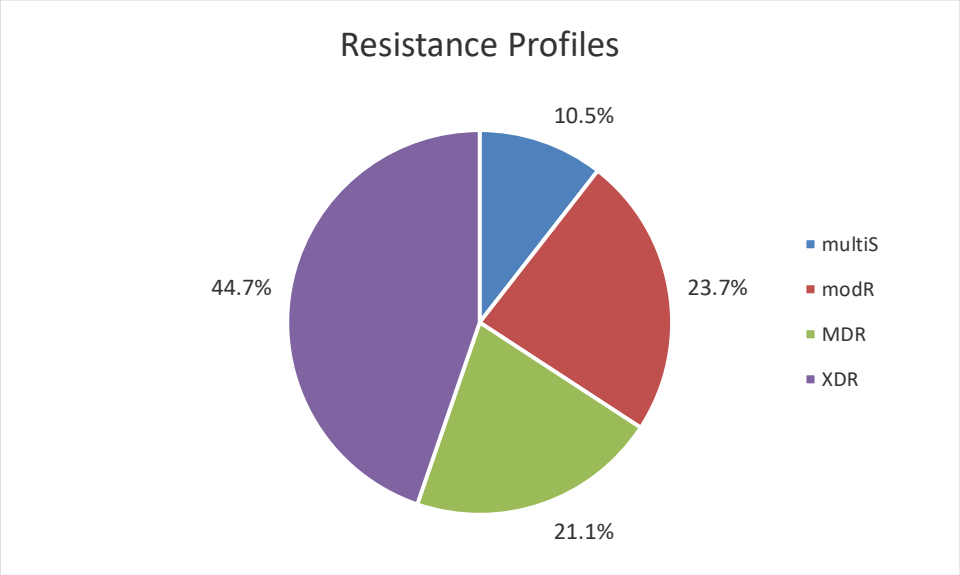

Figure 13: % of the 38 isolates that belong to the 4 previously described resistance profiles.

Results showed that a total of 17 (44.7%) isolates met the extremely-drug-resistant (XDR) criteria, 8 (21.1%) were multi-drug-resistant (MDR), 9 (23.7%) were moderately resistant (modR) and 4 (10.5%) were considered multi-susceptible (multiS).

4.2-Biofilm production

In order to classify biofilm production, the average of the 3 crystal violet measures was then divided by the log10 of the CFU of each isolate so that it was normalized. For the interpretation of biofilm results, isolates were classified as non-biofilm producer ($OD \leq 0.05$), weak-biofilm producer ($OD > 0.05–0.1$), moderate-biofilm producer ($OD > 0.1–0.3$) and strong-biofilm producer ($OD > 0.3$).

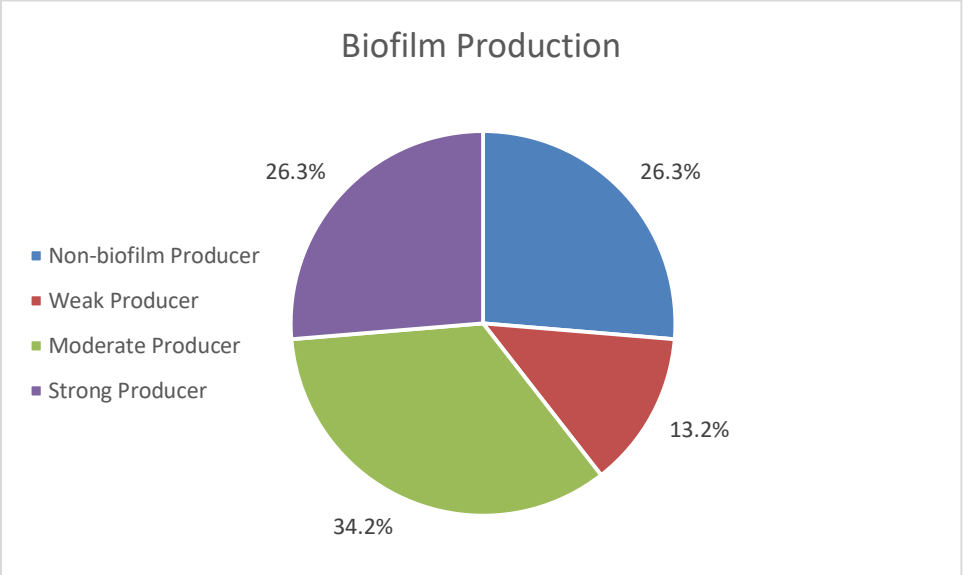

Figure 14: Biofilm production in the different isolates according to the classification explained above.

Moderate-biofilm producers were the most common among our isolates (34.2%). With 26.3% of the isolates each, non-biofilm producers and strong-biofilm producers made up for more than half of the total of isolates. Lastly, weak-biofilm producers were scarcer (13.2%).

4.3-Biofilm Development in Chambers

Biofilm architecture was also evaluated through confocal microscopy. *Figure 15* shows biofilms produced by different isolates. Green colour represents alive bacterial cells within the biofilm, whereas dead cells are shown in red.

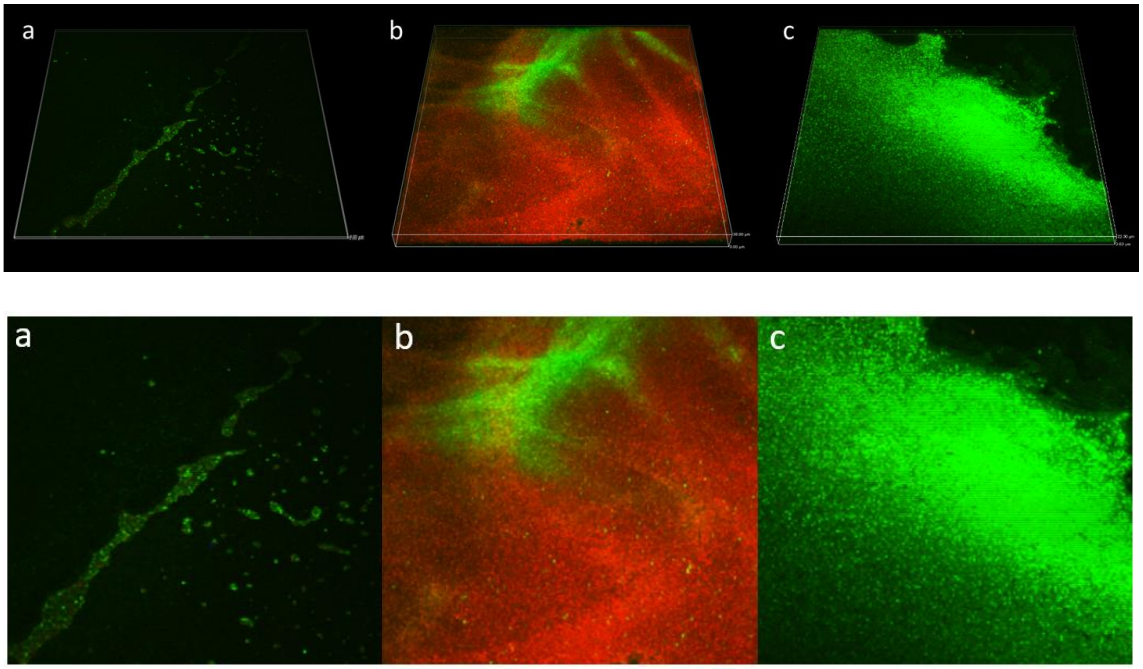

Figure 15: Biofilm formation under confocal microscope. Upper panel shows three-dimensional structure of the biofilms while lower panel shows biofilm in detail. a) PANAVIR 64, b) PANAVIR 117 and c) PANAVIR 180.

As expected, morphology of biofilms was variable. In panel a, PANAVIR 64 represents a weak biofilm producer, which correlates with the results observed by the CV method. Most bacteria within this biofilm were alive (green colour), and we can see how thin the biofilm is. In panel b, PANAVIR 117 shows a robust biofilm morphology. However, most of the bacterial cells within the biofilm structure are dead as they present a red colour. When we compare this with its biofilm production by the CV method, we can see that it belongs to the moderate-biofilm producer category. The biofilm is thicker when compared to PANAVIR 64. In panel c, PANAVIR 180 shows a high biofilm production where large green masses of bacteria can be seen inside these structures, which means that most of the biofilm is composed by alive cells. This isolate was considered as a strong-biofilm producer by the CV method and it is also thicker.

4.4-Interaction of *P. aeruginosa* clinical isolates with A549 cells

Percentage of invasion from the different isolates is presented in *Table 5* below. Isolate numbers in yellow belong to high risk clones, whose percentages of invasion are represented in *Figure 16*.

Table 5: Invasion percentages of the isolates. Only 33 were analysed due to lack of time, PANAVIR 8, 37, 159, 216 and 217 are missing.

Strain	Invasion %	Strain	Invasion %	Strain	Invasion %
PANAVIR 5	0,0	PANAVIR 66	0,0	PANAVIR 173	2,1
PANAVIR 19	0,1	PANAVIR 70	9,7	PANAVIR 175	35,6
PANAVIR 28	1,2	PANAVIR 80	0,9	PANAVIR 178	1,3
PANAVIR 30	7,2	PANAVIR 81	0,8	PANAVIR 180	0,0
PANAVIR 31	0,6	PANAVIR 85	0,2	PANAVIR 185	36,8
PANAVIR 45	0,0	PANAVIR 108	0,0	PANAVIR 187	3,3
PANAVIR 46	25,0	PANAVIR 109	0,0	PANAVIR 195	4,4
PANAVIR 56	0,1	PANAVIR 117	0,0	PANAVIR 196	0,8
PANAVIR 57	56,0	PANAVIR 125	2,1	PANAVIR 206	10,3
PANAVIR 60	0,0	PANAVIR 145	6,8	PANAVIR 211	0,1
PANAVIR 64	37,9	PANAVIR 149	0,7	PANAVIR 213	36,3

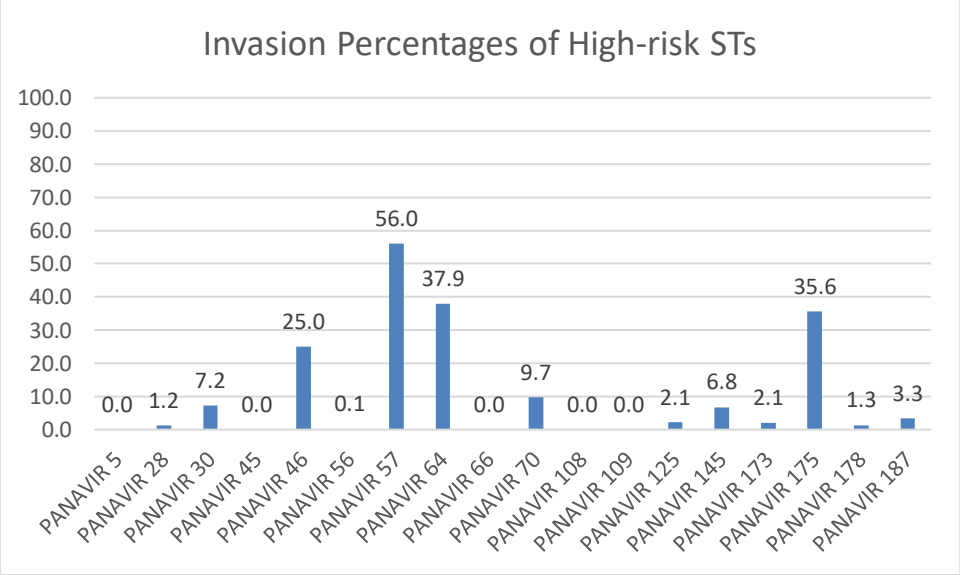

Figure 16: Invasion percentages of the isolates belonging to high-risk clones.

Most of the isolates represented in the figure showed extremely low invasion percentages, 14 out of 18 below 10%, this can be explained due to their high cytotoxicity, that, during the 1h infection time already killed all the cells. PANAVIR 57 with 56% shows the higher invasion rate followed by PANAVIR 64 with 37.9% and PANAVIR 175 with 35.6%.

In order to see the virulence capacity of the isolates and confirm the data from the invasion analysis, cell infection was performed and then seen through the confocal microscope. Cells are seen in green colour with the nucleus in blue, bacteria are shown in red.

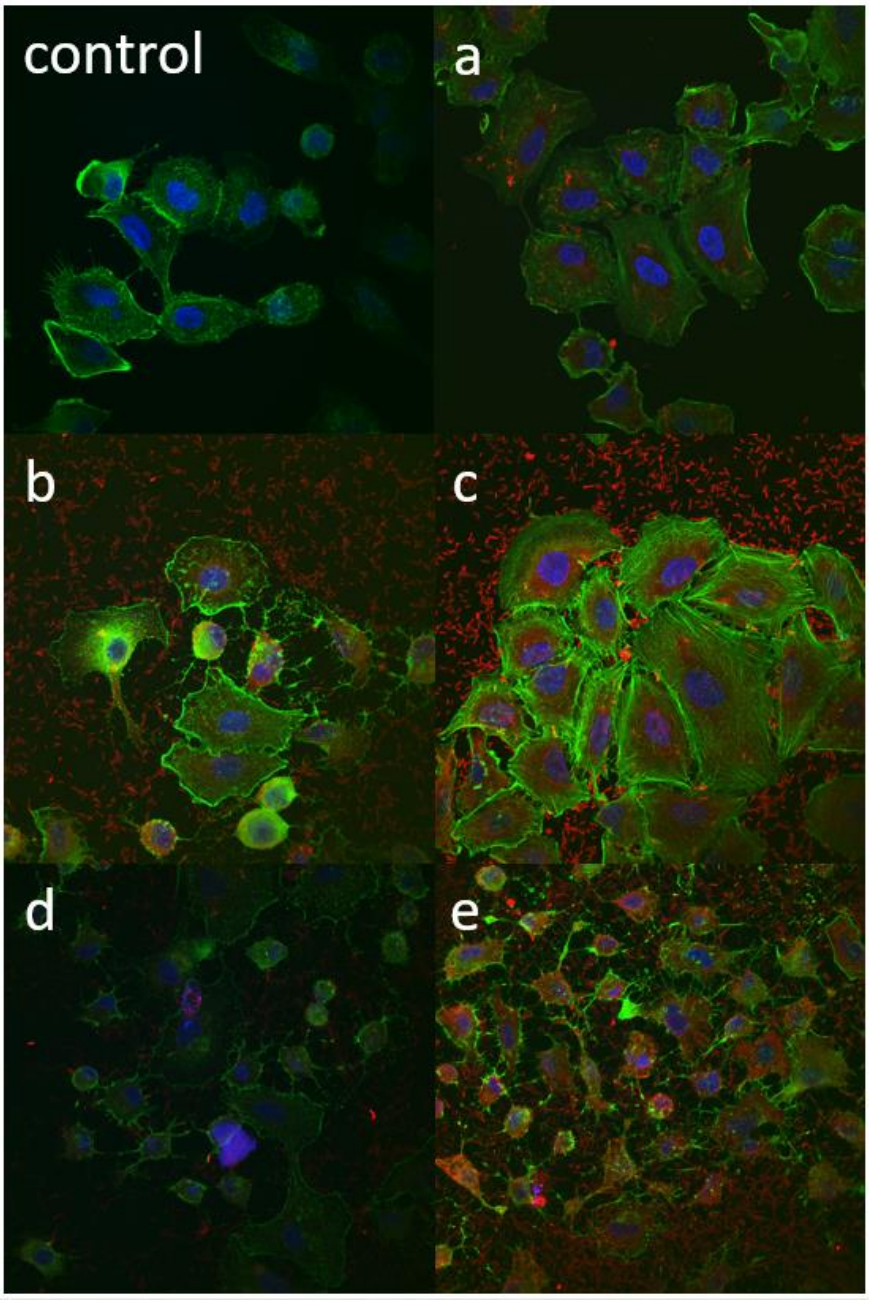

Figure 17: Cell infection through confocal microscope. control) Non-infected cells, a) PANAVIR 19, b) PANAVIR 45 (VIM20+), c) PANAVIR 70, d) PANAVIR 109 (VIM2+) and e) PANAVIR 175 (GES7+).

In *Figure 17*, when comparing panel a, PANAVIR 19, to the control cells, we can appreciate bacteria mostly in the surface of the cells, which remain apparently healthy. This isolate presented a very low percentage of invasion (0.1%). Panel b represents isolate PANAVIR 45. Here, it can be observed an increased number of bacteria surrounding the cells, whose morphology has changed completely, some of them were round-shaped, which indicates cellular damage. In panel c, PANAVIR 70, shows a large number of bacteria, mostly around the cells, which still look healthy, some bacteria seem to be inside the cells. In panel d, PANAVIR 109, a scarce number of bacteria is seen, even though cells look clearly affected, with most of them already round-shaped. This isolate presented a very low percentage of infection but appears to exhibit a cytotoxic phenotype according to the damage caused to A549 cells. Finally, in panel e, PANAVIR 175, displays a huge amount of bacteria, as well as many cells showing clear signs of apoptosis. This isolate presented one if the highest percentages of invasion (35,6%)

Isolates chosen to be represented on *Figure 17* were selected due to their genetic profiles. PANAVIR 19 as an example of a non-high-risk clone with low invasion percentage. PANAVIR 45 as the only isolate with $bla_{\text{VIM-20}}$, PANAVIR 70 as a high-risk clone that seems not to kill the cells. PANAVIR 109 as the only isolate with $bla_{\text{VIM-2}}$, and PANAVIR 175 as an isolate that has both $bla_{\text{VIM-1}}$ and $bla_{\text{GES-7}}$ (See details in the Resistome results section below).

4.5-Clonal Relatedness and Resistance Profiles of Clinical Isolates

After analyzing the sequencing results given by MLST the 38 isolates were grouped in 19 allelic profiles or sequence types (STs). Among these, 19 were considered as high-risk international clones (STs: 111, 155, 175 and 235).

Table 6: Number of isolates that belong to the 4 high-risk clones found in our isolates.

High risk clone (ST)	Number of isolates
111	1
155	6
175	9
235	3

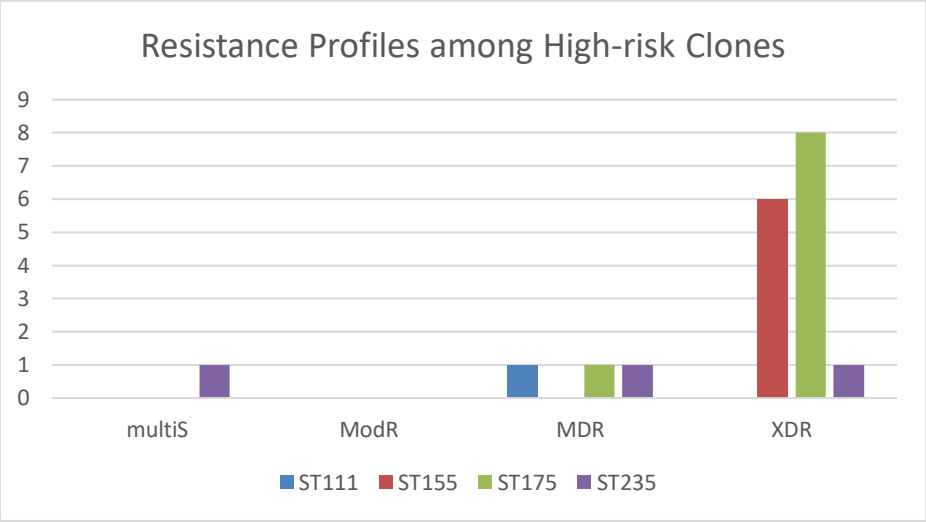

Figure 18: Different resistance profiles seen on high-risk clones found on our isolates.

In *Figure 18*, focusing only on high-risk clones resistance profiles, results showed that 15 (78.9%) of the isolates were XDR while 3 (15.8%) were MDR and only 1 (5.3%) isolate was multiS.

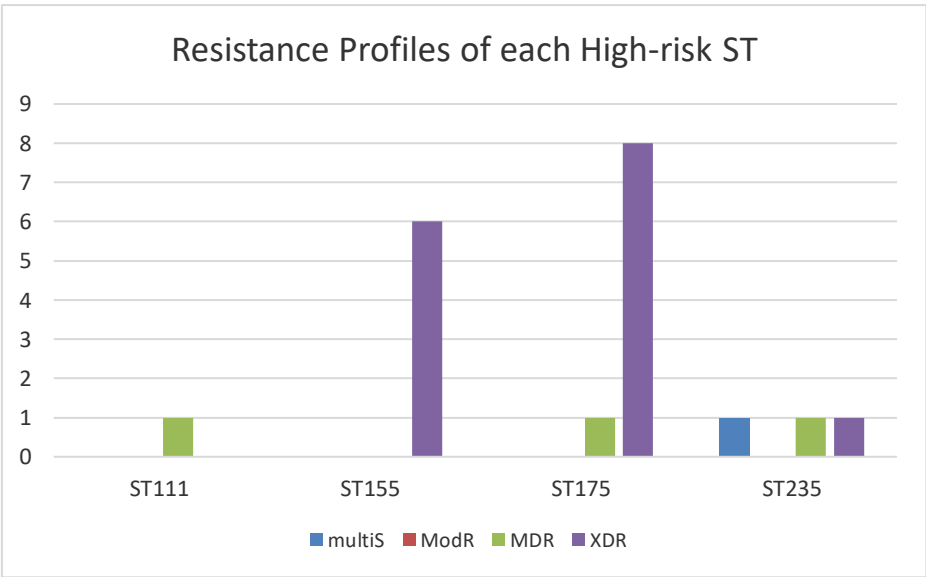

Figure 19: Different resistance profiles seen on each of the high-risk STs found on our isolates.

Focusing on the resistance profile of each high-risk clone, it was observed that ST155 and ST175 were the most resistant clones (and the most common) with a 100% and 89% of extremely-drug-resistant (XDR) isolates, respectively.

4.6-Resistome of the 38 VAP *P. aeruginosa* Isolates

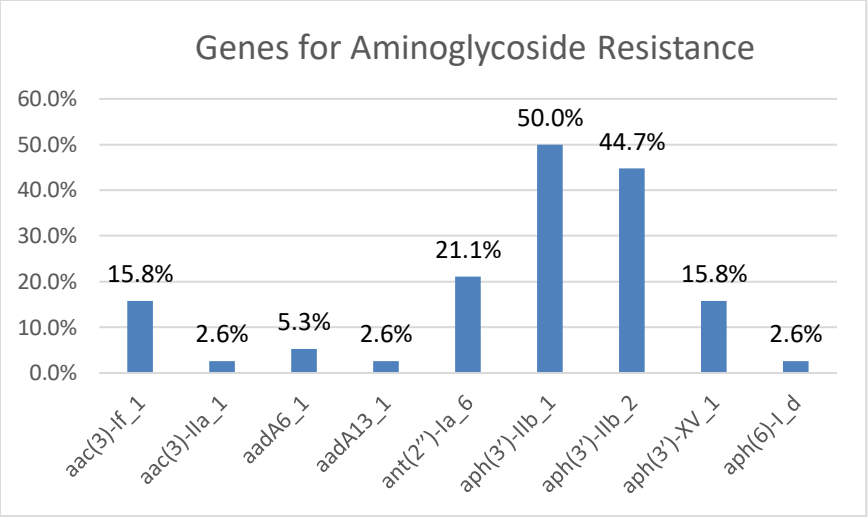

Figure 20: Presence of genes which confer resistance to antibiotics from aminoglycoside family found on our isolates.

According to the DNA sequencing performed to the 38 isolates, 9 well known genes responsible for aminoglycoside antibiotic resistance were found. *aph(3')-IIb_1* and *aph(3')-IIb_2* were the ones with higher presence, 50% and 44.7% respectively. Appearance rates decreased dramatically to 21.1% for *ant(2'')-Ia_6* and 15.8% for *aac(3)-If_1* & *aph(3')-XV_1*. Finally, *aadA6_1*, *aac(3)-IIa_1*, *aadA13_1* and *aph(6)-I_d* were only present in 2 or 1 of the isolates.

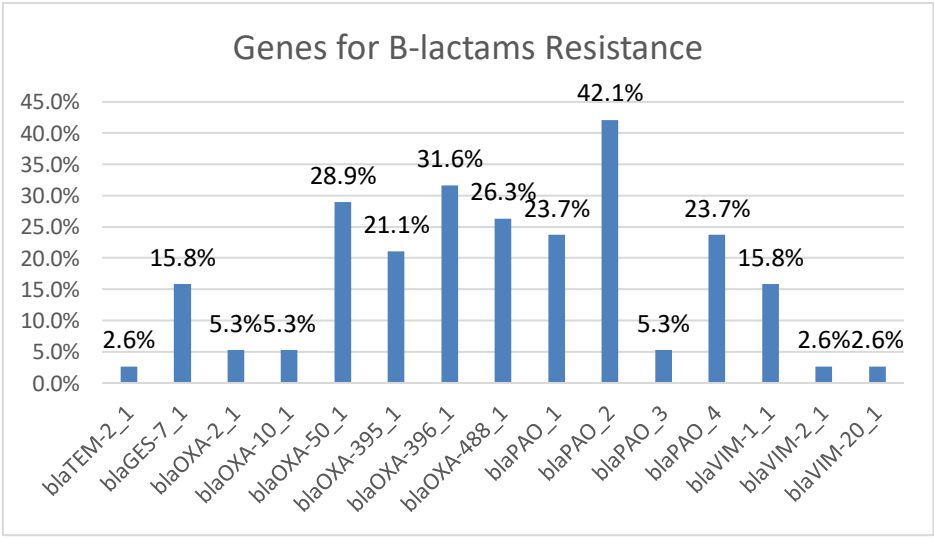

Figure 21: Presence of genes which confer resistance to antibiotics from β-lactam family found on our isolates.

In *Figure 21*, according to the DNA sequencing performed to the 38 samples, 15 well known genes responsible for β-lactamase synthesis thus conferring β-lactam resistance were found. 6 of them belong to the OXA type being *bla*$_{\text{OXA-396\_1}}$ present in 31.6% of isolates followed by *bla*$_{\text{OXA-50\_1}}$ found in 28.9% of them. *bla*$_{\text{PAO\_2}}$ with a 42.1% of presence was the most common among the 15. Two copies of the gen coding for the extended spectrum betalactamase GES-7 and the metallobetalactamase-coding gene *bla*$_{\text{VIM-1}}$ were found within a class 1 integron in 6 isolates and *bla*$_{\text{VIM-20}}$ in one isolate on an class 1 integron together with other antibiotic resistant determinants (Figure 22).

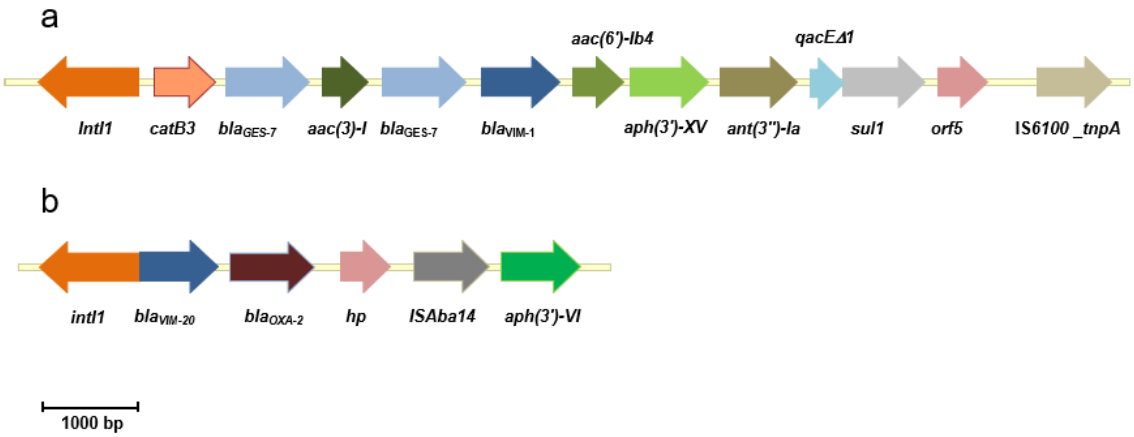

Figure 22: Genetic context of bla$_{\text{GES-7}}$*, bla*$_{\text{VIM-2}}$ *(a) and bla*$_{\text{VIM-20}}$ *(b).*

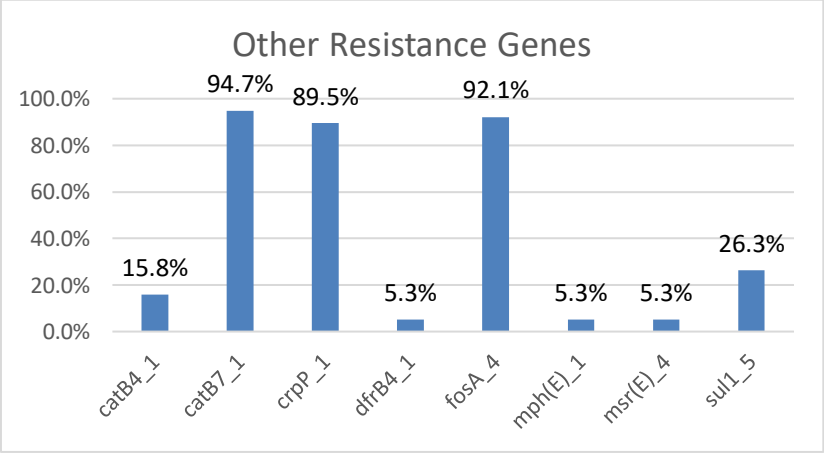

Figure 23: Presence of genes which confer resistance to particular antibiotics used in clinic.

According to the DNA sequencing performed to the 38 samples, other antimicrobial resistance-related genes were found. *catB7_1* is present in most of the isolates (94.7%) giving resistance to Chloramphenicol. *fosA_4* follows with 92.1%, giving resistance to Fosfomycin. Lastly, *crpP_1* is present in 89.5% of the isolates, giving resistance to Ciprofloxacin.

4.7-Virulence Genes

Twenty important different virulence genes are shown in the figures below, these genes were selected in order to cover the different aspects of *P. aeruginosa* virulence, genes from T3SS, T6SS, biofilm or QS are shown.

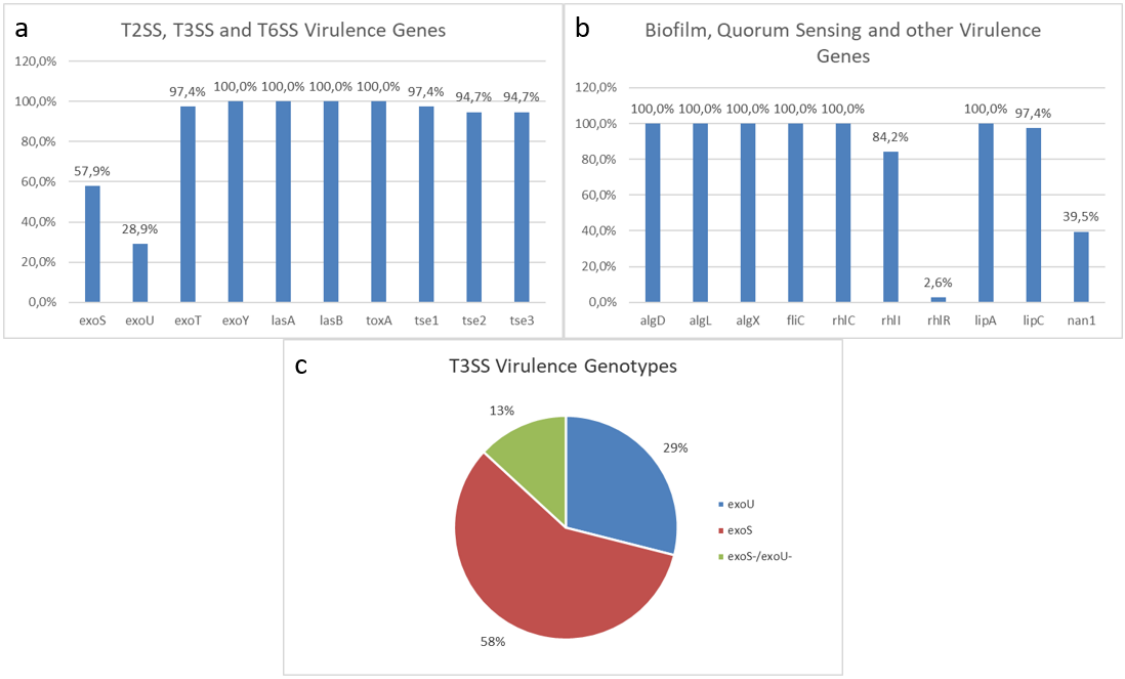

Figure 24: Presence of genes directly related to virulence found on our isolates. a) Genes that belong to the different secretion systems (T2, T3 and T6), b) Genes potentially important for biofilm formation, Quorum Sensing, formation of flagellum and invasion of cells, and c) Distribution of exoS/exoU genotypes on the 38 isolates.

Panel a: Out of the 4 main effectors from T3SS, *exoT* and *exoY* were found on almost all isolates, 97.4% and 100%, respectively. *exoS* was present in 57.9% of the isolates while *exoU* in 28.9%. It is known that these two genes are mutually exclusive, but there are some "rare" isolates that have both genes (Panel c for more detailed information). When infection and invasion rates of isolates with genotypes *exoS* and *exoU* were compared, it was possible to detect the difference between them. The isolates showing *exoS* were more invasive (11.2% vs 6.1% on average) but less virulent towards cells (40% vs 27% of isolates did not affect the cells), while *exoU* isolates invaded less but their attacks provoked more cellular damage to A549 cells. Genes *lasA*, *lasB* and *toxA* were found in all the isolates (100%). T6SS effectors *tse1*, *2* and *3* were found in most of the isolates, 97.4% for *tse1* and 94.7% for *tse2* and *tse3*.

Panel b: The 3 genes related with biofilm production (*algD, algL* and *algX*) were found on the 38 isolates studied. Genes *rhlC* and *rhlI* related with Quorum Sensing were very common, found in 100% and 84.2% respectively, while rhlR was only present in 1 isolate. Lipases *lipA* and *lipC* were also in almost all isolates (100% and 97.4% respectively). *fliC* gene, which encodes for flagellin was present in all isolates while *nan1* related to adhesion to other cell types thus allowing easier infections was only found on 39.5% of the isolates.

5.- Discussion

Ventilator-associated pneumonia is one of the most typical infections occurring on the intensive care units (ICUs) across the globe as well as one of the major causes of morbidity, mortality, and increased funding for ICUs (Joseph et al; 2010). Therefore, it is a critical problem of public health that needs to be addressed in a short period of time, as the number of bacteria resistant to antibiotics will keep rising (WHO; 2015). *P. aeruginosa* is a really versatile bacteria, it possesses many characteristics that facilitate infection, and it is considered one of the main microorganisms causing VAP (Trouillet et al; 1998). For these reasons, this project has focused on describing the different characteristics of *P. aeruginosa* that make it such a problematic pathogen, its virulome and resistome as well as phenotypic characteristics and infection models.

Thanks to whole genome sequencing (WGS) and bioinformatics analysis software we were able to identify 19 high-risk clones among our 38 isolates. High-risk clones are specially linked to a bad prognosis due to the great amount of antibiotic resistance present in these isolates, most of them considered XDR, and their ability to spread (Oliver et al; 2015). Also, we found extended spectrum betalactamase GES-7 in and metallobetalactamase VIM-1 in 6 isolates while VIM-2 and VIM-20 were present in 1 isolate each. The prevalence of these enzymes on Spanish P. aeruginosa clinical isolates was similar to other studies such as Bothelo et al; 2019.

We expected XDR isolates to be less virulent due to a trade-off between pathogenicity and antibiotic resistance capability. However, we were able to find highly virulent isolates that also presented several antibiotics resistant determinants and display a multi-drug resistant phenotype. Out of the 19 high-risk clones, 15 of them were XDR and 3 MDR while presenting at the same time a wide number of virulence genes like *lasA, lasB, exoA, fliC or lipC*. PANAVIR 45, 109 or 175 are really good examples of this finding, all of them are considered XDR isolates, which are also metallobetalactamase VIM-producers (VIM-20, VIM-2 and VIM-1, respectively). Only 1 high-risk clone presented the multiS phenotype, but remained highly virulent when checked on the confocal microscope. We also evaluated the biofilm production, as it is considered an

important factor for the development of VAP. 26.3% of the isolates were strong biofilm producers (PANAVIR 175 included).

These results are preliminary, as we only worked with 38 out of more than 200+ isolates from Spanish VAP patients, which altogether require a lot of analysis. Due to the pandemic situation time was not enough to perform such a thorough evaluation. Further research is needed to better correlate pathogenicity and antibiotic resistance, as results like the ones observed in this work with PANAVIR 175 can give us an insight on the right direction, possibilities of better understanding this relationship on *P. aeruginosa* can be crucial for clinic.

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