Universidade do Minho
Escola de Ciências

Ricardo Filipe de Oliveira Costeira

Genotypic characterization of clinical isolates of *Pseudomonas aeruginosa*

outubro de 2014

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Genotypic characterization of clinical isolates of *Pseudomonas aeruginosa*

Dissertação de Mestrado
Mestrado em Genética Molecular

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Professor Pedro Miguel Santos

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(front page)

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Caracterização genotípica de isolados clínicos de *Pseudomonas aeruginosa*

Resumo

Pseudomonas aeruginosa é um microrganismo patogénico oportunista, responsável por infecções hospitalares frequentemente letais. Com uma grande plasticidade genómica, *P. aeruginosa* tem à sua disposição uma multitude de factores que se traduzem numa grande flexibilidade fenotípica, permitindo-lhe prosperar tanto no ambiente como no hospedeiro. Tais factores são responsáveis pelo elevado número de infecções por *P. aeruginosa* registado na literatura, especialmente em doentes de fibrose cística, e pelo contínuo aumento da frequência de isolados clínicos de *P. aeruginosa* multirresistentes a antibióticos. Estudos epidemiológicos são nesse sentido essenciais para o desenvolvimento de melhores sistemas de vigilância, detecção de epidemias e tratamentos clínicos. A caracterização de microorganismos é fulcral em estudos epidemiológicos.

Em 2010, uma colaboração entre o Centro de Biologia Molecular e Ambiental (CBMA) da Universidade do Minho (Braga, Portugal) e o Hospital de Braga (Braga, Portugal) foi estabelecida, com o intuito de expandir o conhecimento sobre a biologia de *P. aeruginosa*. Esta colaboração resultou numa diversificada colecção de isolados, com mais de 500 amostras actualmente.

Neste trabalho, é apresentado um estudo epidemiológico das infecções por *P. aeruginosa* no Hospital de Braga ao longo de cinco anos, com a caracterização genotípica de 528 isolados por RAPD-PCR. Com este estudo determinou-se que pacientes, serviços hospitalares e fontes anatómicas estavam mais associadas com a ocorrência de infecções por *P. aeruginosa* no Hospital de Braga, assim como qual a prevalência de mono/multinfecções e isolados multirresistentes a antibióticos ao longo dos anos. Após estabelecimento da técnica de RAPD-PCR e desenvolvimento de uma metodologia para a análise genotípica, procedeu-se à divisão dos isolados clínicos de *P. aeruginosa* em 21 grupos genotípicos. A frequência de cada grupo nas situações anteriormente citadas foi determinada, com especial atenção dada a isolados panresistentes a antibióticos e isolados de um doente de fibrose cística. Globalmente, descobriu-se que, apesar de variações de frequência dos grupos genotípicos serem observadas, raramente um grupo genotípico não era representado. Este achado reflecte a ubiquidade e flexibilidade fenotípica de *P. aeruginosa*.

Genotypic characterization of clinical isolates of *Pseudomonas aeruginosa*

Abstract

Pseudomonas aeruginosa is an opportunistic human pathogen, responsible for frequently lethal nosocomial infections. With an out of the ordinary genomic plasticity, *P. aeruginosa* has at its disposal a multitude of traits that allows it to be phenotypically flexible and thrive both in the environment and in the host. Such traits are the ones accounted for the high number of infections by *P. aeruginosa* registered in the literature, especially in cystic fibrosis suffering patients, and for the increasing frequency of multi-drug resistant isolates of *P. aeruginosa,* The epidemiological study of *P. aeruginosa* infections is therefore essential to the development of better surveillance systems, outbreak detection and clinical treatments. The characterization of microorganisms is centre key in epidemiology.

In 2010, a collaborative effort was established between the Centre of Molecular and Environmental Biology (CBMA) of University of Minho (Braga, Portugal) and Hospital de Braga (Braga, Potugal), in order to further expand the knowledge of the biology of *P. aeruginosa*, resulting in a diversified collection of isolates with over 500 samples, currently.

In this work, it is presented an epidemiological study of *P. aeruginosa* infections in Hospital de Braga, with the genotypic characterization of 528 isolates by RAPD-PCR. With this study, it was determined which patients, hospital services and anatomical sources were most associated with *P. aeruginosa* infections in Hospital de Braga, as well as the prevalence of mono/multi-infections and multi-drug resistant *P. aeruginosa* isolates throughout the years. After establishment of the RAPD-PCR technique and development of a pipeline for genotyping analysis, the *P. aeruginosa* isolates were divided into 21 genotypic groups. The frequency of each group in the aforementioned scenarios was determined, with special focus given to pan-drug resistant isolates and isolates from a cystic fibrosis suffering patient. Overall, it was found that, despite variations in genotypic group frequencies, it was rare that a genotypic group was not represented at all. This finding mirrors the ubiquity and phenotypically flexibility of *P. aeruginosa*.

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List of acronyms

AFLP	Amplified length polymorphism
AHLs	N-Acyl homoserine lactones
AP-PCR	Arbitrarily primed polymerase chain reaction
AQs	4-quinolones
bp	base pairs
CF	Cystic fibrosis
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ECM	Extracellular matrix
eDNA	Extracellular deoxyribonucleic acid
ER	Emergency Room
ERIC	Enterobacterial repetitive intergenic consensus
ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
GC	guanine-cytosine
gDNA	Genomic deoxyribonucleic acid
HGT	Horizontal gene transfer
ICUs	Intensive care units
kbp	kilo base pairs (10^3 bp)
LBMG1:100	100 times dilution of Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Germany) prepared in a 6 times concentrated DNA loading buffer
LPS	Lipopolysaccharide
M-CGH	Microarray-based comparative genomic hybridization
Mbp	megabase pairs (10^6 bp)
MDR	Multidrug-resistant
MEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MLVA	Multilocus VNTR analysis
MLVF	Multiple-locus VNTR fingerprinting
MP-ICU	Multi-purpose Intensive care unit
NGS	Next generation sequencing

PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDR	Pandrug-resistant
PFGE	Pulsed-field gel electrophoresis
QS	Quorum sensing
RAPD	Random amplified polymorphic DNA
RAPD-PCR	Random amplified polymorphic DNA polymerase chain reaction
REP	Repetitive extragenic palindromic
rep-PCR	Repetitive extragenic palindromic polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RGPs	Regions of genomic plasticity
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SLST	Single locus sequence typing
TAE	Tris-Acetate-EDTA buffer
TCS	Two-component system
tRNA	Transfer ribonucleic acid
TTSS	Type III secretion system
VNTR	Variable-number tandem repeats
WGM	Whole genome mapping
WGS	Whole genome sequencing
XDR	Extensively drug-resistant

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1. INTRODUCTION

1.1. *Pseudomonas aeruginosa*, a peculiar pathogen

1.2.1. Introducing *Pseudomonas aeruginosa*

Spanning at least 3.8 billion years of evolution, the microbial way of life has established itself as the most pervasive and ubiquitous player in all existing ecosystems (review by DeLong and Pace, 2011). This is not surprising since *Bacteria* and *Archaea* are the most genetically diverse domains of life out of the three-domain system proposed by Carl Woese in 1990 (review by Fraser *et al.*, 2009; Woese *et al.*, 1990).

The genus *Pseudomonas* was first described by German botanist Walter Migula at the end of the nineteenth century and has since then been subject of many taxonomic revisions throughout the years (Özen and Ussery, 2012; review by Palleroni, 2010). Once divided into 5 groups, based on rRNA/DNA homology studies (Fuchs *et al.*, 2001), the genus *Pseudomonas sensu stricto* is now only reserved to the rRNA group I of Palleroni, as polyphasic and genomic studies assigned many strains to new genera (e.g. *Burkholderia* and *Ralstonia*) (Fuchs *et al.*, 2001; review by Palleroni, 2010). According to the List of Prokaryotic Names with Standing in Nomenclature, an online database that exhibits an accurate and up-to-date nomenclature and taxonomy of prokaryotes (Euzéby, 1997), the genus had 216 species assigned on October 14th, 2014.

Pseudomonas spp. are, in general, non-sporulating, aerobic, chemoheterotrophic, motile, rod-shaped, Gram-negative γ proteobacteria (review by Mercado-Blanco and Bakke, 2007; Özen and Ussery, 2012; review by Silby *et al.*, 2011). Many species of this genus are referred in the literature as "fluorescent" (e.g. *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Pseudomonas syringae*), when producers of the yellow-green fluorescent pigments pyoverdines (high-affinity peptidic siderophores) (review by Cornelis, 2010; Fuchs *et al.*, 2001). Non-fluorescent species are, for example, *Pseudomonas alcaligenes* and *Pseudomonas stutzeri* (Fuchs *et al.*, 2001).

Pseudomonads are predominant inhabitants of diverse terrestrial and aquatic habitats, being found both in biofilms or planktonic forms (review by Cornelis, 2010; Özen and Ussery, 2012; review by Silby *et al.*, 2011). *Pseudomonas* spp. are also capable of forming beneficial or pathogenic associations with plants and animals, including humans (review by Mercado-Blanco and Bakke, 2007; Özen and Ussery,

2012). This ubiquity is a consequence of the remarkable metabolic and physiologic diversity of pseudomonads (Özen and Ussery, 2012; review by Silby *et al.*, 2011). Hence, *Pseudomonas* spp. are object of extensive studies due to their impact in human and animal disease, plant physiology and food spoilage, and due to its biotechnological potential (review by Mercado-Blanco and Bakke, 2007; Özen and Ussery, 2012).

The type species of the genus Pseudomonas, *P. aeruginosa*, is a nutritionally undemanding and versatile, opportunistic human pathogen, capable of growing at temperatures up to 42ºC, responsible for frequently lethal nosocomial infections (Fuchs *et al.,* 2001; review by Kerr and Snelling, 2009; review by Kung *et al.,* 2010). In addition to being able to use a wide variety of carbon sources for energy and having minimal nutrient requirements, *P. aeruginosa* is a microorganism that is capable of using nitrogen as a terminal electron acceptor, allowing respiration under anaerobic conditions (review by Kung *et al.,* 2010).

P. aeruginosa is seldom a member of the normal microbial flora of humans (review by Lister *et al.,* 2009). However, colonization rates of this pathogen are increased in immunosupressed patients (e.g. burn victims, cancer patients) or patients with wounds and physical breaches (e.g. patients with catheters) (review by Kerr and Snelling, 2009; review by Lister *et al.,* 2009; review by Silby *et al.,* 2011). Still, by far, the most studied infections caused by *P. aeruginosa* are the ones associated with cystic fibrosis (CF), an autosomal recessive disease, characterized by mutations in a transmembrane protein that controls a chloride and sodium channel present in the epithelial cells of some organs, resulting in viscous secretions that impair cilia clearance, resulting in colonization by bacteria (Damas *et al.,* 2008; review by Folkesson *et al.,* 2012; review by Silby *et al.,* 2011). *P. aeruginosa* is the most commonly found pathogen in the lungs of patients suffering from this disease, leading to increased morbidity and mortality (review by Silby *et al.,* 2011; Damas *et al.,* 2008) (Figure 1).

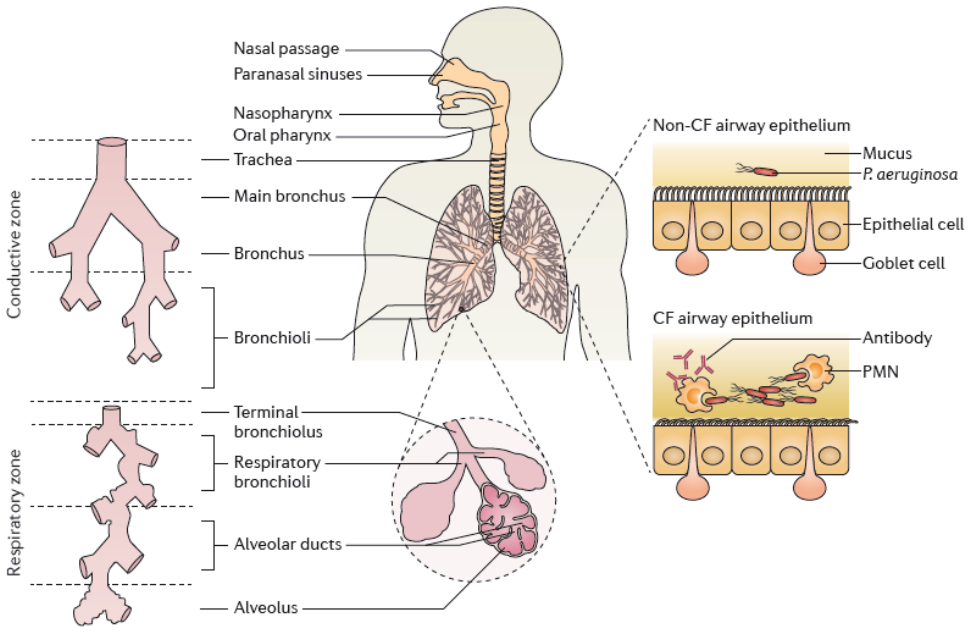


Figure 1. Defects in mucociliary clearance of the CF airaway create opportunities for microbial colonization (review by Folkesson *et al.*, 2012). In CF patients, the cilia cannot clear the viscous and dehydrated mucous layer efficiently, resulting in colonization by bacteria, such as *P. aeruginosa*. The immune responses by polymorphonuclear leukocytes (PMNs) and antibodies lead to scarring of lung tissue and impairment of lung function.

1.2.2. Overview of the genome of *Pseudomonas aeruginosa*

Among the publicly available genomes, almost 90% are from microbes (review by Woods, 2004). The first genome of *P. aeruginosa*, and 25th of all bacteria, to have its sequence completed, was the genome of the strain PAO1 in 2000 (review by Silby *et al.*, 2011), being this strain nowadays considered a major reference for genetic and functional studies in *P. aeruginosa* (Klockgether *et al.*, 2011). Currently, there are 9 strains with completely sequenced *P. aeruginosa* genomes: PAO1 (Refseq. no. NC_002516; Stover *et al.*, 2000), PA14 (NC_008463; Lee *et al.*, 2006), PA7 (NC_009656; Roy *et al.*, 2010), LESB58 (NC_011770; Winstanley *et al.*, 2009), NCGM2.S1 (NC_017549; Miyoshi-Akiyama *et al.*, 2011), M18 (NC_017548; Wu *et al.*, 2011), DK2 (NC_018080; Rau *et al.*, 2012) and B136-33 (NC_020912). More genomes of *P. aeruginosa* are in progress of completion such as the genomes of the strains 2192 (NZ_AAKW00000000), C3719 (NZ_AAKV00000000), 39016 (NZ_AEEX00000000; Stewart *et al.*, 2011), and the genomes of the strains HB13

(NZ_AEVV00000000.3; Soares-Castro *et al.*, 2011) and HB15 (NZ_AEVW00000000.3; Soares-Castro *et al.*, 2001), published by our group in 2011.

The genome of *P. aeruginosa* has a size of 5.5-7 Mbp and a 65-67% GC content, being made up of a single circular chromosome and a variable number of plasmids (Klockgether *et al.*, 2011). With one of the biggest bacterial genomes when compared to other bacteria such as *Eschericia coli* K12 (4.64 Mbp), *Staphylococcus aureus* N315 (2.81 Mbp) or *Haemophilus influenzae* Rd (1,83 Mbp) (review by Lambert, 2002), *P. aeruginosa*'s genome size results from its genetic complexity (many genes with unique functions) rather than gene duplications (review by silby *et al.*, 2011). Evolutionarily speaking, the genome of *P. aeruginosa* is also interesting, considering that it contrasts with the decay of genomes, normally seen when pathogens adapt to a parasitic existence (Mathee *et al.*, 2008). These genomic characteristics aid the metabolic flexibility, pathogenicity and ubiquity of *P. aeruginosa*.

A bacterial species can be described by its pan-genome ('pan', from the greek 'whole'): a sum of its core genome, that encompasses the genes present in all strains, and its accessory genome (or mobilome), that encompasses genes present in some strains or that are unique to single strains (review by Kung *et al.*, 2009; review by Medini *et al.*, 2005). The pan-genome can either be an open pan-genome, typical of microorganisms that colonize multiple environments and have a good range of instruments to exchange genetic material (e.g. *Eschericia coli, Helicobacter pylori, Streptococci, Salmonellae* and *Meningococci),* or a closed pan-genome, typical of microrganisms that live in isolated niches and have a limited access to the global microbial gene pool, resulting in a limited acquisition of novel genes (e.g. *Bacillus anthracis, Mycobacterium tuberculosis and Chlamydia trachomatis)* (review by Medini *et al.*, 2005). The pan-genome of *P. aeruginosa* is an open pan-genome

The core genome of *P. aeruginosa* has a conserved sinteny of genes, and while for many years it was thought that the core genome composed roughly 90% of the total genomic repertoire (review by Kung *et al.*, 2009), that view has radically change with the increasing sequencing of genomes by Next Generation Sequencing (NGS), that exposes how diversified the genome of *P. aeruginosa* is. More recent studies show that the core genome of *P. aeruginosa* might actually only correspond to 78% of the *P. aeruginosa* genome (Ozer *et al.*, 2014). The genome of *P. aeruginosa* has a low interclonal sequence diversity (0.5-0.7%), possessing multiple alleles at the few loci that are subject to diversifying selection (review by Kung *et al.*, 2009; Rau *et al.*, 2012;

Wiehlmann *et al.,* 2007). The genes that form the core genome are the ones responsible for the essential biological features and major phenotypic traits of *P. aeruginosa*, regardless of strain origin (environmental, clinical or laboratorial) (review by Kung *et al.,* 2009; review by Medini *et al.,* 2005).

The accessory genome of *P. aeruginosa,* consists of extra chromosomal elements, like plasmids, and blocks of DNA inserted into the chromosome (Klockgether *et al.,* 2011). These blocks of DNA tend to cluster into specific loci, denominated as regions of genomic plasticity (RGPs) (review by Kung *et al.,* 2009). The genetic sequences that occupy RGPs are usually referred in the literature as genomic islands (> 10 kbp) or islets (< 10 kbp) (review by Kung *et al.,* 2009). Most of the mobilome of *P. aeruginosa* can be sorted into four large groups: a) integrative and conjugative elements; b) replacement islands; c) prophages and phage-like elements; and d) transposons, insertion sequences and integrons (review by Kung *et al.,* 2009).

The accessory genome of *P. aeruginosa* can comprise up to 20-22% of the genome, with elements of variable sizes (Pohl *et al.,* 2014; Ozer *et al.,* 2014). As an example, the *P. aerurginosa* PAO1 genome only contains inserts of 14 kbp or smaller, whereas the LESB58 genome has mobile genetic elements of up to 111 kbp in size (Klockgether *et al.,* 2011; review by Silby *et al.,* 2011). The genes found in the accessory genome are the ones responsible for most inter and intraclonal diversity in *P. aeruginosa* (Klockgether *et al.,* 2011). For instance, there are, in the single *P. aeruginosa* strains 2192, C3719, LESB58, PA7, PACS2 and PAO1, 187, 45, 219, 660, 29 and 58 unique predicted genes, respectively (review by Silby *et al.,* 2011). These genes confer specific phenotypes that are advantageous under selective conditions, such as encoding new catabolic pathways that allow the bacterium to thrive in normally inhospitable environments (e.g. presence of heavy metals and toxic organic compounds) (review by Kung *et al.,* 2009). The accessory genome is also partially responsible for the bacterium persistence in the host, encoding both virulence factors and resistance to multiple classes of antibiotics (review by Kung *et al.,* 2009).

Horizontal gene transfer (HGT) is the central mechanism for genome evolution in microorganisms, particularly those highly adaptable, and *P. aeruginosa* is no exception. For bacterial species to exchange their repertoire via HGT, a recipient needs to be in an environment where donor genetic material is available (Carter *et al.,* 2010). This occurs in nature whether there are different strains of the same species cohabiting a niche or whether a species is part of large and diverse microbial community (Carter *et*

al., 2010). The uptake of new genetic material can occur through transformation, transduction or conjugation (Carter *et al.*, 2010). In addition, bacteria can transfer whole genomic islands, using a variety of pili such as sex pili, type IV pili or the type IV secretion system-related pili (Carter *et al.*, 2010; Filloux *et al.*, 2010). Gaining genomic islands is particularly advantageous to the bacterium, since, this way it is able to acquire a good amount of traits in a single HGT event (Carter *et al.*, 2010). This way, HGT events play a considerable part in the dissemination and persistence of opportunistic infections.

Other than intergenomic comparisons, the most straightforward way to predict the accessory genome of *P. aeruginosa* is looking for an aberrant GC content: the GC content of *P. aeruginosa* is higher than that of the genes from other species, acquired via HGT. Analysing the codon usage, the tetranucleotide usage and looking at the tRNA genes, that are targeted for insertions, are also good approaches (review by Kung *et al.*, 2009). Nonetheless, new elements of the accessory genome undergo the same pressures as other genes upon integration into the chromosome and can, overtime, lose the differential sequences that once distinguished it from the core genome (Klockgether *et al.*, 2011; review by Kung *et al.*, 2009).

1.2.3. Pathogenesis: main mechanisms and outcomes thereof

The genomic plasticity of *P. aeruginosa* is translated into an availability of a multitude of traits that allow the bacterium to be phenotypically flexible and thrive both in the environment and in the host (Figure 2).

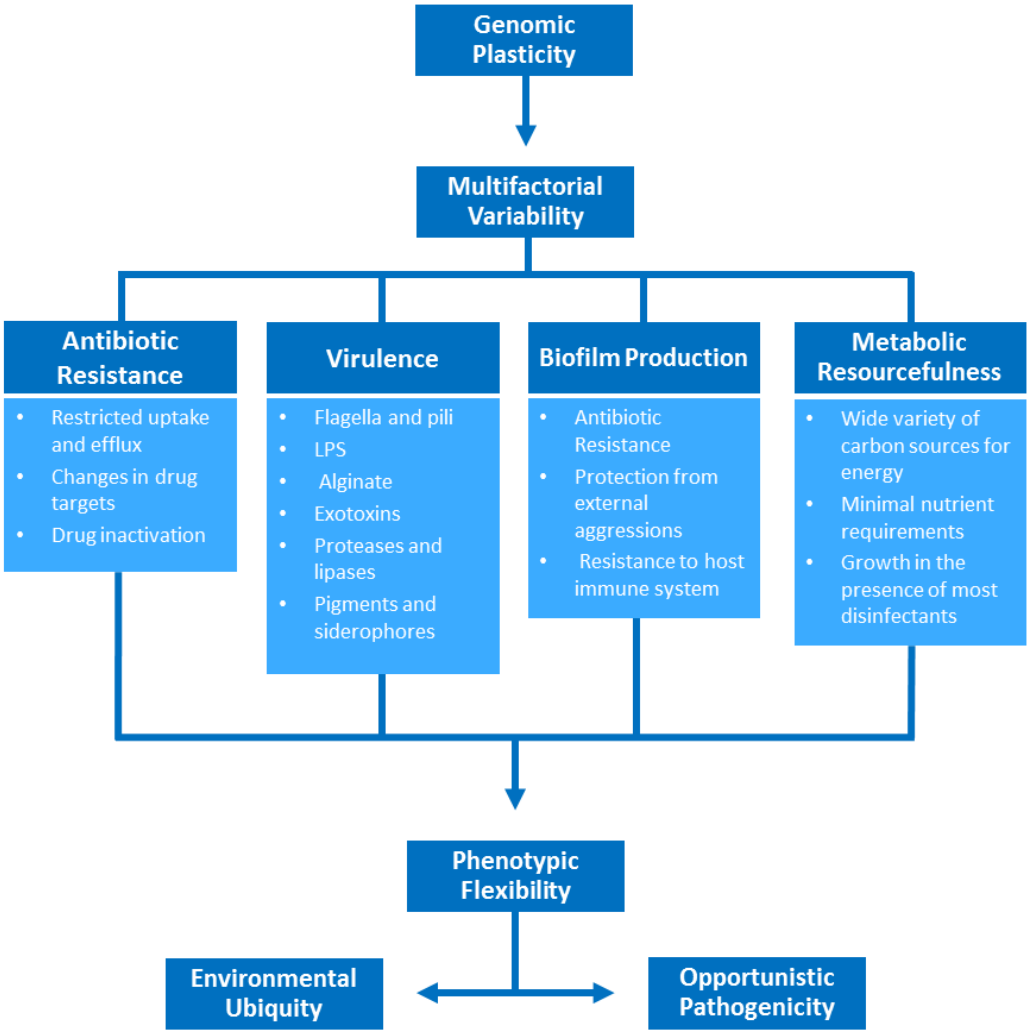


Figure 2. Most studied traits of *P. aeruginosa* that contribute to the ubiquity, pathogenicity and overall fitness of the bacterium.

Up to this date, the traits most studied by scientists to explain the fitness of *P. aeruginosa* are the ones accounted for its ability to form biofilms, its virulence, its capacity to battle most antibiotics and its metabolic resourcefulness (review by Mesquita *et al.*, 2013).

Biofilms

Biofilms are architecturally complex communities of microbes, enclosed in a self-produced extracellular matrix (ECM), growing adhered to each other and/or to a surface (review by López *et al.*, 2010; Schleheck *et al.*, 2009). Conventionally, biofilm communities present an increased resistance to antibiotics, protection from protozoan grazing, resistance to the host innate and adaptive immune system and protection from

external aggressions (review by López *et al.,* 2010; review by Römling and Balsalobre, 2012). The formation of biofilms is almost always associated with chronic and, to some extent, acute infections, being considered a hallmark of persistent infections (review by Römling and Balsalobre, 2012). Diseases, in which biofilms of *P. aeruginosa* were found, include chronic wound lung infection, chronic otitis media, catheter-associated urinary tract infection and the CF lung infection, where host cells and host components can be an integral part of the biofilm together with *P. aeruginosa*. (review by Römling and Balsalobre, 2012).

Biofilm formation varies not only between species but also between strains of the same species (review by López *et al.,* 2010). Still, some characteristics are commonly found, such as the obligatory ECM. Studies in the literature up to this date indicate that, in *P. aeruginosa,* the ECM can be formed by three exopolysaccharides:, PSL (a manose-rich polysaccharide) and PEL (a glucose-rich polymer, encoded by the *pel* gene cluster, that despite being found in most strains, presents a highly variable expression between isolates) and alginate, often found in strains isolated from the lungs of CF patients (review by López *et al.,* 2010). Specifically, alginate, associated with the mucoid phenotype of chronic infections, is proved to protect bacterium from phagocytosis and antibodies, conferring a survival advantage to *P. aeruginosa* (review by Balasubramanian *et al.,* 2013). In addition to the exopolysaccharides, the ECM of *P. aeruginosa* biofilms contains significant amounts of extracellular DNA (eDNA) (review by López *et al.,* 2010).

To form a biofilm, a motile, planktonic cell needs to first approach and adhere onto a surface and then attach irreversibly to it, developing micro-colonies that produce ECM, forming an unstructured biofilm (review by Römling and Balsalobre, 2012; Schleheck *et al.,* 2009). To do this, *P. aeruginosa* uses its flagella, type IV pili, eDNA and PSL, even though not all might be required under specific conditions (review by Harmsen *et al.,* 2010). The next step involves the maturation of the biofilm into a more complex, 3D architecture of microcolonies and void spaces (review by Römling and Balsalobre, 2012; Schleheck *et al.,* 2009). *P. aeruginosa* accomplishes this using its surface-associated motility (15). The pattern of motility, and consequent 3D architecture of the biofilm, is dependent on the environmental conditions (review by Harmsen *et al.,* 2010). For example, twitching motility (a result of a repeated extension, tethering and retraction of the type IV pili) is observed under low iron availability, forming flat biofilms (review by Burrows, 2012; review by Harmsen *et al.,* 2010). In the final stage

of biofilm formation, small clusters of cells can detach from the biofilm, dispersing the community (review by López *et al.*, 2010). In Figure 3, a schematic representation of biofilm development in *P. aeruginosa* can be seen.

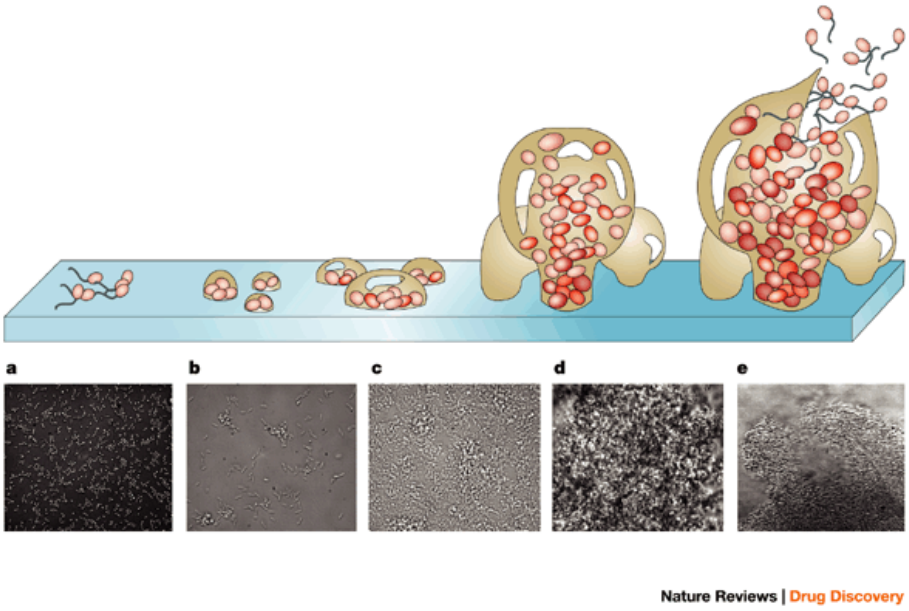


Figure 3. Schematic representation of the stages of biofilm development in *P. aeruginosa*, supplemented by photomicrographs of a developing *P. aeruginosa* biofilm (scale is the same in all images) (review by Davies *et al.*, 2003).

A great number of physiological processes associated with biofilm formation is regulated by quorum-sensing (QS) systems (13). QS systems are a form of cell-cell communication in bacteria, using signals that, upon uptake, regulate cell behaviour (autoinduction) and increase the production of the signal molecule itself (autoregulation) (West *et al.*, 2012). For biofilm-related processes, *P. aeruginosa* responds to a class of autoinducers called acyl homoserine lactones (AHLs), having two QS systems associated with it, *las* and *rhl* (review by López *et al.*, 2010). The importance of these systems always varies according to the strain and environmental conditions, but, as an example, both systems are involved in the lysis of a subpopulation of *P. aeruginosa* PAO1 to generate eDNA (review by López *et al.*, 2010; review by Montanaro *et al.*, 2011). In addition to QS molecules, other signals that trigger the formation of *P. aeruginosa* biofilms are secondary metabolites, such as subinhibitory concentrations of antibiotics, pigments and siderophores (review by López *et al.*, 2010). The GacA/GacS two-component system (TCS), where GacS is a sensor kinase and

GacA is a response regulator, is also described to play a role in biofilm formation in *P. aeruginosa* (review by Gellatly and Hancock, 2013; Workentine *et al.,* 2009). The cyclic dimeric guanosine monophosphate (c-di-GMP) signaling network is present in *P. aeruginosa* and is mentioned to play a role in activating biofilm formation, suppressing motility, and regulating processes associated with the persistence of the infection (review by Römling and Balsalobre, 2012). Notably, *P. aeruginosa* isolates from lungs of patients with CF present elevated c-di-GMP levels (review by Römling and Balsalobre, 2012).

Given the importance of biofilms in the persistence and antibiotic resistance of *P. aeruginosa* infections, biofilms are a clear target of therapeutic interventions (review by Davies *et al.,* 2003; review by Veesenmeyer *et al.,* 2009).

Virulence and secretion systems

There are a multitude of virulence factors in *P. aeruginosa* (review by Gellatly and Hancock, 2013). The most studied virulence factors of *P. aeruginosa* include flagella, type IV pili, lipopolysaccharide (LPS), biofilms and alginate, exotoxin A, lipases and phospolipases, pyocyanin, siderophores, proteases, and exotoxins ExoS, ExoT and ExoU (review by Gellatly and Hancock, 2013; review by Balasubramanian *et al.,* 2013).

Flagella and type IV pili, localized at a cell pole of *P. aeruginosa,* are responsible for the swimming and the twitching motility, respectively (review by Gellatly and Hancock, 2013). Both flagella and type IV pili are responsible for the swarming motility (a highly coordinated motility on semi-solid surfaces) and for the adhesion of the bacterium to epithelial cells (review by Gellatly and Hancock, 2013). Both can also elicit a NFκB-mediated inflammatory response via signalling through the Toll-like receptor 5 (TLR5) (review by Gellatly and Hancock, 2013; review by Veesenmeyer *et al.,* 2009). These cellular components are important virulence factors in acute infections, however a large proportion of isolates from chronic infections present downregulation of flagellin and type IV pili – probably to evade recognition from the host immune system (review by Gellatly and Hancock, 2013; review by Veesenmeyer *et al.,* 2009).

LPS is an integral part of the cell envelope of *P. aeruginosa*, being found in the outer leaflet of the outer membrane (review by Balasubramanian *et al.,* 2013). Composed by three domains (the membrane-anchored lipid A, the core oligosaccharide

and the variable O-antigen), LPS is one of the most important virulence factors of *P. aeruginosa* (review by Balasubramanian *et al.*, 2013; review by Lam *et al.*, 2011). Lipid A is responsible for the binding of the bacterium to host cell receptors, leading to the NfκB-signalling pathway via activation of TLR4 (review by Gellatly and Hancock, 2013). Lipid A recognition can eventually lead to an endotoxic shock (review by Gellatly and Hancock, 2013). Alterations in LPS composition, changes its inflammatory potency and some strains of *P. aeruginosa* are reported to produce neither O-antigen nor lipid A, or produce the O-antigen partially, probably to evade host adaptive immune responses (review by Gellatly and Hancock, 2013).

Exotoxin A is responsible for inhibiting the host elongation factor 2, and consequently, protein synthesis, leading to cell death and repression of the host immune response (review by Gellatly and Hancock, 2013).

Lipases and phospolipases are important to break down surfactant lipids and phospolipids of host cell membranes (review by Gellatly and Hancock, 2013).

Pyocyanin, the blue-green pigment that gives some *P. aeruginosa* colonies their distinct colour, is responsible for many phenomena during infection: it causes oxidative stress on the host cells, disrupts host catalase and mitochondrial electron transport, induces apoptosis in neutrophils, inhibits the phagocytosis of apoptotic bodies by macrophages, modulates the expression of chemokines IL-8 and RANTES by airway epithelial cells, suppresses cilia beating and protects *P. aeruginosa* against the reactive oxygen and nitrogen species produced by phagocytic cells (review by Gellatly and Hancock, 2013).

Enhanced iron uptake in *P. aeruginosa* is provided by the two siderophores produced by *P. aeruginosa*, pyoverdine and pyochelin, as well as siderophores produced by other microorganisms (review by Balasubramanian *et al.,* 2013). The importance of pyoverdine to the maintenance of the expression of some virulence factors in *P. aeruginosa* has been proved (e.g. exotoxin A) (review by Balasubramanian *et al.*, 2013; review by Gellatly and Hancock, 2013). Plus, intracellular iron concentration can impact biofilm development (review by Balasubramanian *et al.*, 2013).

Several proteases are secreted by *P. aeruginosa*: alkaline protease, that is able to degrade the host complement proteins, the host fibronectin and free flagellin monomers, thereby interfering with the flagellin recognition; the elastases LasA and LasB, where LasB has been shown to be able to degrade the opsonizing proteins, while LasA enhances the proteolytic activity of LasB; and protease IV, that degrades complement

proteins, immunoglobulins, fibrinogen and opsonizing proteins (review by Gellatly and Hancock, 2013).

P. aeruginosa secretes many factors to the extracellular environment but one specific set of toxins is injected directly into the cytosol of host cells – a phenomenon that is mediated by the type III secretion system (TTSS) (overall there are present, in *P. aeruginosa,* five of the six secretion systems found in Gram-negative bacteria) (review by Bleves *et al.,* 2002). The TTSS consists in a macromolecular "syringe"/injectisome, formed by a secretion apparatus (a transmembrane cylindrical body) and a translocon to inject the toxins (review by Bleves *et al.,* 2002; review by Veesenmeyer *et al.,* 2009). The toxins (or effectors), once injected into host cells, have the ability to mimic the activity of endogenous proteins and hijack host processes and factors (review by Bleves *et al.,* 2002; review by Veesenmeyer *et al.,* 2009). There are four effectors documented for *P. aeruginosa*: ExoS, ExoT, ExoU and ExoY. The first three are linked to virulence (review by Veesenmeyer *et al.,* 2009). ExoS (present in 70% of all strains) and ExoT (present in all strains) induce disruption of the host cell actin cytoskeleton, block phagocytosis, and eventually cause cell death (review by Veesenmeyer *et al.,* 2009). ExoU (present in 30% of all strains) is the most virulent of the effectors (having a phospolipase activity), leading to a rapid dissolution of the plasma membrane and also starting mechanisms that end up in excessive inflammation, increased tissue damage and dissemination of the infection (review by Veesenmeyer *et al.,* 2009).

Overall, TTSS increases the pathogenicity of *P. aeruginosa* infections, being associated with persistence of the bacterium in lungs and with an increased mortality in patients with respiratory infections (review by Veesenmeyer *et al.,* 2009). Currently, there is an effort in designing agents that disrupt both TTSS and inhibit effectors (review by Veesenmeyer *et al.,* 2009).

The regulation of virulence factors in *P. aeruginosa* is mainly attributed to QS, since it is estimated that QS regulates up to 10% of genes in the genome and more than 20% of the expressed bacterial proteome (review by Gellatly and Hancock, 2013). To regulate its virulence, *P. aeruginosa* produces three autoinducers, two belonging to the AHLs class, regulating the *las* and *rhl* systems, and one belonging to the class of 4-quinolones (AQs), controlled by *las* and regulating the *rhl* system, connecting both (review by Balasubramanian *et al.,* 2013; review by Jimenez *et al.,* 2012; review by Veesenmeyer *et al.,* 2009). As in biofilm formation, c-di-GMP (e.g. controlling motility) and TCSs (e.g. modifications in lipid A can be caused by the presence of antimicrobial

peptides that act either through the ParRS or the CpxRS TCSs) also regulate the QS (review by Gellatly and Hancock, 2013; review by Jimenez *et al.,* 2012

Mechanisms of antibiotic resistance

As a result of a combinatory effect between several factors, *P. aeruginosa* is a microorganism with a remarkable capacity of resistance to antibiotics: *P. aeruginosa* has a low cell wall permeability, has a great genetic capacity to express a wide repertoire of resistance mechanisms (*P. aeruginosa* possesses one of the biggest bacterial genomes, as reviewed earlier), and can acquire resistance genes due to mutations that alter the expression/function of specific chromosomal genes or acquire resistance from other microorganisms, on mobile genetic elements such as plasmids, integrons and transposons via HGT (as reviewed earlier) (review by Lambert, 2002; review by Lister *et al.,* 2009). For example, the transposon Tn6061 is known to carry 10 antibiotic resistance genes (review by Kung *et al.,* 2009). Overall, the mechanisms of drug resistance in *P. aeruginosa* are restricted uptake and efflux, changes in drug targets and drug inactivation (review by Lambert, 2002).

Epidemiologically significant classes of antibiotics for *Pseudomonas aeruginosa* are: (i) aminoglycosides (amikacin, gentamicin, netilmicin and tobramycin), that bind to the 30S subunit of the ribosome, inhibiting protein synthesis (review by Lambert, 2002; review by Magiorako *et al.,* 2011; review by Mesaros *et al.,* 2007); (ii) β-lactams, subdivided into carbapenems (imipenem, meropenem, and doripenem), cephalosporins (ceftazidime and cefepime) and monobactams (aztreonam), that inhibit the peptidoglycan-assembling transpeptidases, located on the outer leaflet of the cytoplasmic membrane (review by Lambert, 2002; review by Magiorako *et al.,* 2011; review by Mesaros *et al.,* 2007); (iii) fluoroquinolones (ciprofloxacin and levofloxacin), that bind to the A subunit of DNA girase, important for chromosomal stability during DNA replication (review by Lambert, 2002; review by Magiorako *et al.,* 2011; review by Mesaros *et al.,* 2007); (iv) phosphonic acids (fosfomycin), that inhibit bacterial cell wall biogenesis by enzyme inactivation (Brown *et al.,* 1995; review by Magiorako *et al.,* 2011; Okuhara *et al.,* 1980); (v) penicillins + β-lactamase inhibitors (ticarcillin-clavulanic acid, piperacillin-tazobactam), that work together by preventing penicillins' inactivation by bacterial β-lactamases before cell death (review by Drawz and Bonomo, 2000; review by Magiorako *et al.,* 2011) ; and (vi) polymyxins (colistin and polymyxin B), last-resort drug antibiotics, that bind to phospholipids in the cytoplasmatic

membrane, destroying its barrier function (review by Lambert, 2002; review by Magiorako *et al.,* 2011). Although there is no consensus in the scientific community regarding the classification of *P. aeruginosa* as multidrug-resistant (MDR), most studies attribute the MDR status to isolates that are resistant to at least 1 agent of at least 3 classes of antibiotics aforementioned (review by Hirsch *et al.,* 2010; Gill *et al.,* 2011). More specifically, for *P. aeruginosa* isolates to be classified as extensively drug-resistant (XDR) they must be resistant to 1 agent in all but 2 of the aforementioned classes, and, to be pandrug-resistant (PDR), they must be resistant to all antimicrobials tested (Gill *et al.,* 2011).

The outer membrane of *P. aeruginosa* is itself a barrier to the penetration of antibiotics due to its low permeability (review by Lambert, 2002). β-lactams and fluoroquinolones must pass the membrane through porins and, qualitative and quantitative losses of the porin OprD are associated with an increase in the minimum inhibitory concentrations of antibiotic treatments (review by Lambert, 2002; review by Mesaros *et al.,* 2007). Aminoglycosides and colistin promote their uptake via LPS binding (review by Lambert, 2002). Resistance to aminoglycosides and polymyxins has been found in lab strains overexpressing *orpH*, an outer membrane protein that prevents binding to LPS, but not many clinical isolates of *P. aeruginosa* have been found with this feature (review by Lambert, 2002). Modification in lipid A of LPS, using the ParR-ParS TCS, has been accounted for polymyxins resistance in *P. aeruginosa* (Fernández *et al.,* 2010). Alginate has been described as a barrier to aminoglycosides, although the fact that many mucoid isolates are highly sensitive to aminoglycosides shows that the effect of this barrier is minimal (review by Lambert, 2002).

Multidrug efflux systems (in *P. aeruginosa,* the systems *mexAB-oprM, mexAB-oprD, mexXY-oprM, mexCD-oprJ* and *mexEF-oprN*) are a cause of innate and acquired resistance to antibiotics and disinfectants (review by Lambert, 2002; review by Mesaros *et al.,* 2007; review by Strateva and Yordanov, 2009). For instance, the overexpression of *mexXY-oprM* is linked to an increased resistance to aminoglycosides, while upregulation of the *mexAB-OprD* and *mexAB-OprM* systems, in combination with the microorganism's low outer membrane permeability, can contribute to decreased susceptibility to penicillins, β-lactams and fluoroquinolones (review by Drawz and Bonomo, 2000; review by Lambert, 2002; review by Mesaros *et al.,* 2007). Even though efflux is considered to infer only a low-to-moderate level of resistance, selection of mutants with an increased pump production induces cross-resistance to other antibiotics

that are also a substrate to the derepressed pump (for example, it is known that exposing the pathogen to fluoroquinolones selects cross-resistance to β-lactams and aminoglycosides) (review by Mesaros *et al.*, 2007). This limits the range of antibiotics available to use in case of infection. Additionally, multidrug efflux systems lower the intra-bacterial antibiotic concentrations, favouring the appearance of target mutations (review by Mesaros *et al.*, 2007).

Altered targets are the rarest mechanism of resistance to β-lactams in *P. aeruginosa* (review by Strateva and Yordanov, 2009). However, resistance to fluoroquinolones can be due to point mutations in the *girA/girB* genes that encode the enzyme DNA gyrase and mutations in the *parC* and *parE* that encode two subunits of topoisomerase IV, a secondary target of fluoroquinolones (review by Lambert, 2002; review by Strateva and Yordanov, 2009). Methylation of 16S rRNA has been recently attributed to induce resistance to aminoglycosides in Gram-negative bacteria, including *P. aeruginosa*, being the responsible genes found in transposons within transferable plasmids (review by Strateva and Yordanov, 2009).

All strains of *P. aeruginosa* have a chromosomal gene encoding for β-lactamase (*ampC*) and, mutations in its regulatory gene, *ampR*, can result in its overexpression, inducing resistance to β-lactams (review by Lambert, 2002). β-lactamase is an extracellular enzyme accountable for reducing local antibiotic concentrations (review by Lambert, 2002). Production of β-lactamases enzymes is the most common and important drug resistance mechanism in Gram-negative bacteria (review by Drawz and Bonomo,2000). Other β-lactamases produced by *P. aeruginosa* include extended-spectrum plasmid-mediated enzymes active against both penicillins and β-lactams (review by Lambert, 2002). Carbapenemases also induce resistance to β-lactams and can be transferred between strains via plasmids (review by Lambert, 2002). Plasmid-mediated modifying enzymes that transfer cetyl, phosphate or adenylyl groups to amino and hydroxyl substituent on antibiotics have been accounted for gain of resistance to aminoglycosides (review by Lambert, 2002). The mutant frequency of *P. aeruginosa* resistance to fosfomycin has been reported as very high both in vitro and in vivo (Rodríguez-Rojas *et al.*, 2010; Rodríguez-Rojas and Blázquez, 2009). Particularly, mutation of *glpT* (essential for uptake) and overexpression of *fosA* (enzyme modification of fosfomycin), have been described as essential for persistence of cells treated with this antibiotic (De Groote *et al.*, 2011). Drug inactivation is not a major mechanism of fluoroquinolone resistance (review by Strateva and Yordanov, 2009).

Phenotypic switching, persister cells and social cheaters

Phenotypic switching is the reversible switch between two phenotypic states that can occur in a variable fraction of a genetically identical bacterial population, creating two subpopulations with altered gene expression (review by Mesquita *et al.,* 2013; review by Sousa *et al.,* 2011). This phenomenon is part of the adaptive response of bacteria to environmental fluctuations, being fundamental for the population fitness and persistence, occurring at higher rates than the frequency of spontaneous mutations (review by Sousa *et al.,* 2011).

In *P. aeruginosa,* phenotypic switching is often associated with the transition to a mucoid phenotype in the CF lung, and with the appearance of small colony variants (or morphotypes) among biofilm growing cells, such as persister cells and social cheaters (review by Häussler, 2004; review by Mesquita *et al.,* 2013; Sandoz *et al.,* 2007; review by Sousa *et al.,* 2011).

Persister cells is the name given to a small percentage of cells that remain in a dormant-like state in the microbial population and that are highly resistant to antibiotic treatments (review by Lewis, 2010; review by Mesquita *et al.,* 2013). The existence of persister cells is higher when the microbial population reaches the stationary state or forms biofilms, since ECM acts as a diffusion barrier to small molecules (e.g. cofactors, nutrients and vitamins) and, as a consequence some cells become metabolically inactive (review by Lewis, 2010; review by López *et al.,* 2010). As we have seen, most of the antibiotics are only effective in metabolically active cells (review by Lewis, 2010). The persister cells are the ones that re-establish the microbial population after an antibiotic treatment (Niepa *et al.,* 2012).

Social cheaters are QS-deficient variants, most of which carry a mutation in the gene encoding *lasR,* which take advantage of the production of quorum-sensing factors by the group of cells surrounding them (Sandoz *et al.,* 2007). QS imposes a metabolic burden on growing bacterial cells under conditions that require QS for growth, and social cheaters, have in this sense a growth advantage compared with the wild type cells (Sandoz *et al.,* 2007). *P. aeruginosa* social cheaters are commonly isolated from chronic and acute infections (Sandoz *et al.,* 2007).

There are, still, only speculating mechanisms explaining the formation of morphotypes, persister cells and social cheaters (Niepa *et al.,* 2012; Sandoz *et al.,* 2007; review by Sousa *et al.,* 2011). Some proposed mechanisms are, for example, the insertion and excision of transposable elements or a stochastic variation, "noise", at the

transcriptional and translational level of specific genes (Niepa *et al.,* 2012; review by Sousa *et al.,* 2011). It was already proved that *P. aeruginosa* persister cells can be formed in response to QS molecules and pyocyanin (Möker *et al.,* 2010).

1.2. Genotyping methods in molecular epidemiological surveillance of clinical infections by *Pseudomonas aeruginosa*

1.2.1. Clinical relevance and epidemiological overview of *Pseudomonas aeruginosa* infections

Environmental reservoirs of *P. aeruginosa* include swimming pools, whirlpools, hot tubs, contact lens solutions, home humidifiers, soil and rhizosphere, and vegetables (review by Lister *et al.,* 2009). In hospitals, *P. aeruginosa* can be found in respiratory therapy equipment, endoscopes, urometers, showers and bathrooms, disinfectants, sanitizers, antiseptics and cleaning equipment, among others (review by Kerr and Snelling, 2009). Humans are, too, reservoirs of this bacterium, stressing the fact that good hygiene is imperative in the control and prevention of *P. aeruginosa* in hospital settings (review by Kerr and Snelling, 2009).

A report from 2008, surveying 463 hospitals in the USA over a 22 month period, positioned *P. aeruginosa* as the top 6th causing agent of nosocomial infections, the 2nd commonest cause of ventilator-associated pneumonia and the 7th commonest cause of catheter-related bloodstream infection (Hidron *et al.,* 2008). *P. aeruginosa* is especially problematic in intensive care units (ICUs) (review by Lister *et al.,* 2009). A European study, representing 40% of all western Europeans ICUs, with data collected during a 24 hour period, showed *P. aeruginosa* to be the 2nd most frequently isolated microorganism in ICU-acquired infections, being responsible for 30% of pneumonias, 19% of urinary tract infections and 10% of bloodstream infections (review by Lister *et al.,* 2009; Spencer, 1996).

The increasing frequency of MDR isolates of *P. aeruginosa* is concerning as efficient microbial options become increasingly limited (Obritsch, *et al.,* 2005). A study made in the United States of America has shown that over a period of 10 years (1993-2002) the number of isolates classified as MDR increased from 4% to 14% in ICUs.

More recently, a new study from the United States of America showed that among 235 bloodstream isolates, obtained from ICU-staying patients between 2005 and 2007, 21% were resistant to aminoglycosides, 91% were resistant to penicillins/cephalosporins, and all were resistant to carbapenems and quinolones (Tam *et al.,* 2010). Coincidently, in this study, 14% of the isolates were also classified as MDR (Tam *et al.,* 2010).

CF patients are particularly susceptible to *P. aeruginosa* infections (review by Kerr and Snelling, 2009). Indeed, 54.4% of the whole CF patient population is believed to be infected with this bacterium, numbers that go up to 80% when considering just the over 18 years old CF patients (Gaspar *et al.,* 2013). Historically, CF patients died within 5 years after the onset of a chronic *P. aeruginosa* infection, but intense early eradication therapy changed the prognostics (review by Høiby, 2011). The population structure of *P. aeruginosa* can be characterized by a few dominant clones wide-spread in disease and environmental habitats (Wiehlmann *et al.,* 2007), however, data collected from more than 50 CF centres in Europe, showed that even though not all clones are found in CF lungs, there is no preponderance of habitat or disease-associated clones (review by Cramer *et al.,* 2010). What has been observed is that the initially acquired clone, persists for many years, and diversifies by *de novo* point mutations and accessory genome composition, adapting to the CF lung and persisting for decades despite host defence mechanisms and antimicrobial treatments (review by Cramer *et al.,* 2010; review by Høiby, 2011).

Being a ubiquitous microorganism, for many years was thought that *P. aeruginosa* infections were caused by unrelated strains, however, it is currently known that epidemic outbreaks from the same strain may occur; such is the case of the drug-resistant, CF-related Liverpool epidemic strain (LES), found in over 60% of patients at a UK paediatric CF center, using genotyping methods (Salunkhe, *et al.,* 2005). Historically, CF patients infected with MDR epidemic strains have had poorer outcomes, requiring greater hospitalization, than patients infected with unique strains of *P. aeruginosa* (Govan *et al.,* 2007).

Characterization of *P. aeruginosa* is center key in epidemiological studies of nosocomial infections, contributing to an increased effectiveness of surveillance systems, outbreak detection and clinical treatment (review by Sabat *et al.,* 2013; review by Tenover *et al.,* 1997). In the next section, a review of the techniques available to genotype *P. aeruginosa* isolates is presented.

1.2.2. Bacterial characterization methods

Nowadays, a multitude of characterization methods is available to distinguish pathogens and isolates, being usually divided into two main groups: phenotypic methods, which characterize products of gene expression in order to differentiate strains, and genotypic methods that involve the analysis of DNA and RNA-based genetic elements of bacteria (Arbeit, 1995; Loreen *et al.*, 2001; review by Tenover *et al.*, 1997). An ideal method must follow 3 criteria: (i) provide unambiguous results (typeability); (ii) always provide the same result for the same isolate (reproducibility); (iii) distinguish epidemiological unrelated strains (discriminatory power) (Loreen *et al.*, 2001).

Phenotypic methods are the oldest and most conventional ones and include typing methods such as: (i) biotyping, where the genus and species of a microorganism can be distinguished based on its response to a panel of biochemical agents (Loreen *et al.*, 2001); (ii) serotyping, where series of antibodies are used to detect different antigenic determinants on the bacteria's cell surface (review by Ranjbar *et al.*, 2014); (iii) antimicrobial susceptibility patterns, where resistance profiles to antibiotics are assayed (review by Ranjbar *et al.*, 2014); (iv) phage typing, where susceptibility of bacteria (lysis) to different phages is observed in agar plates (Marples and Roosdahl, 1997); (v) polyacrylamide gel electrophoresis (PAGE) with or without immunoblot typing, where cellular proteins are run on gel and profiles compared (Loreen *et al.*, 2001); and (vi) multilocus enzyme electrophoresis (MEE), where enzyme polymorphisms between strains are detected depending on the electrophoretic motilities of the encoded proteins (review by Cooper and Feil, 2004).

While having historically provided data for many epidemiological studies, phenotypic methods are, as a whole, too variable, labor intensive and time-consuming to be applied to large epidemiological studies (review by Ranjbar *et al.*, 2014). In addition, phenotypic typing methods are best applied to the typing of microorganisms with marked variations in gene expression (review by Ranjbar *et al.*, 2014). This is not the case in most infections, where only a small number of strains of a species are present (review by Ranjbar *et al.*, 2014).

In recent years, genotyping tools have revealed to be very powerful to discriminate isolates of the same pathogen (review by Lin *et al.*, 2014). Here, we present the most common genotyping methods divided into 4 main categories:

restriction-based methods, PCR-based methods, sequencing-based methods, and novel and prospective technologies.

Restriction-based methods

Pulsed-field gel electrophoresis (PFGE), a technique first developed by Schwartz and Cantor in 1984 (Schwartz and Cantor, 1984), is still considered the golden standard for genotyping up to these days (review by Goering, 2010). In this method, bacterial chromosomal DNA is digested into several fragments, including Mb-sized fragments, which are separated in 1.5% agarose gels based on a time-associated size-dependent reorientation of DNA migration, accomplished by periodically changing the electric field in different directions (review by Goering, 2010; Schwartz and Cantor, 1984). The restriction enzymes used must be unusual cutters, and its choice is critical for PFGE success (review by Goering, 2010). *SpeI, XbaI, DraI* and *SspI* are the most used in *P. aeruginosa* PFGE studies (review by Goering, 2010). The success of this technique, and the explanation to why it remains a top choice after many years, is based on its excellent discrimination power, high epidemiological concordance, excellent typeability and intra and inter-laboratory reproducibility, all with relatively low costs (review by Sabat *et al.,* 2013). In addition, thanks to initiatives such as PulseNet (Swaminathan *et al.,* 2001), international fingerprint databases were constructed, allowing a fast detection of emerging clones and monitoring of strains in different regions and countries (review by Sabat *et al.,* 2013). On the other hand, this method is technically demanding, labor-intensive, time-consuming and lacks resolution power to distinguish bands of nearly identical size (differing by less than 5%) (review by Sabat *et al.,* 2013).

Restriction fragment length polymorphism (RFLP) is a technique wherein genomic DNA is cut with one or more restriction endonucleases, generating a number of DNA fragments with different lengths (Panneerchelvam and Norazmi, 2003). These fragments are then separated using Southern blot analysis in which the digested genomic DNA is subjected to electrophoresis through an agarose gel, transferred to a membrane, and visualized by specific label DNA probe hybridization, determining the length of the fragments complementary to the probe (review by Lin *et al.,* 2014; review by Liu and Cordes, 2004). A RFLP occurs when the length of a fragment varies between individuals (review by Lin *et al.,* 2014). RFLP is a reproducible and high discriminatory technique for species but has proven to be insufficient to distinguish

between isolates within a species (review by Lin *et al.*, 2014). Moreover, it is difficult and time consuming to develop markers for species lacking molecular information (review by Liu and Cordes, 2004).

rRNA can be used for RFLP analysis (review by Koçak *et al.*, 2011), called ribotyping. This method uses conserved regions of the 16S and 23S rRNA genes for probing the different RFLP patterns, allowing determination of the DNA sequence fragments, as rRNA operons are universal (review by Koçak *et al.*, 2011). Ribotyping is time-consuming and demands skilled personnel. However, this has been surpassed by the use of automated ribotyping systems, making ribotyping a good option for a first step analysis in epidemiological studies (review by Ranjbar *et al.*, 2014).

PCR-based methods

PCR-RFLP is a variation of the RFLP method, where fragments of a specific gene are amplified with specific primers, restricted and then separated by gel electrophoresis (review by Koçak *et al.*, 2011; review by Ranjbar *et al.*, 2014). For proper discrimination after amplicon cutting, the amplified gene must have variable sequences flanked by highly conserved sequences, targeted by universal primers (review by Ranjbar *et al.*, 2014). This method can be used to distinguish different genera and species in epidemiological studies, like RFLP, with the advantage of being less laborious (review by Ranjbar *et al.*, 2014).

Random amplified polymorphic DNA (RAPD) is a PCR-based technique that allows the detection of genetic polymorphisms without previous knowledge of the nucleotide sequence (Williams *et al.*, 1990). This technique uses short primers (normally 10 bp primers) that anneal at different places in the genome under non-stringent temperature conditions, generating variable-length amplicons, when two primer binding sites are within a 0.1-3 kb range (Ranjard *et al.*, 1999; review by Sabat *et al.*, 2013) The amplicons, or genetic markers, are then resolved by gel electrophoresis (Ranjard *et al.*, 1999). Due to its capacity to generate random markers for the entire genome of the microorganism, RAPD allows the assessment of proper genetic diversity (review by Lin *et al.*, 2014). Multiplex RAPD-PCR, a variation of the RAPD-PCR, where a considerable number of primers are used, can be applied when there is a necessity to increase the number of informative genetic markers (e.g. 11 primers have been used to assess the diversity of *Mycobacterium tuberculosis*) (Hatta *et al.*, 2014). Although RAPD-PCR is a simple, inexpensive and rapid typing method, intra and inter-

laboratory reproducibility issues have been reported due to its high sensitivity to primer variation, DNA concentration, DNA template quality, DNA polymerase, the use of non-stringent annealing temperature conditions, and gel electrophoresis technique (Hatta *et al.*, 2014; review by Sabat *et al.*, 2013). In arbitrarily primed PCR (AP-PCR), other variation of the original RAPD-PCR, the amplification is conducted in three parts (each with its own stringency and component concentration), higher primer concentrations are employed during the first PCR cycles and primers of variable length (sometimes designed for other purposes) are used (review by Sabat *et al.*, 2013). The same advantages and disadvantages of RAPD-PCR apply to AP-PCR.

Repetitive extragenic palindromic-PCR (rep-PCR) is a genotyping method that, using oligonucleotide primers complementary to rep sequences, amplifies diverse regions of the bacterial genome lying between spacer fragments (Mohapatra *et al.*, 2007; review by Ranjbar *et al.*, 2014). The PCR products are then separated by gel electrophoresis, generating a pattern. The rep sequences can be divided into four types: the repetitive extragenic palindromic (REP) sequences, the enterobacterial repetitive intergenic consensus (ERIC) sequences, the BOX elements and the polytrinucleotide $(GTG)_5$ sequences (Mohapatra *et al.*, 2007; Versalovic *et al.*, 1994). REP sequences are 38 bp palindromic units conserved in all Gram-negative enteric bacteria (Versalovic *et al.*, 1994); ERIC sequences are 126 bp elements with central, conserved palindromic structures, conserved in all Gram-negative enteric bacteria, and appearing to be restricted to transcribed regions of the genome (review by Lin *et al.*, 2014; Versalovic *et al.*, 1994); BOX elements are modular in nature, with three differentially conserved subunits, namely boxA (57 bp), boxB (43 bp) and boxC (50 bp) (Versalovic *et al.*, 1994); the polytrinucleotide $(GTG)_5$ sequences that can be between 15 and several hundred bp of length, and are found to be highly repetitive in bacterial genomes (Versalovic *et al.*, 1994). Like RAPD, rep-PCR is relatively cheap, can obtain results in a short period of time and has a good discriminatory power (although REP-PCR has been described to not be as good as RAPD and ERIC-PCR) (review by Lin *et al.*, 2014; review by Sabat *et al.*, 2013). However, like RAPD, this method can be susceptible to irreproducibility due to variability in reagents and gel electrophoresis systems (review by Sabat *et al.*, 2013).

Variable-number tandem repeats (VNTR), often referred in the literature as micro or minisatellite DNA, are regions of the genome with tandem repetitive sequences ranging from a few to more than 100 bp in length (Smittipat *et al.*, 2005;

review by Ranjbar *et al.,* 2014). The number of tandem repeats can be highly variable among strains of the same species, being prone to higher-than background mutation rates due to DNA strand slippage during replication (review by Ranjbar *et al.,* 2014). In VNTR typing, primers are designed to anneal to flanking non-repetitive sequences, amplifying the DNA that encompasses short tandem repeats of a DNA sequence (review by Ranjbar *et al.,* 2014). The products are then separated by gel electrophoresis, sized and compared (Smittipat *et al.,* 2005; review by Ranjbar *et al.,* 2014). When many VNTR loci are used to characterize a population, the assay is called as multilocus VNTR analysis (MLVA), or multiple-locus VNTR fingerprinting (MLVF), when the assay does not provide an unambiguous calculation of the individual number repeats per locus (review by Ranjbar *et al.,* 2014; review by Sabat *et al.,* 2013). MLVA typing is cheap, fast and easy, whereas MLVF does not allow inter-laboratory comparisons since the exact number of repeat units in the obtained amplicons cannot be determined (review by Sabat *et al.,* 2013). Other intrinsic limitation of MLVA typing is that it is not a universal method, with primers needing to be designed for the targeted pathogens (review by Sabat *et al.*, 2013).

Amplified length polymorphism (AFLP) is a technique based on the selective amplification of restricted DNA fragments cut with two enzymes (review by Sabat *et al.,* 2013; Vos *et al.,* 1995). First, genomic DNA is cut, and oligonucleotide adaptors are specifically ligated to the sticky ends of the DNA fragments, followed by PCR amplification, using primers complementary to the adaptor sequence, restriction site and a few nucleotides at the end of the unknown template (review by Sabat *et al.,* 2013; Vos *et al.,* 1995). The products of amplification are run on gel and the band profiles compared (Vos *et al.,* 1995). AFLP is robust and reliable, since it combines the reliability of RLFP with the power of the PCR technique, using stringent reaction conditions (Vos *et al.,* 1995). Since its development, AFLP, has been broadly applied due to its high discriminatory power, however, AFLP is labor-intensive and expensive (review by Eberle and Kiess, 2012; review by Sabat *et al.,* 2013).

Sequencing-based methods

Multilocus sequence typing (MLST) is based on the more classic and well-tested MEE tests mentioned before, assigning alleles at each gene locus directly by nucleotide sequencing of internal fragments of ~ 450 bp, instead of indirectly from the electrophoretic mobilities of their gene products (review by Cooper and Feil, 2004;

review by Enright and Spratt, 1999). Since genes possess varying degrees of genetic drift, housekeeping genes are the most often sequenced due to their presence in all isolates within a species (review by Ranjbar *et al.*, 2014). However, the genes selected must be under strong selective pressures for genetic variability to be significant, which is not the case for most housekeeping genes (review by Ranjbar *et al.*, 2014). Genes that fall under such specifications are for example, virulence-related genes, with the typing method being called many times as multivirulence-loci sequence typing (MVLST) (review by Ranjbar *et al.*, 2014). For *P. aeruginosa*, the 7 genes used for MLST studies are: *acsA* (acetyl coenzyme A synthetase), *aroE* (shikimate dehydrogenase), *guaA* (GMP synthase), *mutL* (DNA mismatch repair protein), *nuoD* (NADH dehydrogenase I chain C, D), *ppsA* (phosphoenolpyruvate synthase) and *trpE* (anthralite synthetase component I) (Curran *et al.*, 2004). MLST is a technique that combines PCR and automated DNA sequencing, reducing labor and analysis time (review by Lin *et al.*, 2014), with the major advantage that the DNA sequencing data can be shared between different laboratories, with platforms/databases designed especially for this purpose (review by Lin *et al.*, 2014). Gene sequences that differ at even one nucleotide are assigned as different alleles, making this technique highly discriminatory (review by Enright and Spratt, 1999). Despite all these advantages, MLST is still a complex and expensive technique to perform (review by Eberle and Kiess, 2012).

Single locus sequence typing (SLST) is a typing mechanism that borrows its name from the technique mentioned above, and describes the sequencing of a single gene or gene locus, which displays enough polymorphism to be used in a typing scheme (Andreoletti *et al.*, 2013). Entailing the same operational steps as MLST, the only difference is in the number and selection of the target loci (e.g. the flagelin gene, *flaA*, short variable region may be used for typing of *Campylobacter*) (Andreoletti *et al.*, 2013). This technique is often complemented with other typing methods such as PFGE (review by Sabat *et al.*, 2013).

Novel and prospective technologies

Microarray-based comparative genomic hybridization (M-CGH) techniques have been used to extensively characterize bacterial intra-species genetic diversity (review by Kuboniwa and Amano, 2012). Here the principle of the microarray, where labelled cDNA molecules are hybridized on thousands of microscopic spots with specific complementary oligonucleotides (probes), is applied by generating probes

identifying genomic markers representing small nucleotide polymorphisms (review by Ranjbar *et al.,* 2014). Simultaneously, this technology allows the profiling of microorganisms based on multiple gene products such as antibiotic resistance determinants and virulence factors (review by Ranjbar *et al.,* 2014). Initially expensive, this technology has become more cost-effective and the SNP analysis can be used to study the bacteria population genetics (review by Ranjbar *et al.,* 2014). Genotyping microarray kits have been developed for various bacterial species, including *P. aeruginosa* (review by Sabat *et al.,* 2013). While promising, M-CGH needs to have its reproducibility established between different laboratories prior to broad use (review by Sabat *et al.,* 2013).

Whole genome mapping (WGM), or optical mapping, is a typing method that generates a barcode-like 'map' of genomic restriction sites of the genome of an isolate (Andreoletti *et al.,* 2013). Its discriminatory capability, reproducibility and repeatability are described as high (Andreoletti *et al.,* 2013). At this point the use of this technique is limited by the costs of the experiments and the availability of the needed specialized equipment (review by Sabat *et al.,* 2013).

Whole genome sequencing (WGS) uses NGS systems to rapidly sequence whole genomes (Andreoletti *et al.,* 2013). As prices of WGS drop, some researchers state that in a few years WGS could become the sole diagnostic and molecular epidemiological tool, taking care of identification, genetic characterization and drug susceptibility testing (review by Ranjbar *et al.,* 2014). However, the key challenge will be to rapidly compute and interpret relevant information from large datasets (review by Sabat *et al.,* 2013): depending on the technology, the reads obtained from NGS systems can be relatively short, making *de novo* genome assembly challenging and time consuming. In addition, well-trained bioinformaticians are needed, as well as heavy computer resources. If WGS becomes the top choice for genotyping in the future, it must be performed under rigid standard operating procedures for identification of variations over time and between laboratories (review by Ranjbar *et al.,* 2014).

Other genotyping methods can be found in the literature, such as plasmid fingerprinting, terminal restriction fragment length polymorphism (t-RFLP) and molecular serotyping, but they will not be discussed in this work.

In Figure 4, an overview summarizing the methods presented in this section is shown.

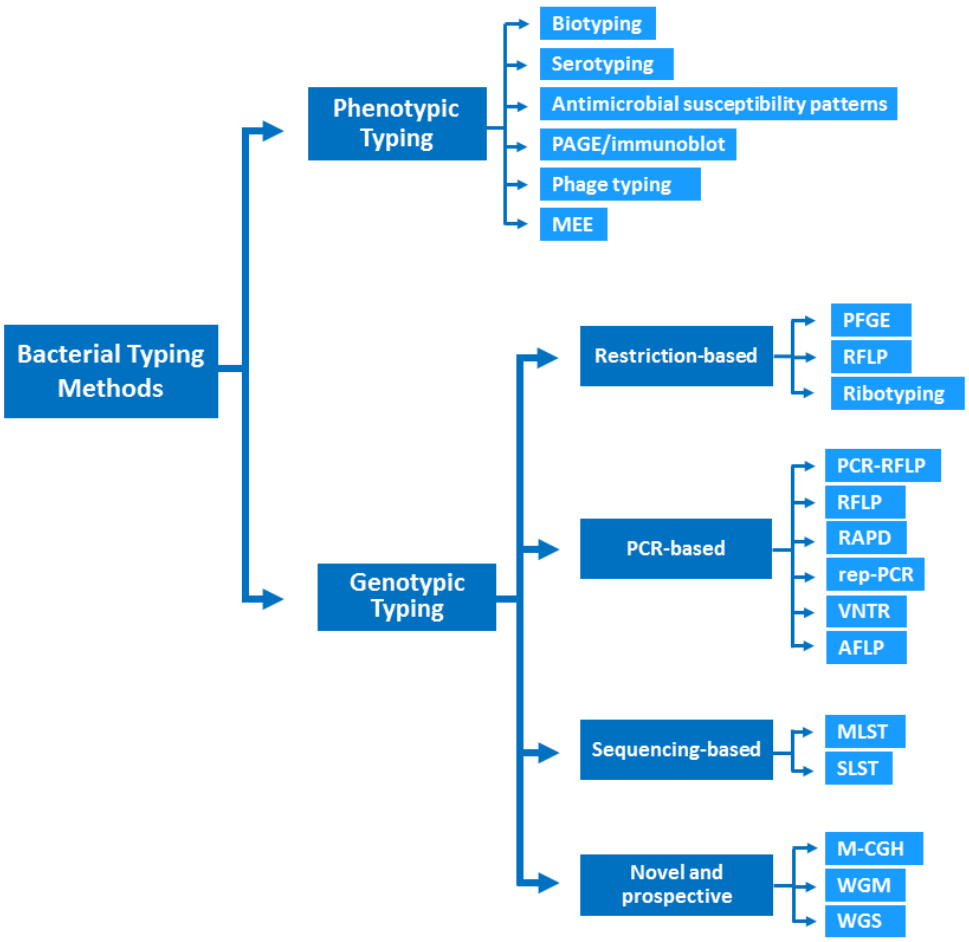


Figure 4. Summary of methods available for bacterial typing.

As stated, there is no perfect genotyping method, and when choosing one, all cons and pros must be taken into account. For *P. aeruginosa* epidemiological studies, the most commonly found genotyping methods in the literature are PFGE, RAPD, rep-PCR (specifically, ERIC and BOX) and MLST.

1.3. Aims of the work

Since 2010, a collaborative effort, led by Professor Pedro Santos, has been established between Hospital de Braga (Braga, Portugal) and the Centre of Molecular and Environmental Biology (CBMA) of University of Minho (Braga, Portugal) to further gain knowledge in the biology of *P. aeruginosa* nosocomial infections. This effort resulted in a diverse and growing collection of *P. aeruginosa* clinical isolates that, on May of 2014, reached 520 samples to study.

The present work was developed in the scope of my 2nd year in the Master's Degree in Molecular Genetics, at the Department of Biology of the University of Minho. The experimental work was performed at the Laboratory of Molecular Genetics and the Laboratory of Molecular Biotechnology at CBMA, under the supervision of Professor Pedro Santos. The main aims of the present work were:

(i) Perform an epidemiological study on *P. aeruginosa* nosocomial infections.

(ii) Characterize the genomic variability among *P. aeruginosa* clinical isolates, using RAPD-PCR as a genotyping method.

2. MATERIALS AND METHODS

2.1. Clinical isolates of *Pseudomonas aeruginosa*

From March of 2010 to May of 2014, 520 isolates of *P. aeruginosa* were cultured from the fluid and tissue of 325 patients of Hospital de Braga (Braga, Portugal). The isolates were confirmed by the hospital services as *Pseudomonas aeruginosa* using either the VITEK® II system (BioMerieux, France) or the Microscan WalkAway® system (Dade MicroScan, Inc., USA).

Information regarding the date and source of isolation, age and sex of the patient, associated hospital service, type of infection (mono or multi) and antibiotic susceptibility profile was kindly provided by Dra. Alberta Faustino, from Hospital de Braga. This information will be referred from now on as metadata. An epidemiological study was performed with the metadata. The antibiotic susceptibility profiling carried out by the hospital was assayed using the Kirby-Bauer disk diffusion susceptibility test (Wayne, 2006) for 7 antibiotics of 5 classes of antipseudomonads: the third-generation cephalosporin ceftazidime, the carbapenem imipenem and the extended spectrum β-lactam piperacillin with bacterial β-lactamase inhibitor tazobactam, aminoglycosides amikacin, gentamicin and tobramycin, and the fluoroquinolone ciprofloxacin. The PDR isolates collected after 2012 were tested for resistance to the polymixin colistin by the hospital. The PDR isolates were tested again in the lab for resistance to colistin, using the broth dilution method to determine minimal inhibitory concentrations of antimicrobial substances described by Wiegand et al. (2008).

The clinical isolates were kept at -80ºC at the CBMA installations. When culturing the isolates in the lab, 5 of the isolates showed 2 morphotypes under no selective media pressure, expanding the collection to 525 samples. Morphotypes were assigned a letter A or B in this work, in addition to their standard ID (e.g. HB89A and HB89B). Moreover, our study used 3 worldwide *P. aeruginosa* reference strains: PAO1, isolated in 1955 from the wound of a patient in Melbourne, Australia (Holloway, 1955); PA14, isolated before 1995 from a burn patient in Massachusetts, USA (Rahme *et al.*, 1995); and LESB58, isolated in 1988 from a CF patient in Liverpool, England (Winstanley *et al.*, 2009).

2.2. *Pseudomonas aeruginosa* cultures and gDNA purification

The clinical isolates were thawed to PIA (*Pseudomonas* Isolation Agar) (see Table I) and grown overnight at 37ºC. The next day, picking biomass from the PIA plate, the bacteria was streaked in LB Lennox Agar (see Table I) and grown overnight at 37ºC. The LB plates were carefully observed to check for the presence of distinct morphotypes and when seemingly present, a single colony from each morphotype was streaked in LB Lennox Agar and grown overnight at 37ºC to confirm the differential phenotypes.

Table I. Composition of the culture media used in this study.

Culture media	Composition
LB Lennox Broth (Sigma-Aldrich, USA)	0.5% NaCl, Bacto Tryptone, 0.5% Yeast Extract and ddH20
LB Lennox Agar (Sigma-Aldrich, USA)	0.5% NaCl, Bacto Tryptone, 0.5% Yeast Extract, 2% Agar Noble and ddH_20
PIA (Difco, USA)	10 mM NaCl, 2% Bacto Tryptone, 0.5% Yeast Extract, 2.5 mM KCl and ddH_20

For the purification of genomic DNA (gDNA) of *Pseudomonas aeruginosa* isolates, the Wizard® Genomic DNA Purification Kit (Promega, USA) was used and its protocol optimized.

First, biomass was picked from a plate harboring the overnight-grown isolate and inoculated in 10 mL of LB Lennox Broth. The bacteria was grown overnight at 37ºC with orbital shaking (200 RPMs). The next morning the cultures were refreshed, diluting 1:10 the overnight-grown. The bacteria were left growing until the culture reached an OD_{600nm} of 0.8-1.0 ABS (achieved after 3.5-4 hours for most *P. aeruginosa* isolates). This refresh step has proven to be advantageous during the purification process, especially for isolates with a mucoid phenotype. Alternatively to culturing the bacteria in liquid medium, biomass can be scrapped from the LB or PIA plate and ressuspended in 1mL of sterile 0.9% NaCl, using a vortex.

Bacterial cells corresponding to 1 mL of culture were pelleted by centrifugation at maximum speed (13-16.000 x *g*) for 2 minutes, using Eppendorf Microcentrifuge 5415D and MiniSpin® Plus microcentrifuges (Eppendorf, Germany). The supernatant

was removed and 600 µL of Nuclei Lysis Solution was added to the cell pellet, while gently ressuspending the cells. The samples were incubated at 80ºC for 5 minutes in a thermoblock (FALC Instruments, Italy) and allowed to cool to room temperature afterwards. Once cooled, 3 µL of RNAse A (4 mg/µL) were added and the samples were incubated for 1 hour at 37ºC. At that time, 200 µL of Protein Precipitation Solution were added to the RNAse-treated cell lysate and the samples were incubated on ice of 10 minutes, being then centrifuged at maximum speed (13,000-16,000 x *g*) for 10 minutes (when needed this time was extended, to precipitate any debris still remaining in suspension). The supernatant was kept and transferred to a clean microtube containing 600 µL of room temperature isopropanol, inverting the mixture until thread-line strands of DNA formed a visible biomass. The microtubes were centrifuged at maximum speed (13,000-16,000 x *g*) for 2 minutes, the supernatant carefully discarded and the microtubes drained in clean absorbent paper. Then, 600 µL of room temperature 70% ethanol were added to the microtube, and the DNA pellet washed by inversion. The microtubes were centrifuged at maximum speed (13,000-16,000 x *g*) for 2 minutes, the supernatant carefully discarded carefully, the microtubes drained in clean absorbent paper and the pellet allowed to air dry for 15 minutes. After 15 minutes, 200 µL of DNA Rehydration Solution were added to microtubes and the DNA was left to rehydrate overnight at 4ºC. When not immediately used, the DNA was stored at -20ºC.

2.3. Normalization of gDNA concentration by gel electrophoresis

The DNA was initially quantified by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA), however, after comparison of concentration values with DNA intensities in 1% agarose gels, no correlation was observed. Therefore, after gDNA purification, normalization of the DNA concentration was achieved by loading and running the samples in 1% agarose gels and comparing the DNA content of each sample to a standardized scale (or ruler). Subsequently, the samples were diluted to the desired concentration.

To decide which sample was going to work as a scale, a few samples were loaded and run in a 1% agarose gel and the one with the highest DNA quantity was selected. The scale was constructed using a 25 µL mixture of DNA, DNA stain and

buffer. The sample used as a scale was diluted 5 times in upH_2O and 1-15 µL of that dilution were loaded together with 5 µL of a 100 times dilution of Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Germany) prepared in a 6 times concentrated DNA loading buffer (10 mM Tris-HCl pH 7.6, 0.03% bromophenol blue, 60% glycerol and 60 mM EDTA), called from here on as LBMG1:100. TAE buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA pH 8.0) was added to each sample of the scale to achieve a final isovolume of 25 µL. The samples were kept in the dark for 15 minutes, at room temperature, before gel loading in order to let a fully DNA staining take place.

The samples to be compared with the scale were diluted 5 times in upH_2O and 5 µL of the dilution were loaded together with LBMG1:100 and TAE buffer, as described above.

The samples were loaded in 1% agarose gels and run at 15 V/cm using Bio-Rad PowerPac™ 300 and PowerPac™ Basic power supplies (Bio-Rad, USA). Gel images were acquired using the imaging system Chemidoc XRS (Bio-Rad, USA). The normalization of the DNA content of each sample was done by diluting the desired sample to a concentration similar to the first/second scale sample. The dilutions were made with upH_2O, in a final volume of 25 µL. After DNA sample normalization, the isolates were genotyped as followed.

2.4. RAPD-PCR and gel electrophoresis of the PCR products

Amplification reactions for RAPD-PCR using the 10-base primer P272 (5'-AGCGGGCCAA-3') were performed in a total volume of 25 µL, being based on RAPD-PCR assays with P272 seen in the literature (Eftekhar *et al.*, 2013; Clode *et al.*, 2000; Hafiane and Ravaoarinoro, 2011; Mahenthiralingam *et al.*, 1996; Nanvazadeh *et al.*, 2013; Saitou *et al.*, 2010). The normalized DNA samples were diluted 5 times and, in each reaction mixture, 5 µL of DNA were mixed with 1x Taq Buffer with $(NH_4)_2SO_4$ (Thermo Scientific, USA), 2.5 mM of $MgCl_2$ (Thermo Scientific, USA), 0.2 mM of dNTPs (Roche, Switzerland), 3 µM of primer P272, 3U of GoTaq DNA Polimerase (Promega, USA) and upH_2O.

DNA amplifications were accomplished in Bio-Rad T100™ and Bio-Rad MyCycler™ thermal cyclers (Bio-Rad, USA), using the following temperature profile: (i) an initial denaturation of 5 minutes at 94ºC; (ii) 4 cycles with 1 cycle corresponding to 5 minutes at 40ºC (annealing), 5 minutes at 72ºC (extension) and 5 minutes at 94ºC (denaturation); (iii) 30 cycles with 1 cycle corresponding to 1 minute at 94 ºC (denaturation), 1 minute at 40ºC (annealing) and 2 minutes at 72ºC (extension); (iv) and a final extension of 10 minutes at 72ºC.

After amplification, the DNA was stained with 1:4 of LBMG1:100 for 15 minutes, in the dark, at room temperature. The RAPD products were then separated by gel electrophoresis, loading 15 µL of the mixture in 1.5% agarose gels (20 wells, 0.5 cm of thickness) run at 9 V/cm, using Bio-Rad PowerPac™ 300 and PowerPac™ Basic power supplies (Bio-Rad, USA). At least two lanes with molecular weight standards were present in each gel for an improved band molecular weight approximation. The molecular weight standards used in this study were the BenchTop 1kb DNA Ladder (Promega, USA) and the O'GeneRuler 1 kb DNA Ladder (Thermo Scientific, USA). In addition to the molecular weight standards, each gel was loaded with 3 positive controls with an expected, well-known profile (RAPD products of PAO1, LESB58 and HB15) and a negative control (RAPD-PCR performed with no template DNA).

Gel images were acquired in the imaging systems Chemidoc XRS (Bio-Rad, USA) and VWR GenoSmart (VWR, USA) using standardized measures, applied to all gels in this study. After the first round of pictures was taken, the RAPD products were stained again, incubating the gels in 100 mL of TAE buffer with 10 µL of Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Germany) for 30 minutes in the dark, at room temperature, with agitation. Pictures of the gels were taken as before. This secondary staining ensured that fainter bands became clearly visible. If, upon gel analysis, a RAPD profile showed to be unclear regarding band(s) presence(s), new RAPD-PCRs would be set for that sample.

2.5. Genotyping analysis

2.2.1. Gel anaylsis

All gel images used for genotyping analysis were color inverted using GIMP 2.8 and had its contrast enhanced using the automatic command "Stretch Contrast". The images were exported from GIMP 2.8 using the JPEG format and imported into Gel Analyzer 2010a (Lazar, 2010) for band calling.

After automatic lane detection by Gel Analyzer 2010a (Lazar, 2010), the gel background was removed using the "rolling ball" command, using a 25 ball radius. Automatic band detection followed. Addition or removal of bands was only done when the pixel intensity spectrum delivered by the program corroborated the presence or absence of bands. The molecular weight standards were defined and, using the linear log fitting, the molecular weight of the bands composing the RAPD patterns were determined. The band weight information of all samples was copied to a spreadsheet in Microsoft® Excel® 2010, and organized vertically in a single column.

2.2.2. Data analysis

In order to obtain a binary matrix, where "0" corresponds to absence and "1" to presence of bands in a sample, the Microsoft® Excel® 2010 macro ClassMaker 2.0.ß (Cardinali *et al.*, 2003) was used. Using the "Start" command, the molecular weight data saved in the single column of the spreadsheet mentioned can be loaded. After automatically segregate the bands per sample, the CL1 algorithm was used to create the classes range, using a similarity threshold of 95%. Other algorithms can be used but it was found that CL1 was the one that worked better with our data. The resulting binary matrix was saved to a tab-delimited text file.

To create the dissimilarity matrix and the hierarchical clustering, different combinations of methods were tested, with a few samples, in SPSS 22: Dice's coefficient (Dice, 1945), Jaccard's coefficient (Jaccard, 1912), Euclidean distance and squared Euclidean distance were tested as dissimilarity methods and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sokal and Michener, 1958) and Ward's Method (Ward *et al.*, 1963) were tested as hierarchical clustering methods. The combination of methods chosen was Dice's coefficient/UPGMA. The data was analysed in RStudio, as more flexibility in handling data is available with this program.

In RStudio, "read.delim" was used to load the binary matrix and the "t(x)" function was used to transpose the data, so that each line in the matrix corresponded to a different sample/isolate. After installing and loading the "arules" package, together with

the “Matrix” package, the distance matrix was computed with the “dissimilarity” function, using Dice's coefficient (Dice, 1945). Then, “hclust”, from the pre-loaded package “stats”, was used for hierarchical cluster analysis of the distance matrix, using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sokal and Michener, 1958), also known as “average”. For better handling of tree-like structures, the “as.dendrogram” function from the “stats” package was used and, with the function “plot” from the pre-loaded “graphics” package, the dendrogram relating all 528 isolates was drawn. With “cut” from the pre-loaded package “base”, the dendrogram was cut at a 0.75 distance (“h”), in order to split the *Pseudomonas aeruginosa* collection into large genotypic groups to be studied jointly with the metadata available. A 0.75 distance was selected in order not to have more than 25 genotypic groups to work with. Using “labels”, from that same package, it was registered which isolate belonged to each group. To get the number of genotypes within the *P. aeruginosa* collection, the cut value was changed to a lower one (e.g. “h = 0.30”, to see the number of genotypes at 70% similarity). The R script developed during the course of this analysis in RStudio can be found below.

```
inputR<-read.delim('txt file location')
t<-t(inputR)
library("arules", lib.loc='package location')
d<-dissimilarity(t, method = "dice", args = NULL)
c<-hclust(d, method = "average", members = NULL)
as<-as.dendrogram(c)
plot(as, ylab="Distance", cex.lab=1.2, cex.axis=0.75)
c075<-cut(as, h=0.75)
plot(c075$upper, ylim=c(0.0, 1), ylab="Distance", cex.lab=1.2,
cex.axis=0.75)
c075
labels(c075$lower[['branch number']])
```

In Figure 5, the pipeline developed for the genotyping analysis is shown.

Other data analysis displayed in this work was attained using Microsoft® Excel® 2010 (i.e. the epidemiological metadata analysis and the analysis correlating the metadata with genotypic analysis).

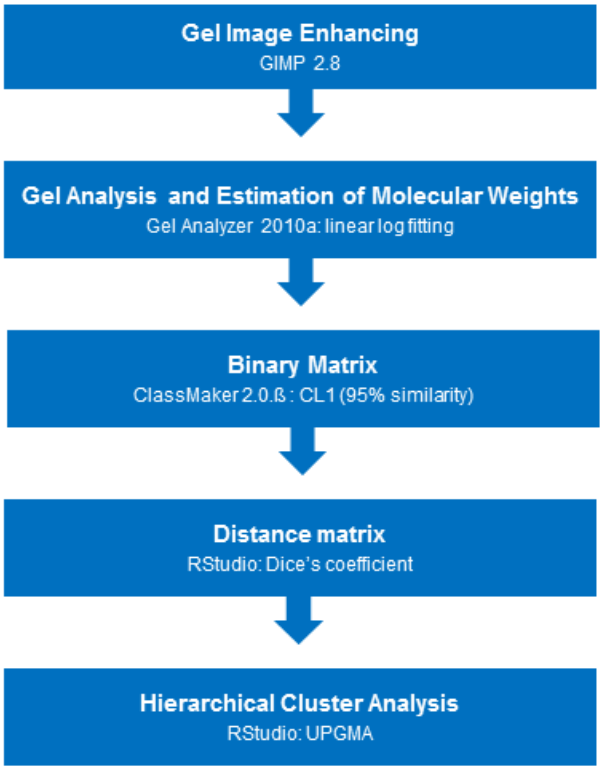


Figure 5. Overview of the pipeline developed for the genotyping analysis.

3. RESULTS AND DISCUSSION

3.1. Epidemiological study of *Pseudomonas aeruginosa* infections

Epidemiological studies are fundamental in public health, leading to better disease surveillance, discovery of risk factors and improved clinical treatments (review by Bartlett and Judge, 1997). With a 5-year collection of *P. aeruginosa* isolates comprising 525 samples (having 519 metadata available), the first assignment of this work was to perform an epidemiological study of *P. aeruginosa* infections in Hospital de Braga. This way, it was hopped to provide simple answers with a potentially great clinical relevance.

3.2.1. Distribution of *Pseudomonas aeruginosa* infections according to the age and gender of the patients and hospital service

The age and gender of the patients infected with *P. aeruginosa*, as well as the hospital service they were treated in, are exposed in Figure 6. Three age groups were selected. Thirteen hospital services were assayed: Cardiology, Emergency Room (ER), General Medicne, Multi-purpose ICU (MP-ICU), Neurosurgery, Oncology, Orthopaedics, Paediatrics, Pneumology, Rehabilitation Medicine, Surgery, Urology and Others.

From the results in Figure 6-A, it is seen that most *P. aeruginosa* infections occurred in patients older than 60 years old (64% of cases), with no gender bias (males over 60 years old represented 34% of infected patients and female 30%). Curiously, in the second most represented age group, there was exactly the double of male patients (with a total of 33% of cases, 22% infected patients were male between 6 and 60 years old and 11% female). The most underrepresented age group corresponded to newborns and patients up to 5 years old (3% of all patients, 1% male and 2% female). This is unusual; *P. aeruginosa* infections are often associated with infants due to their not fully developed immune system and due to the fact that many infants are intubated, catheterized with intravascular catheters/devices in-situ, and/or receive parenteral nutrition, increasing the risk of infection (review by Jefferies, 2012).

The most common places in Hospital de Braga where patients were diagnosed with *P. aeruginosa* infections were the ER, the General Medicine services, the Multi-Purpose ICU (MP-ICU) and the Urology services, with 30%, 15% 11% and 9% of all *P.*

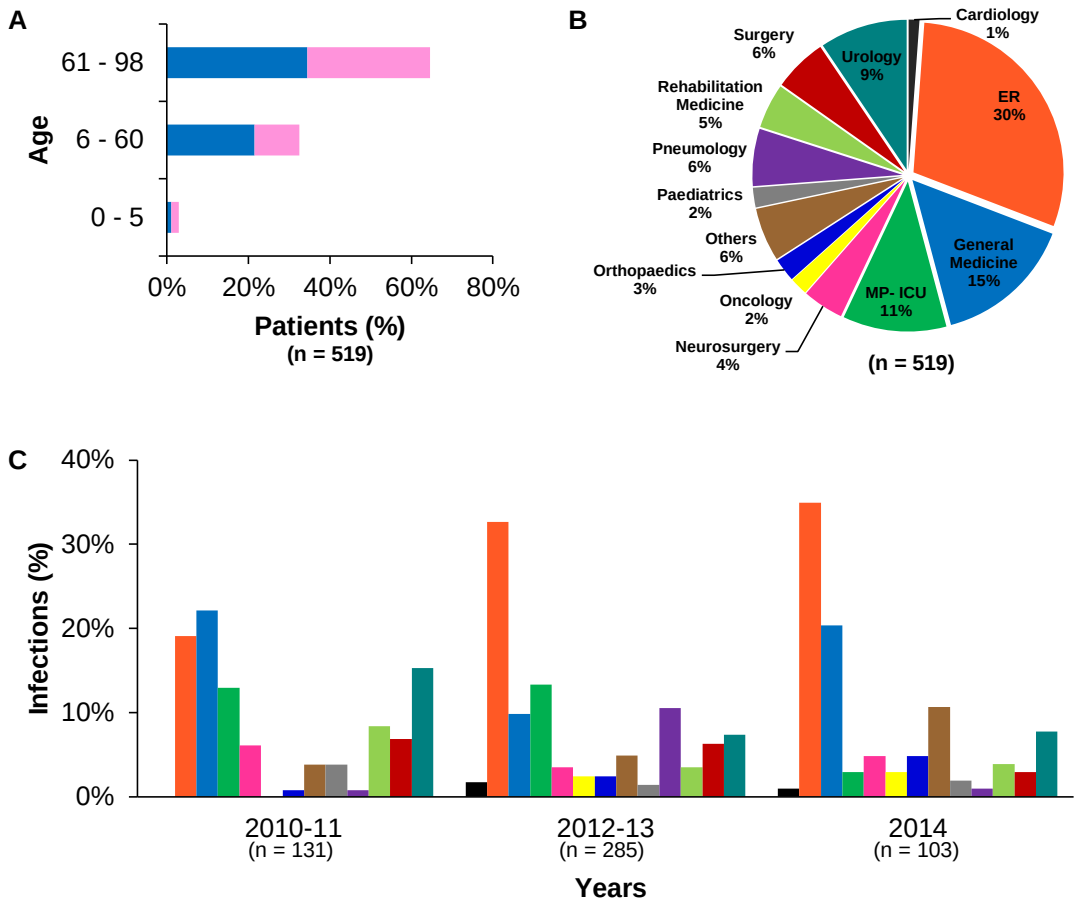


Figure 6. Age and gender of patients infected with *P. aeruginosa* and prevalence of infections per hospital service. In A, infected patients are sorted by age and gender, with blue bars corresponding to male patients and pink bars to female patients. In B, the occurrence of infections is sorted by hospital service and, in C, the temporal occurrence of infections in each hospital service is shown. Each bar color in C corresponds to the hospital service with that same color in B (e.g. orange, ER). In all graphics, "n" represents the number of cases.

aeruginosa infections, respectively (Figure 6-B). There are, indeed, several reports of *P. aeruginosa* infections in ICUs and Urology services in the literature (Croughs 2013; review by Jefferies 2012; Kayabas 2008; review by Lister 2009; Peña, 2003), however, no dedicated studies to *P. aeruginosa* infections in ER and General Medicine designated services have been found. For this epidemiological study, *P. aeruginosa* infections were also grouped by years of occurrence. In Figure 6-C, it is noticeable that, although relatively rare, infections in Cardiology and Oncology were first documented in 2012-13. In 2012-2013, infections in ER, increased considerably, from 19% of all cases in 2010-11 to 33% of all cases in 2012-13, values maintained in 2014, with 35% of infections being found in ER services. In 2012-13, the number of infections found in

Pneumology services also increased greatly, from 1% of all cases in 2010-2011 to 11% of all cases in 2012-13. Nevertheless, contrary to ER, *P. aerguinosa* infections in Pneumology had its values re-established in 2014 (1% of all infections, as in 2010-11). In General Medicine, *P. aeruginosa* infections were at its lowest in 2012-13, with 10% of all cases whereas in 2010-11 and 2014, had 22% and 20% of all cases assigned, respectively. In Urology, *P. aeruginosa* infections decreased from 15% to 7%, in 2010-11 and 2012-13, respectively. This decreased abundance was maintained in 2014, with 8% of all cases being attributed to Urology services. In the MP-ICU, the number of *P. aeruginosa* infections also decreased significantly in 2014, with only 3% of all cases being attributed to this service whereas, in 2010-11 and 2012-13, this service was responsible for 13% of all cases. As mentioned earlier, *P. aeruginosa* infections have been documented in the literature as being particularly problematic in ICUs (review by Jefferies 2012; review by Lister 2009), being noteworthy its relative decrease observed in Hospital de Braga. In 2014, the number of *P. aeruginosa* infections in Surgery also decreased, from 7% and 6% in 2010-11 and 2012-13, respectively, to 3% in 2014. In all other services, the relative number of *P. aeruginosa* infections was roughly maintained throughout the years.

3.2.2. Distribution of *Pseudomonas aeruginosa* isolates according to source of isolation

The *P. aeruginosa* isolates presented in this work were cultured from blood, bronchial aspirates, catheters, ears, pus/exudates/fluids, skin, sputum, ulcers, urine and others sources of isolation (such as surgical wounds and eye). In Figure 7, the isolates comprising the *P. aeruginosa* collection were distributed per source of isolation, and time of isolation.

As it can be seen in Figure 7-A, most isolates were collected from urine (44%) and sputum (31%), comprising together 75% of all isolates. The following most popular sources were bronchial aspirates (7%) and pus/exudates/fluids (7%), with all other sources comprising only 1-3% of all isolates. From 2010-11 to 2012-13 there is a clear increase in isolates collected from sputum, going from 16% to 39% of all isolates collected that years. In 2014, the numbers decreased to 31%, although that still corresponds to almost the double found in 2010-11. One could say that this trend could be associated with an increase in respiratory infections in patients. However, there is no

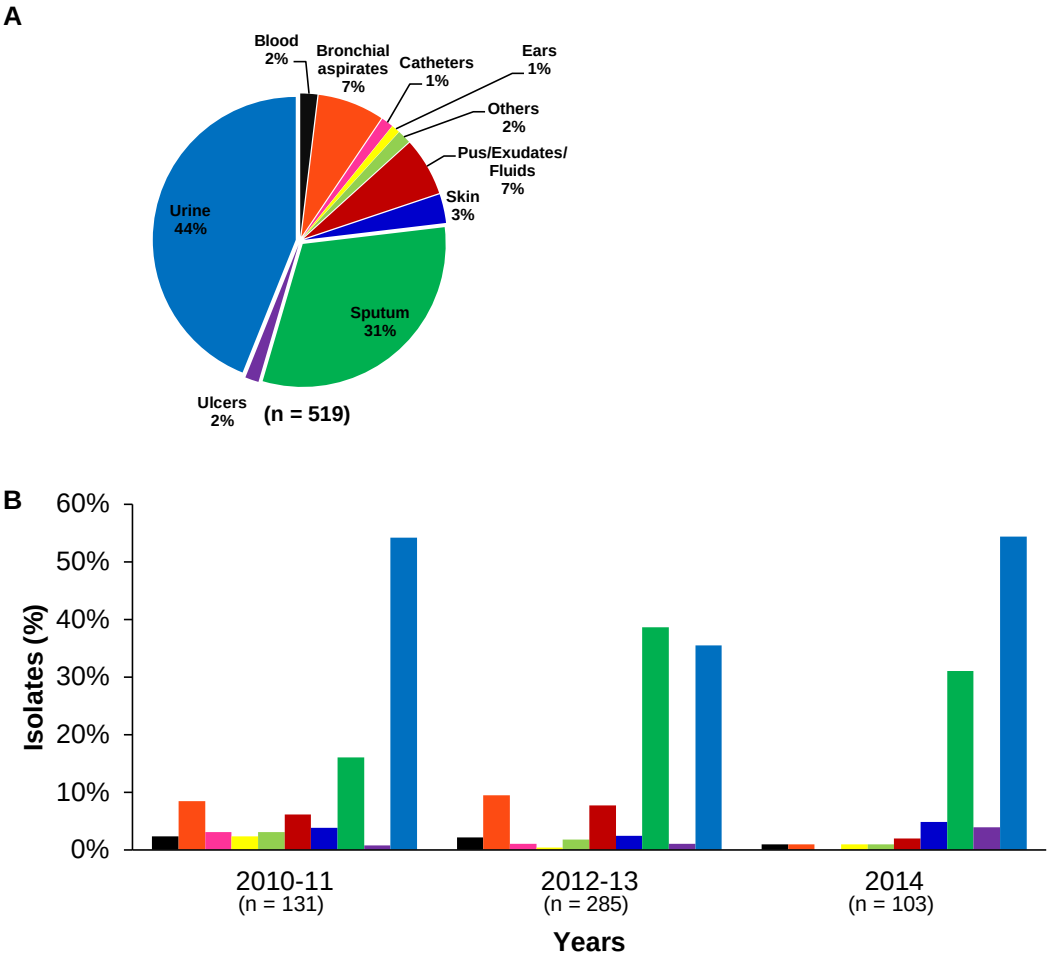


Figure 7. Distribution of *P. aeruginosa* isolates according to source of isolation. In A, the isolates are sorted by source of isolation. In B, isolates of *P. aeruginosa* from different sources are sorted by years. Each bar color in B corresponds to the source of isolation with that same color in A (e.g. black, blood). In both graphics, "n" represents the number of isolates.

data available regarding the disease status of most patients to confirm this hypothesis. In 2012-13, there was a significant decrease in isolates from urine (35%), although, in 2014, the numbers became equal to those of 2010-11 (54%). No isolates were collected from ears in 2012-13 and from catheters in 2014, although, these groups were already part of the groups underrepresented in other years. In 2014, there was a clear decrease in isolates from pus/exudates/fluids from 6% to 8% in 2010-11 and 2012-13, respectively, to 2%. The same applies for isolates from bronchial aspirates, which in 2010-11 represented 8% and 9% of all isolates, respectively, and in 2014 only represented 1% of all isolates. Regarding other sources of isolation, no other significant fluctuations of relative abundance could be seen.

3.2.3. Distribution of the occurrence of mono and multi-infections and the occurrence of MDR *Pseudomonas aeruginosa* isolates

In Figure 8, the distribution of multi-infections with *P. aeruginosa* is shown, as well the occurrence of MDR isolates. Isolates of *P. aeruginosa* were considered as MDR if resistant to at least 3 classes of the antibiotics tested.

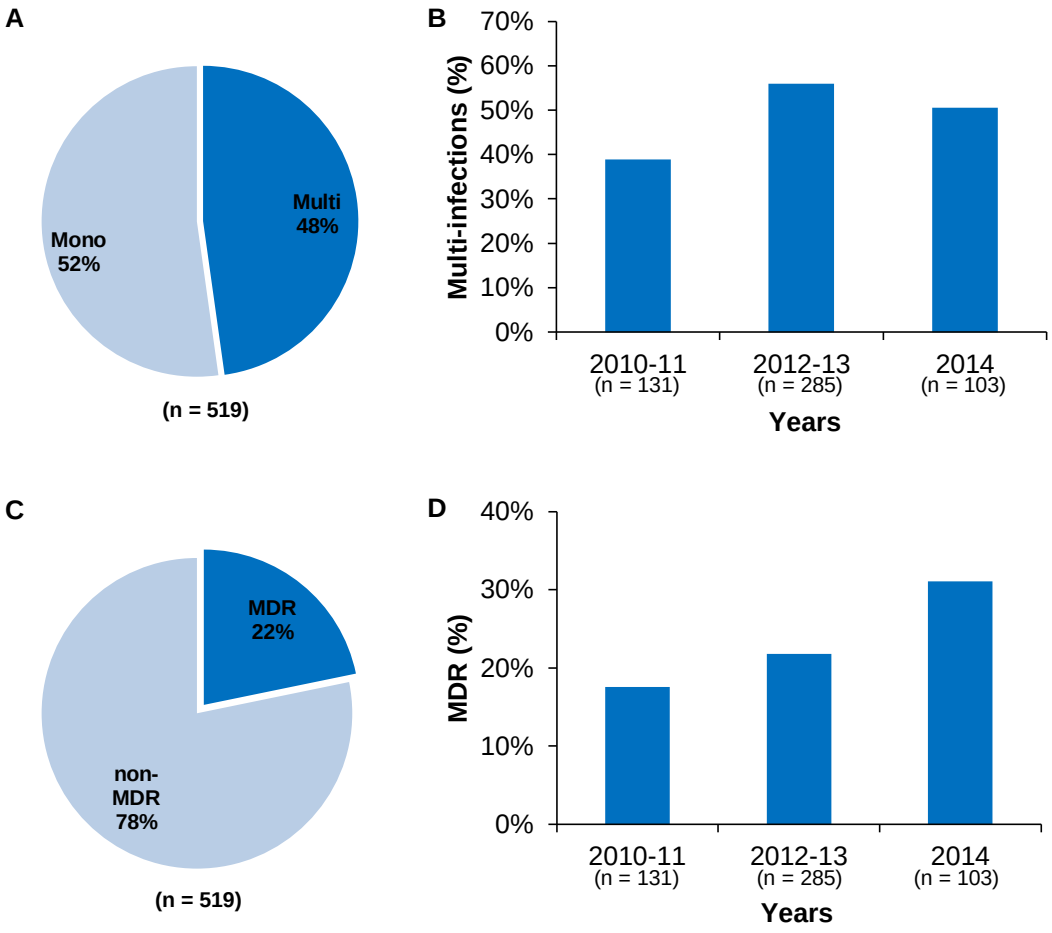


Figure 8. Distribution of *P. aeruginosa* isolates according to occurrence of mono and multi-infections and MDR. In A, the isolates are sorted whether they were found in scenarios of mono-infection or multi-infection and, in B, isolates found in scenarios of multi-infection are sorted by the year they were isolated in. In C, the isolates are sorted according to the antimicrobial resistance profile, and in D, MDR isolates are sorted by year of isolation. In all graphics, "n" represents the number of isolates.

Almost half of the collection of *P. aeruginosa* isolates (48%) was found in scenarios of multi-infection (Figure 8-A), with numbers varying throughout the years: in 2010-11, 39% of all *P. aeruginosa* isolates were found in scenarios of multi-infection, a number that increased in 2012-13, with 56% of all *P. aeruginosa* isolates

being found in that same scenarios; in 2014 the number of *P. aeruginosa* isolates found in scenarios of multi-infection decreased to 50%, but, still, it is considerably higher than the number of isolates in 2010-11 (Figure 8-B). Multi-infection scenarios in Hospital de Braga, include a broad range of microorganisms such as *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Aspergillus fumigatus* and *Candida albicans,* among others. Despite interspecies interactions in mixed species biofilms being still poorly understood, some data from the literature associate *P. aeruginosa* multi-infections with traits favourable to disease progression and persistence, depending on the species (Bragonzi *et al.,* 2012; Høiby *et al.,* 2009). For instance, *P. aeruginosa* and *Burkholderia cenocepacia* interactions lead to increased biofilm formation and host inflammatory response in murine models of CF infection (Bragonzi *et al.,* 2012) and *P. aeruginosa* and *Candida albicans* interactions lead to an enhancement of the production of virulence factors and increased mutability in both pathogens (Trejo-Hernández, 2014). Multi-infections might also be advantageous, as they can increase the opportunity for HGT events to occur between *P. aeruginosa* and other species.

Almost a quarter of the collection of *P. aeruginosa* isolates (22%) was classified as MDR (Figure 8-C). A clear trend was detected, with the percentage of MDR *P. aeruginosa* isolates increasing throughout the years: in 2010-11, 18% of all isolates were classified as MDR, in 2012-13, 22%, and in 2014, the percentage of MDR isolates reached 31%, almost the double of MDR isolates found in 2010-11 (Figure 8-D). In 2013, the USA Center for Disease Control and Prevention, classified *P. aeruginosa* as a 'serious threat level', partially due to the fact that 13% of all *P. aeruginosa* infections were caused by MDR isolates (Center for Disease Control and Prevention, 2013). Moreover, the lack of antimicrobial options to treat patients infected with MDR *P. aeruginosa* isolates is a cause of concern in the scientific community (Obritsch, 2005). In Hospital de Braga, the frequency of MDR *P. aeruginosa* isolates was considerably higher than 13% in all years, and in 2014 even more so. Patients infected with MDR isolates have increased mortality rates, increased hospital stays and present higher costs to the hospital (review by Slama, 2008). The usage of antibiotics always results in "selective pressure" in the host receiving the antibiotic, even when appropriately administered (review by Alanis, 2005; review by Livermore, 2005). Thus, in addition to good clinical practices, new antibiotic development must be re-invigorated in order to maintain the current ability to fight pathogenic infections (review by Livermore, 2005). The increase in MDR isolates seen here could also not be a trend but rather a result from

an increased seasonal consume of antibiotics, especially since most of the *P. aeruginosa* affected patients are elderly (Figure 6). The percentage of *P. aeruginosa* isolates found in the context of mono-infections that are MDR is exactly the same as the number of MDR isolates found in the context of multi-infections (data not shown). This is not surprising considering that *P. aeruginosa* isolates found in the context of multi-infections corresponded to 48% of all isolates (Figure 8-A). The dissemination of *P. aeruginosa* infections and occurrence of MDR isolates in different sources was also investigated (Figure 9).

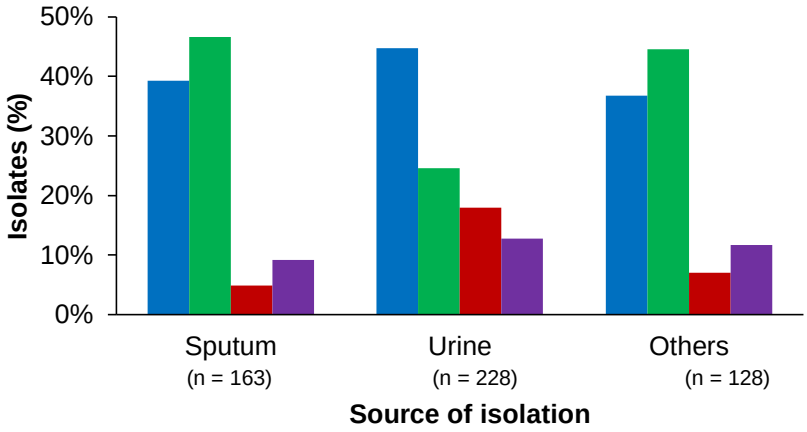


Figure 9. Distribution of *P. aeruginosa* isolates from sputum, urine and other sources (bronchial aspirates, ulcers, etc.) according to occurrence of mono and multi-infections and MDR isolates. Blue bars represent non-MDR isolates found in the context of mono-infections, green bars represent non-MDR isolates found in the context of multi-infections, red bars represent MDR isolates found in the context of mono-infections and purple bars represent MDR isolates found in the context of multi-infections. "n" represents the number of isolates.

Isolates from sputum and other sources (excluding urine) showed a similar frequency profile. *P. aeruginosa* isolates from urine, while having similar frequencies for non-MDR isolates found in the context of mono-infections (45% in urine, and 39% and 37% in sputum and other sources) and MDR isolates found in the context of multi-infections (13% in urine, and 9% and 12% in sputum and other sources), had a significantly smaller frequency of non-MDR *P. aeruginosa* isolates found in the context of multi-infections (25% in urine, whereas in sputum and other sources the frequency was of 47% and 45%, respectively) and had more than a double frequency of MDR *P. aeruginosa* isolates found in the context of mono-infections (18% in urine, whereas in sputum and other sources the frequency was of 5% and 7%, respectively). Nonetheless, no explanation for this bias was found in the literature.

3.2. Genotypic characterization of 528 clinical *Pseudomonas aeruginosa* isolates by RAPD-PCR and association of genotypic groups to epidemiological profiles

All 525 *P. aeruginosa* clinical isolates obtained until May 2014, and 3 worldwide reference strains (PAO1, PA14 and LESB58) were genotyped by RAPD-PCR. After genotyping analysis and grouping of the isolates, a holistic study associating genotypic groups of isolates to epidemiological profiles was made.

3.2.1. Genotypic characterization of 528 clinical isolates of *Pseudomonas aeruginosa* by RAPD-PCR

The RAPD-PCR assays performed in this work were done using the P272 primer. When first performing the RAPD-PCR assays, the quantity of DNA template used was 40 ng, after assessment of DNA concentration by quantification on NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA). However, imperfect genotyping profiles were always obtained despite changes in components of the RAPD solution mix and RAPD-PCR protocol (Figure 10).

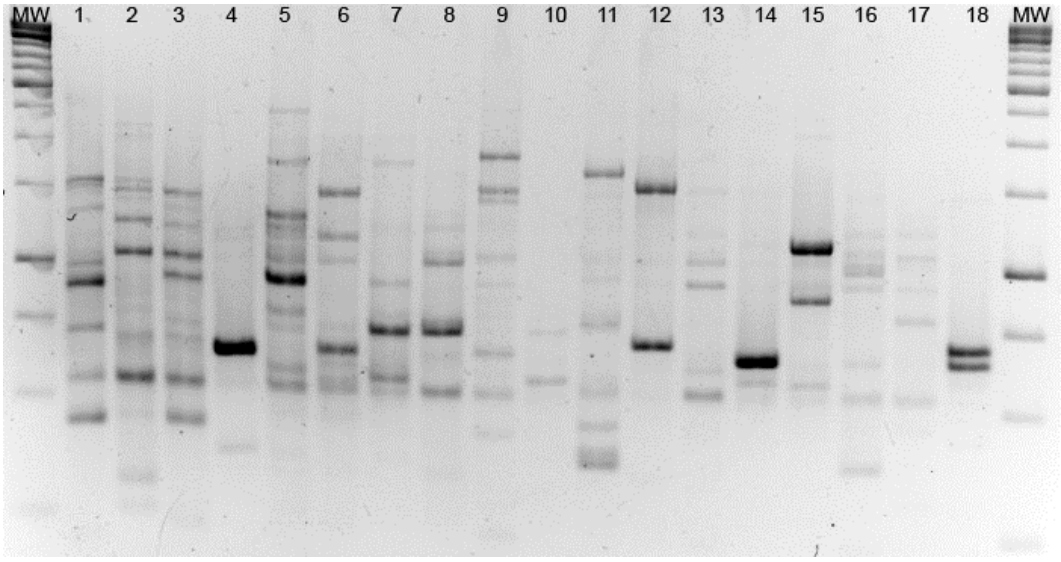
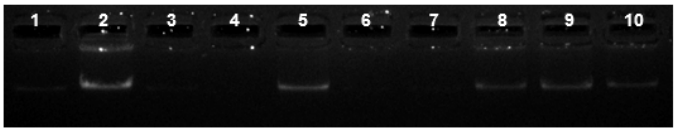


Figure 10. Example of RAPD profiles obtained when assessing the template DNA input quantity with NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA). The first and last lane (MW) correspond to the molecular weight used – O'GeneRuler 1 kb DNA Ladder (Thermo Scientific, USA) – and all others to RAPD profiles of samples of *P. aeruginosa*.

After loading and running the DNA samples in a 1% agarose gel, it was observed that the quantity of DNA obtained between samples did not correlate with the concentration values obtained from NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA) (Figure 11).

A



B

Sample	DNA (ng/µL)
1	179.8
2	308.4
3	21.5
4	338.0
5	247.6
6	302.4
7	689.1
8	222.1
9	131.2
10	144.2

Figure 11. Assessment of DNA quantity by gel electrophoresis and spectrophotometry. In A, different samples of DNA of *P. aeruginosa* were loaded on a 1% agarose gel. In each sample, 5 µl of a 5 times dilution of a DNA sample were loaded with 5 µL of LBMG1:100 and 15 µL of TAE buffer (final volume of 25 µL between samples). In B, the values obtained after quantification on NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA) for that same samples are shown.

The lack of correlation between the visualization of the DNA samples on gel and the values obtained using NanoDrop™ 1000 Spectrophotometer were not due to lack of DNA rehydration in the sample: the DNA samples were always centrifuged before quantification; and, when quantifying the DNA, at least two replicate values per sample were registered, with little to no variation between replicates observed (data not shown). It could be hypothesized that, since *P. aeruginosa* is a microorganism that produces a plethora of exoproducts, some could stay present upon gDNA purification and interfere with DNA quantification. However, it is not known if that is indeed the case.

Given the results observed in Figure 11, it was chosen to perform a normalization of DNA concentration between samples, recurring to gel electrophoresis. To this end, a DNA scale was constructed. To decide which sample was going to work as a scale, a few samples were loaded and run in a 1% agarose gel and the one with the highest DNA quantity was selected (data not shown). Then, by comparing samples with the scale, normalization of DNA concentrations was made (Figure 12). The DNA

concentration was normalized to the first/second band intensity of the scale. For instance, while the DNA sample number 1 from gel B was ready to use, the DNA sample number 5 from gel B needed to be diluted 5 times, since its intensity was similar to the scale sample number 5 from gel A.

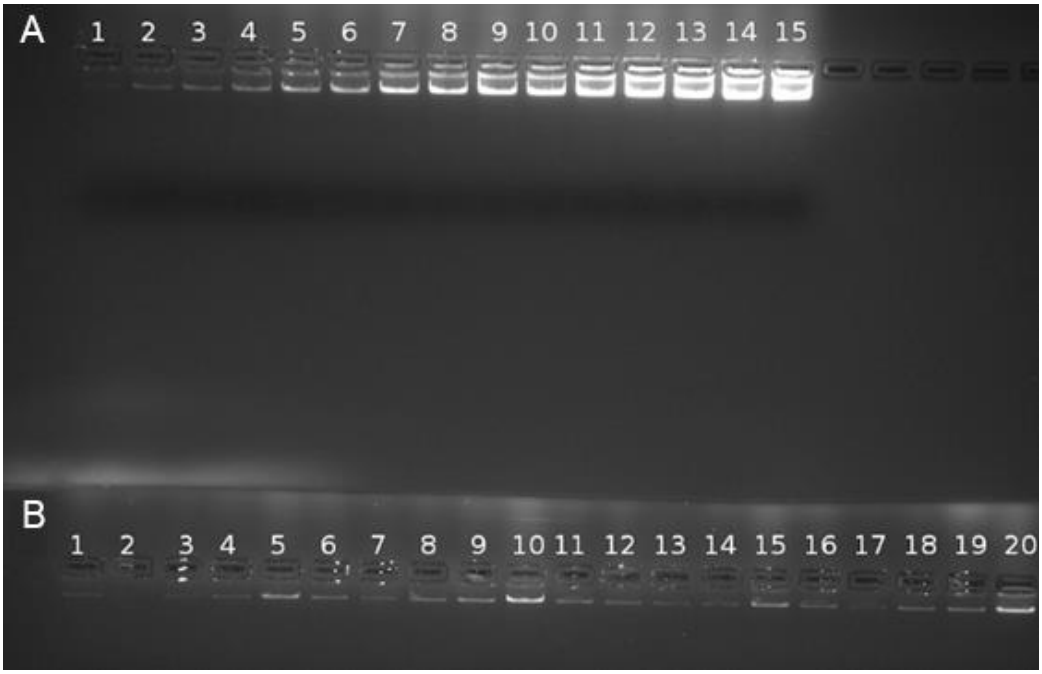


Figure 12. Evaluation of relative DNA concentration by gel electrophoresis. In gel A, a DNA sample used as scale can be seen. In this gel, 1-15 µl of a 5 times dilution of DNA were loaded with 5 µL of LBMG1:100 and TAE buffer to a final volume of 25 25 µL. In gel B with DNA samples for concentration normalization can be seen. In this gel 5 µl of a 5 times dilution of a DNA sample were loaded with 5 µL of LBMG1:100 and 15 µL of TAE buffer (final volume of 25 µL between samples).

After normalization of all 528 samples, each sample was diluted 5 times and 5 µL were used for RAPD-PCR. As it can be seen in Figure 13, successful RAPD profiles were obtained.

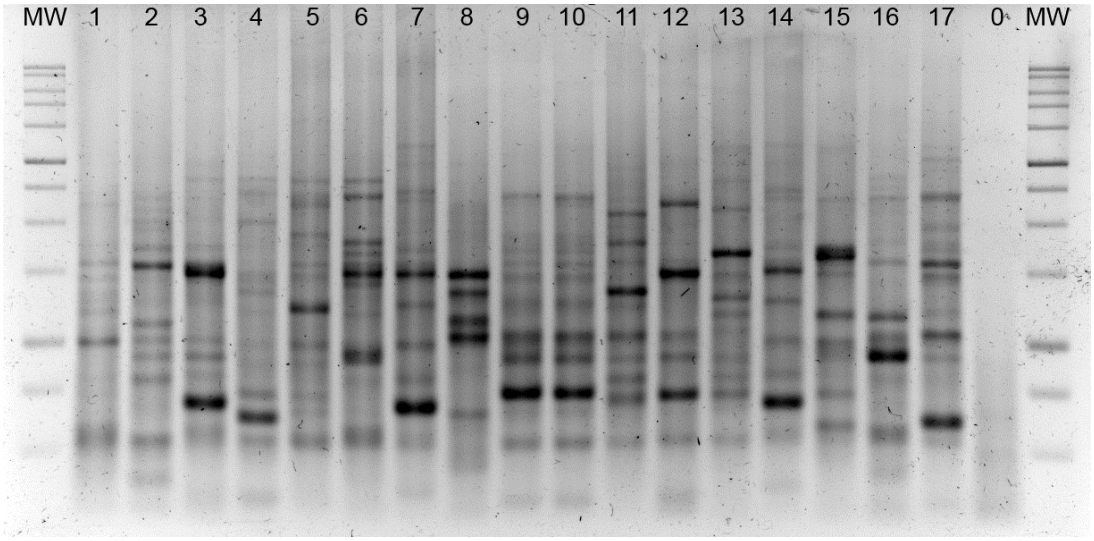


Figure 13. Example of a gel with RAPD profiles, used for genotyping analysis. MW stands for molecular weight standard, BenchTop 1kb DNA Ladder (Promega, USA) being the one pictured. "1", "2" and "3" correspond to the positive controls, PAO1, LESB58 and HB15, respectively. "0" corresponds to the negative control (no template DNA) and "4-17" are the RAPD profiles of the genotyped isolates, HB463-HB476, respectively.

The RAPD-PCR assays performed in this work generated bands ranging from 154 bp to 7.6 kbp, as determined by Gel Analyzer 2010a (Lazar, 2010). This is a broader range than others found in the literature in *P. aeruginosa* genotyping studies using P272 (e.g. 150 bp to 4.4 kbp in Saitou *et al.*, 2010; 300 bp to 3.1 kbp in Rao *et al.*, 2014; and 180 bp to 2.7 kbp in Nanvazadeh *et al.*, 2013). The fingerprinting profiles had between 5 to 16 bands, showing a similar number of bands per profile to most studies found in the literature with P272 in *P. aeruginosa* genotyping (e.g. 3 to 18 bands in Saitou *et al.*, 2010; 5 to 20 bands in Mahenthiralingam *et al.*, 1996; and 3 to 15 bands in Rao *et al.*, 2014; the study by Nanvazadeh *et al.*, 2013 differed from most results with only 3-6 bands per profile). A total of 72 different bands were found after analysis with ClassMaker 2.0.ß (Cardinali, 2003), using the CL1 algorithm and a 95% coefficient of band similarity.

In order to decide which dissimilarity and hierarchical clustering method were the most suited, trials were made using data from PAO1, LESB58, PA14, HB13 and HB15. These trials were then visually compared with the band profiles of each isolate (Appendix – Figure A). Dice's and Jaccard's coefficients, togheter with UPGMA, were the most accurate when looking at the presence and absence of bands between isolates. Ward's clustering method was incompatible with Dice's and Jaccard's coefficients.

Since Dice's coefficient is more used in genotyping studies in the literature than Jaccard's coefficient, Dice's coefficient, together with UPGMA, was used. The dissimilarity and clustering analysis of *P. aeruginosa* isolates were performed in RStudio.

The isolates presented a distance range between 0.824 (maximum) and 0.058 (minimum). For genotype attribution, different cut-off values can be found in the literature. In studies involving RAPD-PCR with P272 for *P. aeruginosa* typing, similarity coefficients greater than 0.90 (Silva *et al.,* 2014), greater than 0.89 (Saitou *et al.,* 2010) and greater than 0.80 (Mahenthiralingam *et al.,* 1996; Salimi *et al.,* 2009) have been used. When selecting similarity coefficients greater than 0.80, 511 RAPD types were attributed to the 528 isolates (a number that dropped to 474, when a similarity coefficient of 0.70 was selected). Normally, in the literature, a smaller ratio of genotypes/collection is found (e.g. in Saitou *et al.,* 2010, 12 genotypes were found for 66 clinical isolates; and in Salimi *et al.,* 2009, only 8 genotypes were found for 126 clinical isolates). However, most *P. aeruginosa* genotyping studies published in the literature focus on specific groups of isolates (e.g. Saitou *et al.,* 2010, used isolates from cockroaches and human urine collected for 7 months; and Salimi *et al.,* 2009, used isolates from a hospital burn unit collected for 6 months). Here, we present a much diversified collection of clinical isolates: for 5 years and 2 months, *P. aeruginosa* was isolated from more than 10 sources in more than 13 different locations (hospital services) of Hospital de Braga. Therefore, when working with more diversified collections of isolates, the ratio of genotypes/collection tends to increase. Such is the case of Silva *et al.,* 2014, that collected *P. aeruginosa* for 10 years, from different anatomical sources of patients staying at different ICUs in Brazil, and found 86 genotypes for 96 isolates. The fact that the range of bands that resulted from the RAPD-PCR was much broader than others found in the literature (as exposed earlier) helps fundament the hypothesis that the high number of genotypes found here resulted from high genomic diversity. To test this hypothesis, the isolates could be genotyped by other typing methods, such as rep-PCR, that as RAPD is inexpensive and fast, or PFGE, the gold standard for genotyping. Alternatively, less discriminatory primers could be used for RAPD, such as P208, that generates fewer bands in fingerprints than P272 (Eftekhar *et al.,* 2009). To guarantee that the high genotypic number is not biased by the chosen genotyping analysis, multiple programs should be used and compared, in addition to the pipeline presented here (e.g. GelCompar II and Phoretix 1D Pro).

For further analysis, the *P. aeruginosa* collection was divided into 21 genotypic groups (A-U), based on a distance cut-off of 0.75 (Figure 14-A). The clinical isolates that compose each group can be consulted in Table A of the Appendix. Specifically, the worldwide references PAO1, PA14 and LESB58 were found to be in group Q, M and R, respectively, and the already sequenced isolates of the collection, *P. aeruginosa* HB13 and HB15 (Soares-Castro *et al.*, 2011), were attributed to group T and N, respectively. The most represented genotypic group among the *P. aeruginosa* isolates is group R, with 14% of all isolates, whereas the most underrepresented groups were C and F with only 1% of all isolates (Figure 14-B). The other genotypic groups were represented with 3-7% of all isolates (Figure 14-B).

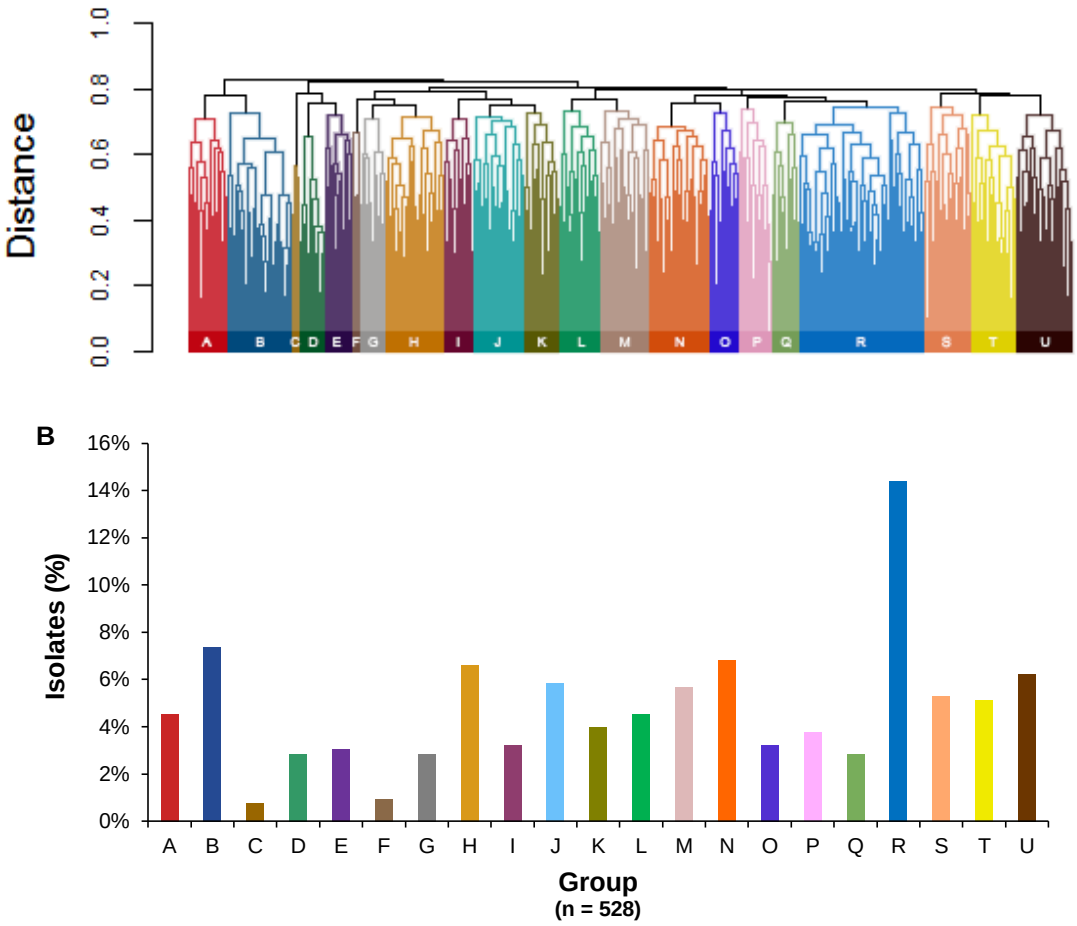


Figure 14. Genotypic groups of the clinical isolates of *P. aeruginosa*. In A, a dendrogram representing the 21 genotypic groups created at a 0.75 cut distance is shown. In B, the relative abundance of each group is represented. "n" represents the number of isolates.

3.2.2. Distribution of the *Pseudomonas aeruginosa* genotypic groups throughout the years

The relative abundance of each genotypic group of *P. aeruginosa* has not been the same throughout the years (Figure 15).

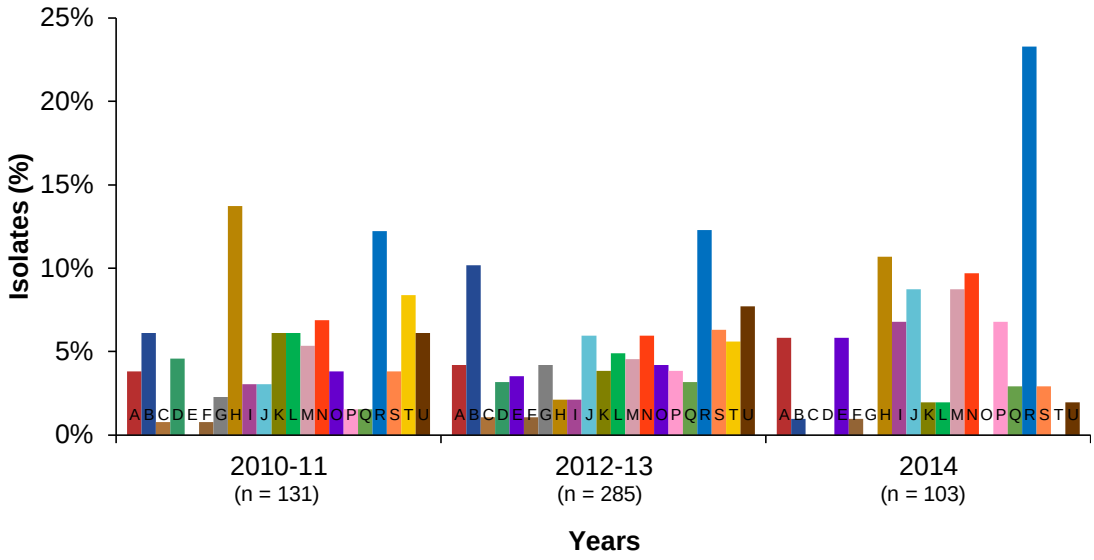


Figure 15. Relative abundance of the genotypic groups of *P. aeruginosa* clinical isolates throughout the years ("n" represents the number of isolates).

In 2010-11, clinical isolates belonging to the genotypic groups H and R were the most commonly found, with a frequency of 14% and 12%, respectively. In 2012-13, while the abundance of isolates belonging to the group R stayed the same (12%), isolates from group H were underrepresented, corresponding to only 2% of all isolates. On the other hand, isolates from group B were ones of the most frequent (10%). Moreover, in 2012-13, a new genotypic group appeared, designated as group E (4%). In 2014, group R almost doubled its representativeness, compared to previous years (23%), and isolates from group H were again among the most present (11%). Groups I, J, M and N were more represented in 2014, when comparing with previous years, having a frequency of 7%, 9%, 9% and 10%, respectively. On the other hand, in 2014, no isolates belonging to groups C, D, G, O and T were found. With the exception of isolates from group T, that in 2010-11 had a frequency of 8% and in 2012-13 had a frequency of 6%, the groups not represented in 2014, were already underrepresented in previous years.

3.2.3. Distribution of the *Pseudomonas aeruginosa* genotypic groups according to age and gender of the patients, hospital service and source of isolation

The relative frequency of the majority of the genotypic groups did not significantly fluctuate considering age and gender of patients above 6 years old (Appendix – Figure B): apart from group E, that was not represented in females between 6 and 60 years old, and group C, that was not represented in males above 6 years old and females between 61 and 98 years old, all genotypic groups were represented in all classes. Groups C and F were not represented in the aforementioned classes due to the fact that they corresponded to only 1% of all samples isolated (Figure 15). Genotypic groups that evidenced differences between classes were, for example, group R, which varied between 9% and 16% between classes and group N, which varied between 4% and 11% between classes (Appendix – Figure B). For new-born patients and patients up to 5 years old, 5 and 8 genotypic groups were attributed for males and females, respectively (Appendix – Figure B). Since these classes corresponded to only 3% of all patients, with 5 cases for males and 9 for females between 0 and 5 years old, it could be said that *P. aeruginosa* infections in this age group were not associated with any specific genotypic group. However, to prove this hypothesis, a larger sample group for this age class is necessary. This could be accomplished as more *P. aeruginosa* isolations will happen throughout the years.

In underrepresented hospital services (no more than 30 isolates) – Cardiology, Neurosurgery, Orthopaedics, Paediatrics, Rehabilitation Medicine, Surgery, and Others – it wasn't possible to associate genotypic groups to service due to the observed variability (Appendix – Figure C). In other hospital services, despite the significant variability of genotypic groups within each service, some stood out (Appendix – Figure C): in ER, there was a dominance of isolates from group R, with 19% of all isolates; in General Medicine and Urology, both groups R and H stood out, corresponding to 15% and 10% of all isolates in General Medicine and to 18% and 12% of all isolates in Urology, respectively; in the Multipurpose ICU, groups B and M prevailed, with 14% and 12% of all isolates; and, in Pneumology, the groups most represented were groups J and N with 16% of all isolates. Though these results were interesting, the assumptions made for Urology and Pneumology need further confirmation, as their sampling groups were not as large as others (only 49 samples were isolated in Urology and 32 in

Pneumology). This will be achieved as more *P. aeruginosa* isolations will occur in the coming years.

As with hospital services, some sources of *P. aeruginosa* were underrepresented – blood, catheters, ears, ulcers and "others" (Appendix – Figure D). Therefore, any tendency of specific genotypic group dominance needs further confirmation with increased sampling numbers. In other sources, despite the diversity of genotypic groups observed, some groups prevailed (Appendix – Figure D): among isolates from sputum and urine, isolates from the genotypic group R were the most frequent ones, corresponding to 15% and 18% of all isolates, respectively; among isolates from pus/exudates/fluids, the most prevalent genotypic groups were groups B, D and G, corresponding to 15%, 12% and 12% of all isolates, respectively; among isolates from bronchial aspirates, the most represented groups were B and T, corresponding to 21% and 19% of all isolates, respectively. Interestingly, among isolates from pus/exudates/fluids and isolates from bronchial aspirates, isolates from group R had only a relative abundance of 3% (Appendix – Figure D), despite being the group with the highest isolate frequency (Figure 15).

3.2.4. Association of *Pseudomonas aeruginosa* genotypic groups to the occurrence of multi-infection and abundance of MDR isolates

In Figure 16, the frequency of the genotypic groups associated with the occurrence of multi-infections and a MDR phenotype is shown.

A great diversity of genotypic groups of *P. aeruginosa* isolates was found in scenarios of multi-infection (Figure 16-A), with all genotypic groups being represented. Isolates from group R were the most abundant ones, with a frequency of 13%, which is not surprising since this was the group harbouring most isolates of the *P. aeruginosa* collection (Figure 15). *P. aeruginosa* isolates found in scenarios of multi-infection corresponded to 48% of all isolates (as seen in Figure 8), and most genotypic groups presented a similar frequency of isolates found in scenarios of multi-infection (Figure 16-B). Apart from the genotypic group Q, which had only 33% of isolates from multi-infection sites, all genotypic groups had a frequency of isolates from multi-infection sites equal or greater than 41%. Groups F, G and K, were composed by 60%, 60% and 62% of isolates from multi-infection sites. Group C was composed by 75% of isolates from multi-infection sites; however this genotypic group is underrepresented (with only

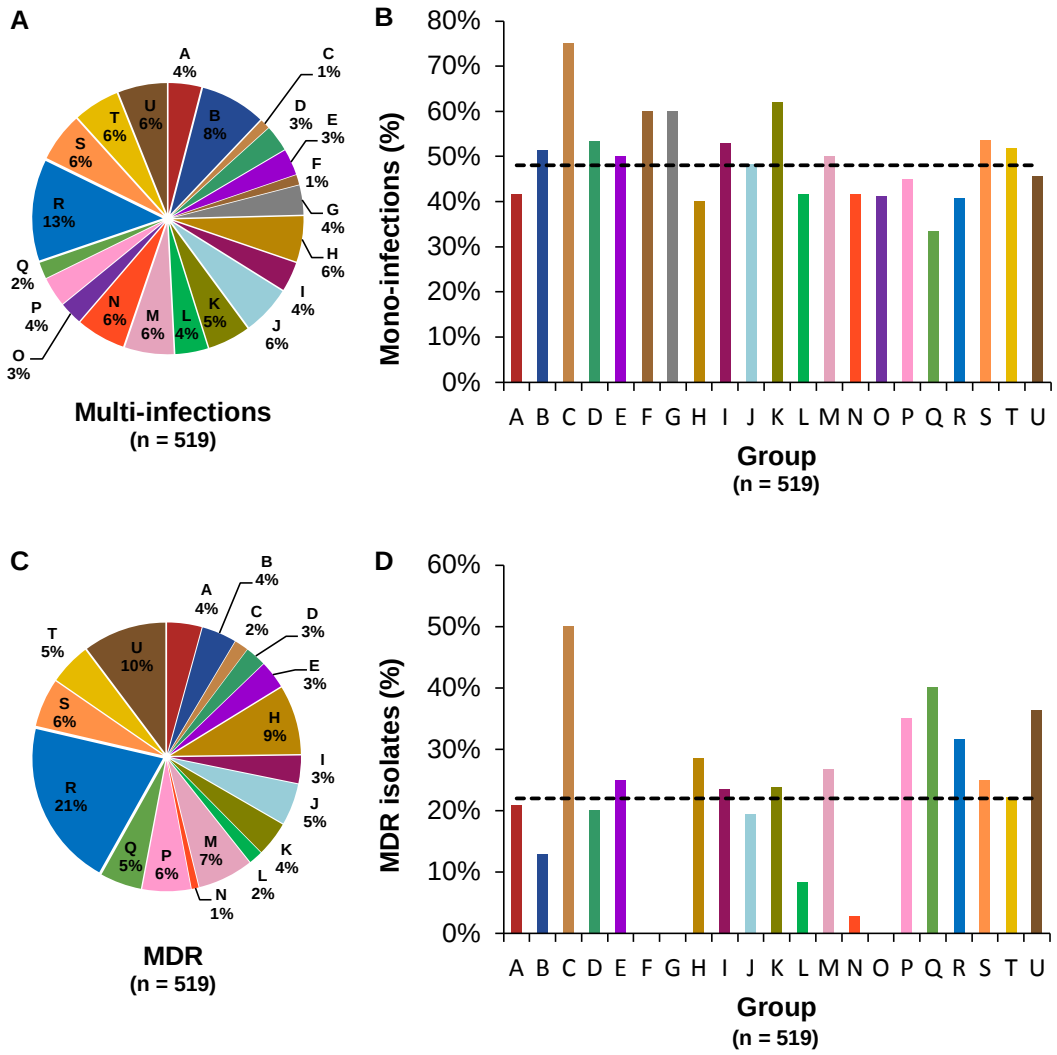


Figure 16. Genotypic groups associated with the occurrence of multi-infections and MDR. In A, the frequency of genotypes associated with the occurrence of multi-infections with *P. aeruginosa* can be seen. In B, the relative abundance of *P. aeruginosa* isolated from scenarios of multi-infection in each genotypic group is showed. In C, the distribution of each genotypic group among MDR isolates can be seen. In D, the relative abundance of MDR isolates in each genotypic group is showed. In B and D, the horizontal doted bar represents the mean value for the entire *P. aeruginosa* collection.

1% of all isolates, Figure 15). All other genotypic groups had a frequency equal or smaller than 54% isolates from multi-infection scenarios.

A good diversity of genotypic groups attributed to *P. aeruginosa* MDR isolates was observed (Figure 16-C). Nonetheless, 40% of all MDR isolates were found to be from groups R, U and H, with an abundance of 21%, 9% and 10%, respectively. Groups

F, G and O were not represented among MDR isolates, but these groups were also found to not be abundant among all isolates, with a frequency of only 1%, 3% and 3%, respectively (Figure 15). MDR *P. aeruginosa* isolates corresponded to 22% of all *P. aeruginosa* isolates (Figure 8). Intra-genotypic group, that frequency not always applies (Figure 10-D). The groups P, Q, R and U were composed by more MDR isolates than normal, with a frequency of MDR isolates equal to 35%, 40%, 32% and 36%, respectively. Group C was composed by 50% of MDR isolates; however this genotypic group is underrepresented (with only 1% of all isolates). On the other hand, groups B, L and N only had 13%, 8% and 3% of MDR isolates composing them. All other genotypic groups had a frequency of 10-29% of MDR *P. aeruginosa* isolates.

3.2.5. Genotypic variability of pan-resistant *Pseudomonas aeruginosa* isolates

P. aeruginosa infections with PDR isolates are associated with even worse outcomes than those already described for MDR isolates, as resistance is found for all antimicrobials tested (review by Magiorako *et al.*, 2011). For example, a study by Wang *et al.* (2005), showed that patients suffering PDR *P. aeruginosa* infections had incredibly high mortality rates (74%) and those who died had an average survival duration of only 19 days after diagnosis (Wang *et al.*, 2005). Given the especially high morbidity and mortality associated PDR *P. aeruginosa* infections, it was questioned whether PDR isolates were part of a specific genotypic group or not, as determined by RAPD-PCR.

In this study, *P. aeruginosa* isolates were considered PDR when resistant to all 7 antibiotics tested by Hospital de Braga, using the Kirby-Bauer disk diffusion susceptibility test (Wayne, 2006). Using this criterion, 16 isolates were found to be PDR. Resistance to the polymyxin colistin was also tested by Hospital de Braga for PDR *P. aeruginosa* isolated after 2012. The PDR isolates were tested for resistance to colistin again in the lab, using a broth dilution method to determine minimal inhibitory concentrations of antimicrobial substances, as described by Wiegand *et al.* (2008) (data not shown). In Table II, the 16 PDR *P. aeruginosa* isolates and information regarding their resistance to colistin and genotypic group, as determined by RAPD-PCR, is shown.

Table II. List of PDR *P. aeruginosa* isolates, resistance to colistin (R – resistant; S – susceptible), and genotypic group attributed by RAPD-PCR. The genomes of the isolates in bold are currently being sequenced in the scope of "The 1000 *Pseudomonas aeruginosa* Genomes Project".

Isolate	Resistance to colistin	Genotypic Group
HB13	S	T
HB136	S	D
HB158	R	R
HB159	R	J
HB167	S	R
HB225	S	S
HB254	S	T
HB288	R	P
HB290	R	U
HB298	S	J
HB332	S	P
HB392	R	D
HB401	S	L
HB405	S	D
HB410	S	H
HB428	S	R

Despite some genotyping groups being more present than others, there was a significant variability of genotypic groups among PDR *P. aeruginosa* isolates. More specifically, the 5 *P. aeruginosa* isolates that showed resistance to colistin were all from different genotypic groups, indicating a genotypic distance superior to 0.75 among them. As mentioned earlier, colistin is a last-resort antibiotic (review by Lambert, 2002), and a resistant phenotype to this drug is especially problematic, even more so, if genetically distant isolates can be resistant to the drug, as presented here. Genotypic diversity among PDR isolates was also found in the literature (Hsueh *et al.*, 2005). A future task in this work should involve the genotyping of the PDR *P. aeruginosa* isolates with a variety of genotyping methods such as of rep-PCR and PFGE, in order to confirm that the genomic diversity observed is not an artifact caused by the usage of only one genotyping method.

Earlier this year, a worldwide consortium of clinical and basic researchers launched "The 1000 *Pseudomonas aeruginosa* Genomes Project", an ambitious project that aims at: (i) the reconstruction of 1000 *P. aeruginosa* genomes using NGS technologies; (ii) the creation of a standardized genome sequence database representative of *P. aeruginosa* populations collected from all over the world; (iii) and the development of new platforms that promote the synergy between genomics and clinical data, improving patient treatments. In that scope, the current genotyping work has enabled the selection of a panel of 14 PDR *P. aeruginosa* isolates to be integrated in the project and have its genome sequenced, using NGS. The isolates currently being sequenced are represented in bold in Table II.

3.2.6. Genotypic variability of *Pseudomonas aeruginosa* isolates from a CF patient

As mentioned before, *P. aeruginosa* infections are particularly problematic in CF patients, even leading to death (review by Kerr and Snelling, 2009). Among the *P. aeruginosa* isolates genotyped in this work, 6 were isolated from one CF patient suffering from CF. In Table III, the isolates and their genotypic group are shown.

Table III. List of *P. aeruginosa* isolates from one CF patient and genotypic group attributed by RAPD-PCR.

Isolate	Genotypic Group
HB14	M
HB36	N
HB363	R
HB406	S
HB407	S
HB436	J

Four out of the six *P. aeruginosa* isolates belonged to different genotypic groups with only HB406 and HB407 sharing the same. This indicates a genotypic distance superior to 0.75 between the four of them. HB406 and HB407 belonged to the same

genotypic group, sharing a genotypic distance of only 0.1 between them; however both were isolated from the same patient, on the same day.

The finding that *P. aeruginosa* isolates from CF patients are genotypically diverse is consistent with other genotyping studies available in the literature (Vosahlikova *et al.,* 2007; Wahab *et al.,* 2013). This finding is also consistent with the fact that the success of colonization of the CF lung by *P. aeruginosa* results from long-term adaptation of the bacterium, and not from disease-specific clones (review by Cramer *et al.,* 2010).

For the establishment of a chronic infection, *P. aeruginosa* diversifies into several morphotypes in the CF lung, undergoing several physiological changes that include loss of functions that were once beneficial to invade and injure the host, such as motility, type III secretion, O antigen biosynthesis, exotoxin, and protease and phenazine production (e.g. pyocianin) (review by Nguyen and Singh, 2006). Other changes include the alteration of surface antigens, the overproduction of alginate, an increased biofilm formation and antibiotic tolerance (review by Hogardt and Heesemann, 2013). Therefore, following work with these CF isolates should involve a phenotypic characterization of their infection status, using *in vitro* tests, and assessment of their overall virulence, using animal models such as *Caenorhabditis elegans* and *Galleria mellonella* (Miyata *et al.,* 2003; Tan *et al.,* 1999). With a possibility to track the CF isolates in the lungs of the patients over time, in addition to a continuous phenotypic and virulent characterization, a genomic characterization of their microevolution in the CF lung should be assessed by NGS. Thus, it would be possible to further expand the knowledge of which point mutations in genes are the most essential for a change in phenotype and persistence of *P. aeruginosa* in the CF lung.

4. CONCLUDING REMARKS

P. aeruginosa is an opportunistic human pathogen, responsible for frequently lethal nosocomial infections (review by Kerr and Snelling, 2009).

In this work, we presented a study involving a diversified collection of over 500 clinical isolates of *P. aeruginosa*, recovered for a period of 5 years and 2 months, in Hospital de Braga (Braga, Portugal). This is of relevance, as most studies in the literature choose to focus on isolates from specific hospital services (e.g. ICUs, urology and burn units), diseases (e.g. CF, urinary tract infections and pneumonia), age groups (e.g. newborns) or traits (e.g. MDR). The isolates presented here were found in patients from all ages, being treated in over 13 different hospital services, and isolated from over 10 different anatomical sources. Moreover, the *P. aeruginosa* collection here presented, includes both MDR and non-MDR isolates, isolates found in the context of mono and multi-infections and isolates from patients suffering from CF.

The first aim of this work was to perform an epidemiological study on *P. aeruginosa* infections in order to provide answers relevant to health care personnel. We were able to successfully find which age/gender groups were the most affected by *P. aeruginosa* infections in Hospital de Braga, which hospital services had the most patients diagnosed with *P. aeruginosa* infections, which anatomical sources most isolates were collected from, and how many isolates were found in the context of multi-infections. We were also able to notice a trend in which the number of MDR *P. aeruginosa* isolates has been growing throughout the years. Different patterns regarding type of infection and drug resistance status in different sources of isolation were also found.

Genotyping is center key in epidemiological studies involving *P. aeruginosa* as it can contribute to better surveillance systems, outbreak detection and more adequate clinical treatment options (review by Sabat *et al.*, 2013; review by Tenover *et al.*, 1997). Therefore, the second aim of this work was to perform a genotypic characterization of all *P. aeruginosa* clinical isolates, using RAPD-PCR as a typing method. We successfully established RAPD-PCR as a typing method in our lab, allowing for the continuous motorization of clinical isolates. We also successfully established a pipeline for genotyping analysis, based on freeware programs. With RADP-PCR we were able to divide the *P. aeruginosa* collection into 21 different genotypic groups. The frequency of isolates in each group was determined, as well as their relative abundance throughout the years, finding it variable. We found that age and gender did not seem to play a differentiating role in the frequency of each genotypic group. On the other hand, some

significant variations of frequency of genotypic groups were observed depending on the hospital service and source of isolation of the *P. aeruginosa* isolates. We also found that some groups were under or overrepresented in scenarios of multi-infection and among MDR isolates. We found that PDR isolates, including colistin resistant isolates, evidenced a high genotypic variability, indicating that the acquisition of multiple drug resistances may not be a trait more favorable to occur in genotypically close *P. aeruginosa* isolates, but rather a capacity wide-spread among all *P. aeruginosa*. Lastly, we also found isolates from CF patients to be genotypically diverse among them, being consistent with data from the literature that states that the colonization of the CF lung is derived from long-term adaptation and not disease-specific clones.

Overall, it was found that, despite variations in the relative abundance of some genotypic groups, a group was rarely not represented at all. This only mirrors the well-known fact that *P. aeruginosa* is a ubiquitous microorganism with a remarkable genomic plasticity, that allows for a phenotypic flexibility indispensable for it to thrive in the most diverse surroundings, making *P. aeruginosa* a top of the list microorganism when the subject is pathogenicity.

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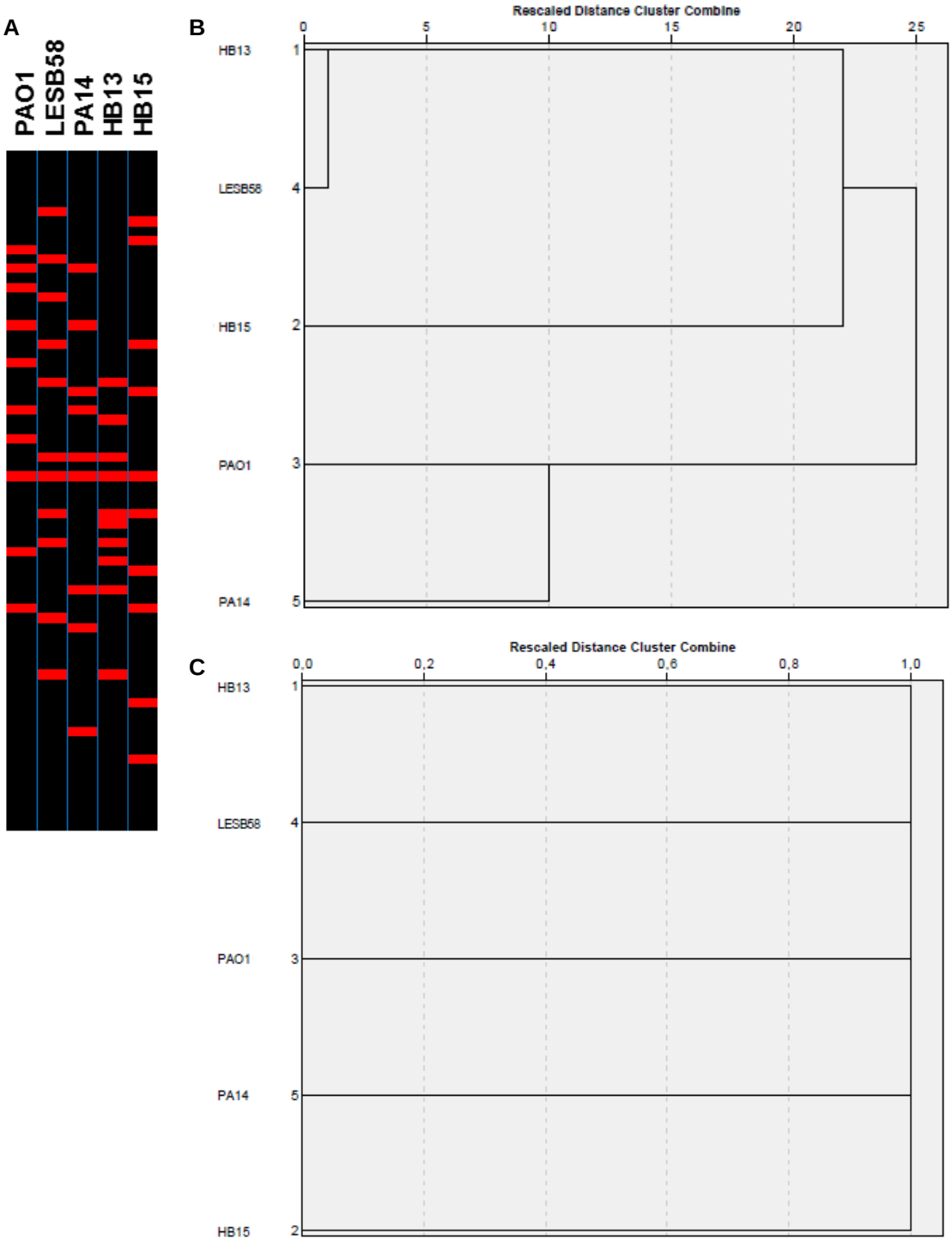
6. APPENDIX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PA14
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HB15
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HB13 1
LESB58 4
HB15 2
PAO1 3
PA14 5
C
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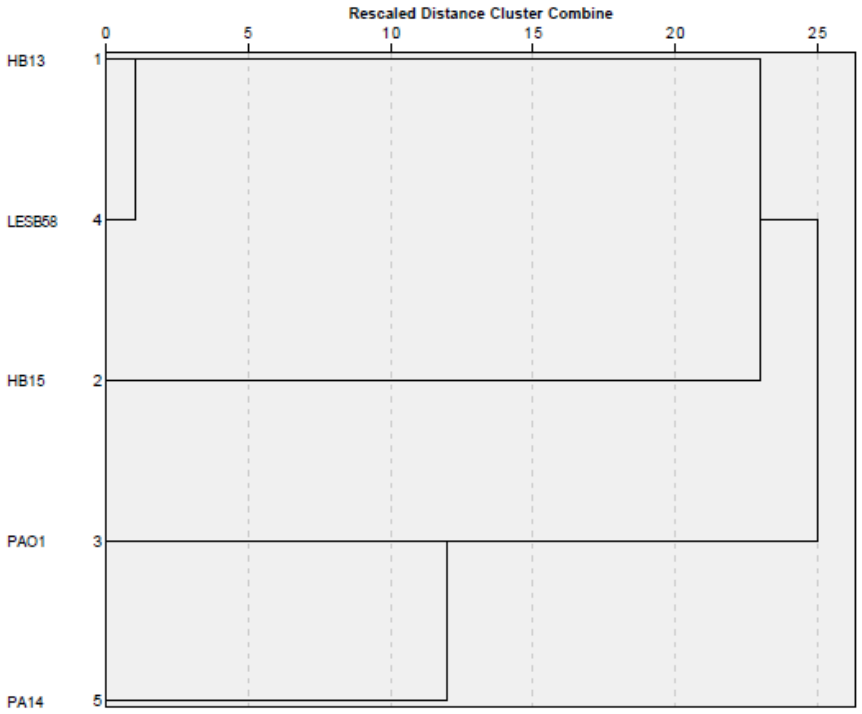
PA14 5
HB15 2

D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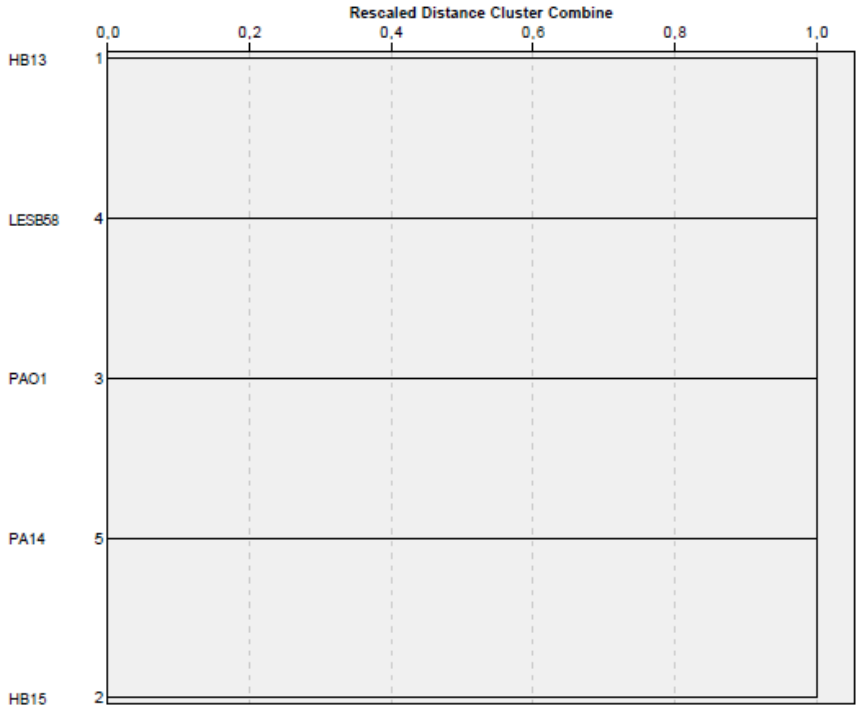
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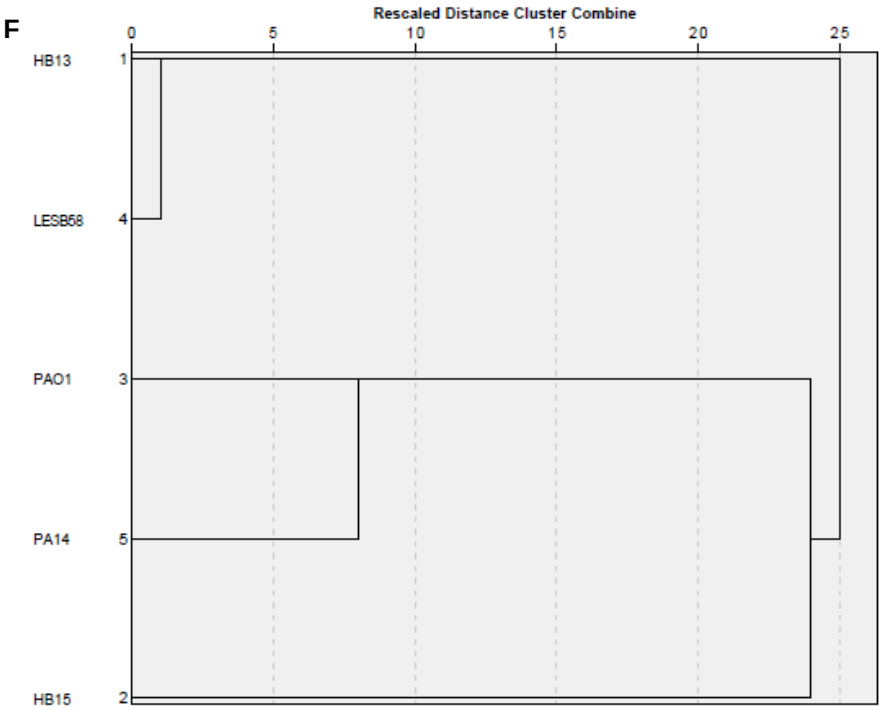
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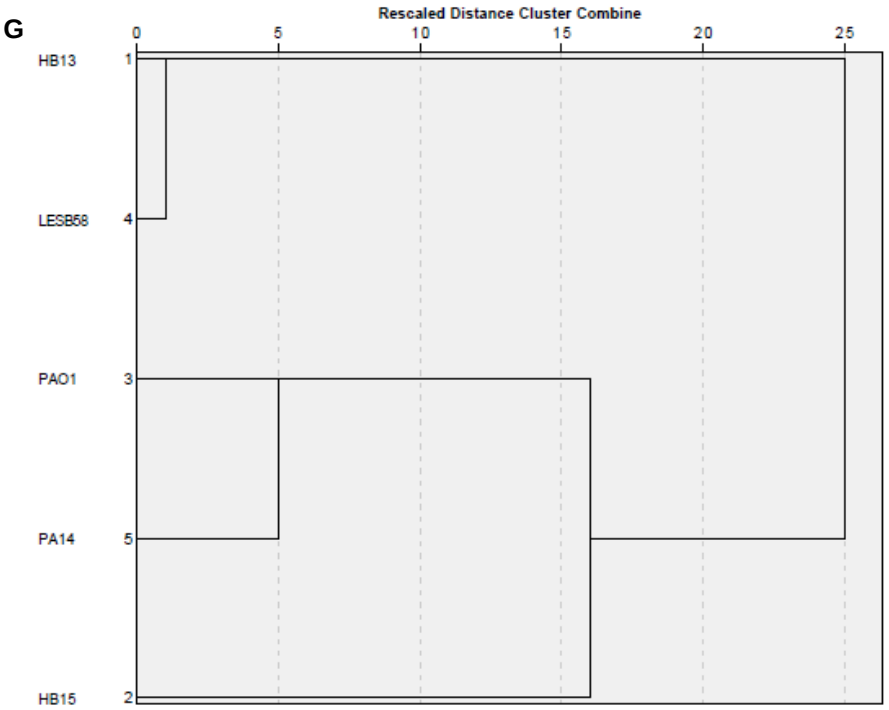
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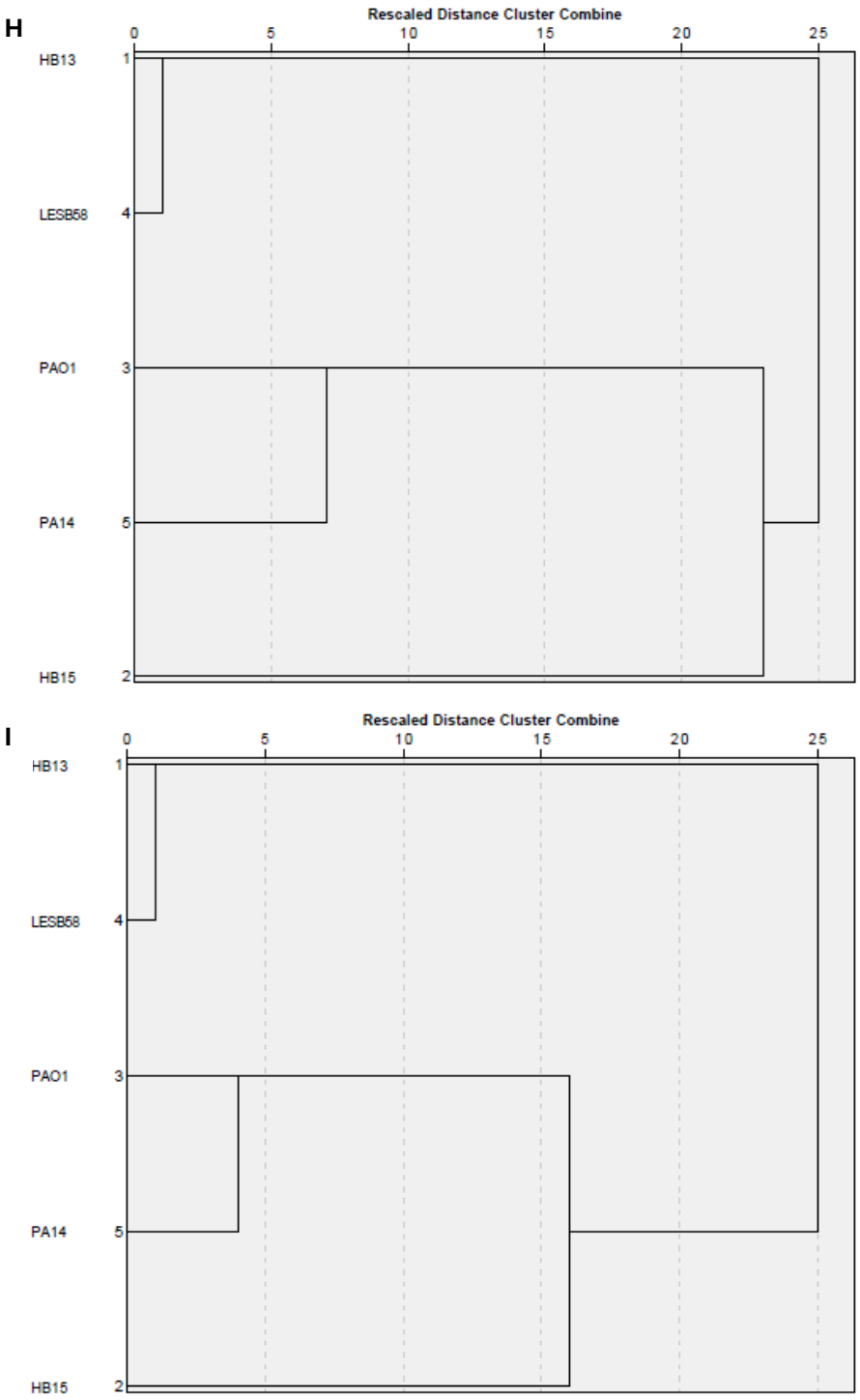


Figure A. Trials for the selection of a dissimilarity and hierarchical clustering method for genotyping analysis. In A, a scheme of the band profiles of *P. aeruginosa* PAO1, LESB58, PA14, HB13 and HB15 can be seen. In B-I, dendrograms from different dissimilarity and hierarchical clustering methods can be seen (B – Dice's coeficient/UPGMA; C – Dice's coeficient/Ward; D – Jaccard's coeficient/UPGMA; E – Jaccard's coeficient/Ward; F – Euclidean Distance/UPGMA; G – Euclidean Distance/Ward; H – Squared Euclidean Distance/UPGMA; I – Squared Euclidean Distance/Ward).

Table A. Distribution of the *Pseudomonas aeruginosa* clinical isolates in genotypic groups.

Group	*Pseudomonas aeruginosa* clinical isolates								
A	HB29	HB54	HB65	HB75	HB76	HB87	HB127	HB226	HB227
	HB228	HB249	HB261	HB270	HB283	HB337	HB398	HB404	HB415
	HB447	HB450	HB476	HB477	HB478	HB488			
B	HB33	HB51	HB57	HB64	HB88	HB91	HB95	HB124	HB130
	HB157	HB170	HB182	HB193	HB199	HB205	HB211	HB216	HB239
	HB241	HB244	HB247	HB267	HB285	HB302	HB303	HB305	HB350
	HB351	HB352	HB358	HB367	HB377	HB378	HB396	HB397	HB400
	HB402	HB403	HB495						
C	HB101	HB207	HB223	HB338					
D	HB39	HB47	HB48	HB82	HB85	HB126	HB136	HB151	HB263
	HB276	HB297	HB355	HB374	HB392	HB405			
E	HB11	HB150	HB190	HB200	HB237	HB287	HB292	HB340	HB361
	HB383	HB429	HB440	HB500	HB501	HB507	HB520		
F	HB58	HB257	HB319	HB343	HB514				
G	HB77	HB92	HB105	HB188	HB195	HB230	HB235	HB251	HB264
	HB278	HB310	HB344	HB347	HB369	HB399			
H	HB4	HB17	HB21	HB28	HB30	HB34	HB35	HB55	HB67
	HB69	HB81	HB89A	HB97	HB118	HB120	HB122	HB163	HB172
	HB196	HB218	HB313	HB315	HB316	HB362	HB410	HB411	HB417
	HB430	HB431	HB433	HB435	HB505	HB506	HB508	HB515	
I	HB41	HB53	HB83	HB103	HB231	HB245	HB268	HB356	HB386
	HB412	HB418	HB451	HB452A	HB479	HB491	HB509	HB517	
J	HB68	HB86	HB144	HB153	HB154	HB156	HB159	HB176	HB179
	HB191	HB201	HB220	HB232	HB255	HB272	HB289	HB298	HB359
	HB366	HB373	HB379	HB419	HB420	HB436	HB446	HB448	HB457
	HB466	HB473	HB513	HB519					
K	HB8	HB38	HB40	HB71	HB90	HB94	HB119	HB125	HB133
	HB219	HB238	HB274	HB286	HB311	HB318	HB320	HB357A	HB376
	HB382	HB455	HB481						
L	HB7	HB12	HB20	HB25	HB45	HB49	HB89B	HB107	HB109
	HB110	HB113	HB155	HB162A	HB162B	HB177	HB184	HB203	HB330
	HB368	HB375	HB390	HB401	HB432	HB467			
M	HB2	HB14	HB32	HB59	HB63	HB99	HB114	HB132	HB140
	HB148	HB171	HB175	HB206	HB209	HB210	HB222	HB300	HB301
	HB380	HB425	HB464	HB468	HB469	HB471	HB472	HB492	HB494
	HB499	HB516	PA14						

N	HB6	HB15	HB18	HB36	HB46	HB96	HB108	HB115	HB138
	HB165	HB168	HB233	HB265	HB277	HB281	HB306	HB322	HB329
	HB333	HB339	HB357B	HB360	HB365	HB393	HB409	HB414	HB434
	HB437	HB438	HB453	HB454	HB474	HB482	HB484	HB487	HB489
O	HB22	HB24	HB27	HB44	HB52	HB56	HB204	HB236	HB291
	HB317	HB372	HB387	HB388	HB394	HB424	HB426	HB427	
P	HB50	HB102	HB185	HB187	HB208	HB221	HB262	HB288	HB293
	HB323	HB326	HB332	HB348	HB463	HB496	HB497	HB498	HB502
	HB503	HB504							
Q	HB104	HB137	HB143	HB145	HB178	HB181	HB197	HB217	HB260
	HB331	HB391	HB408	HB483	HB486	PAO1			
R	HB1	HB3	HB10	HB16	HB26	HB31	HB37	HB61	HB74
	HB84	HB98	HB106	HB112	HB116	HB128	HB131	HB135	HB142
	HB146	HB158	HB160	HB166	HB167	HB180	HB202	HB215	HB243
	HB246	HB252	HB253	HB269	HB271	HB284	HB295	HB299	HB308
	HB309	HB312	HB314	HB321	HB324	HB327	HB334	HB345	HB346
	HB353	HB363	HB364	HB371	HB384	HB421	HB422	HB423	HB428
	HB439	HB441	HB442	HB443	HB444	HB445	HB449	HB456	HB458
	HB459	HB460	HB461	HB462	HB465	HB470	HB475	HB480	HB485
	HB510	HB518	HB521	LESB58					
S	HB5	HB19	HB42A	HB72	HB100	HB147	HB149	HB169	HB183
	HB186	HB194	HB212	HB213	HB224	HB225	HB229	HB294	HB328
	HB342	HB349	HB381	HB406	HB407	HB413	HB416	HB452B	HB490
	HB493								
T	HB13	HB43	HB62	HB70	HB73	HB78	HB93	HB111	HB117
	HB129	HB152	HB161	HB164	HB174	HB189	HB198	HB214	HB254
	HB256	HB259	HB266	HB275	HB279	HB282	HB304	HB307	HB341
U	HB9	HB23	HB42B	HB60	HB66	HB80	HB121	HB123	HB134
	HB139	HB141	HB173	HB192	HB234	HB240	HB242	HB248	HB250
	HB258	HB273	HB280	HB290	HB296	HB325	HB335	HB336	HB354
	HB370	HB385	HB389	HB395	HB511	HB512			

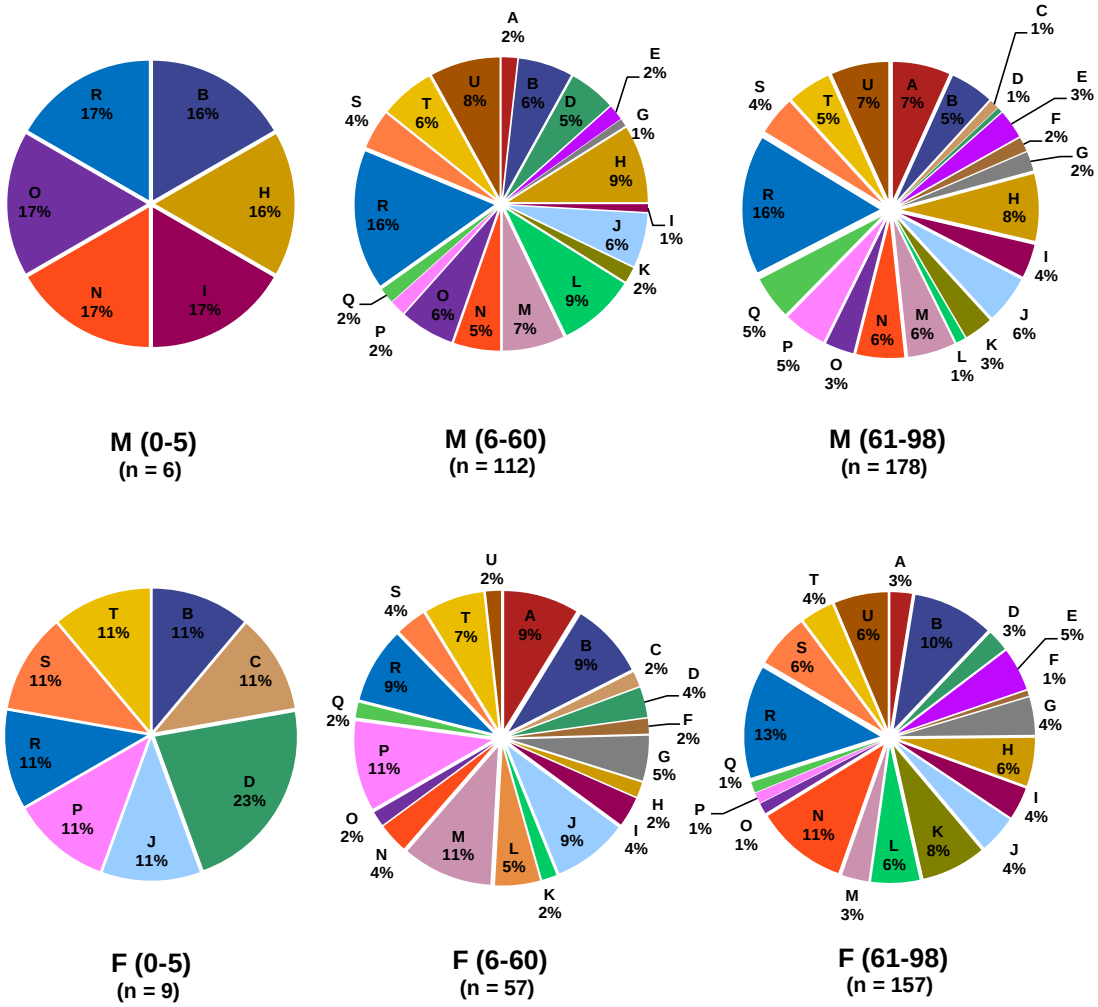
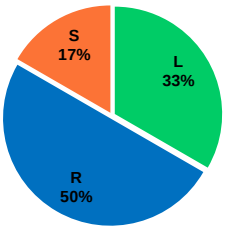


Figure B. Frequency of *P. aeruginosa* genotypic groups among patients. "n" represents the number of isolates.



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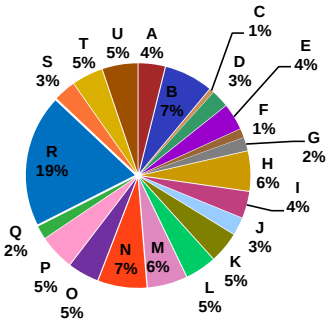

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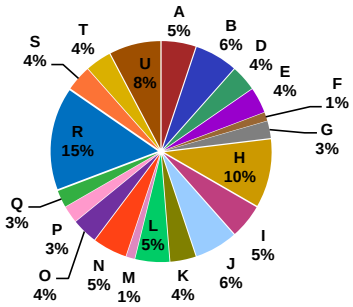

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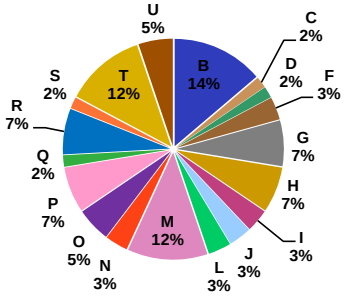

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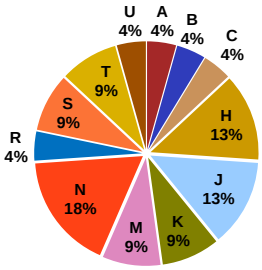
(n = 58)



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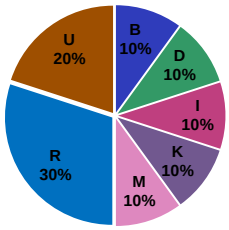

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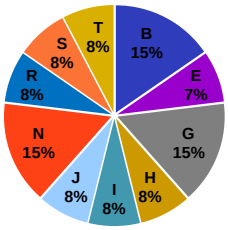

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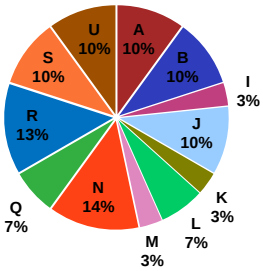
(n = 13)



Others
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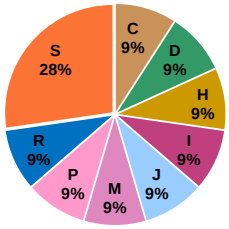

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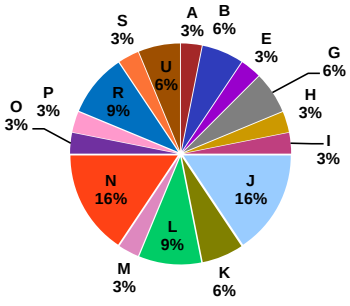

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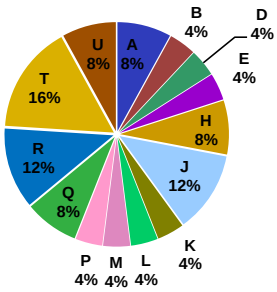

Rehabilitation Medicine
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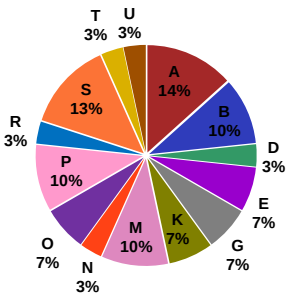

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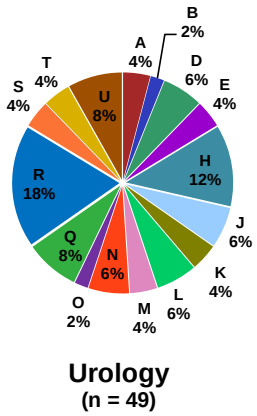

Figure C. Frequency *P. aeruginosa* genotypic groups in patients from different hospital services. "n" represents the number of isolates.

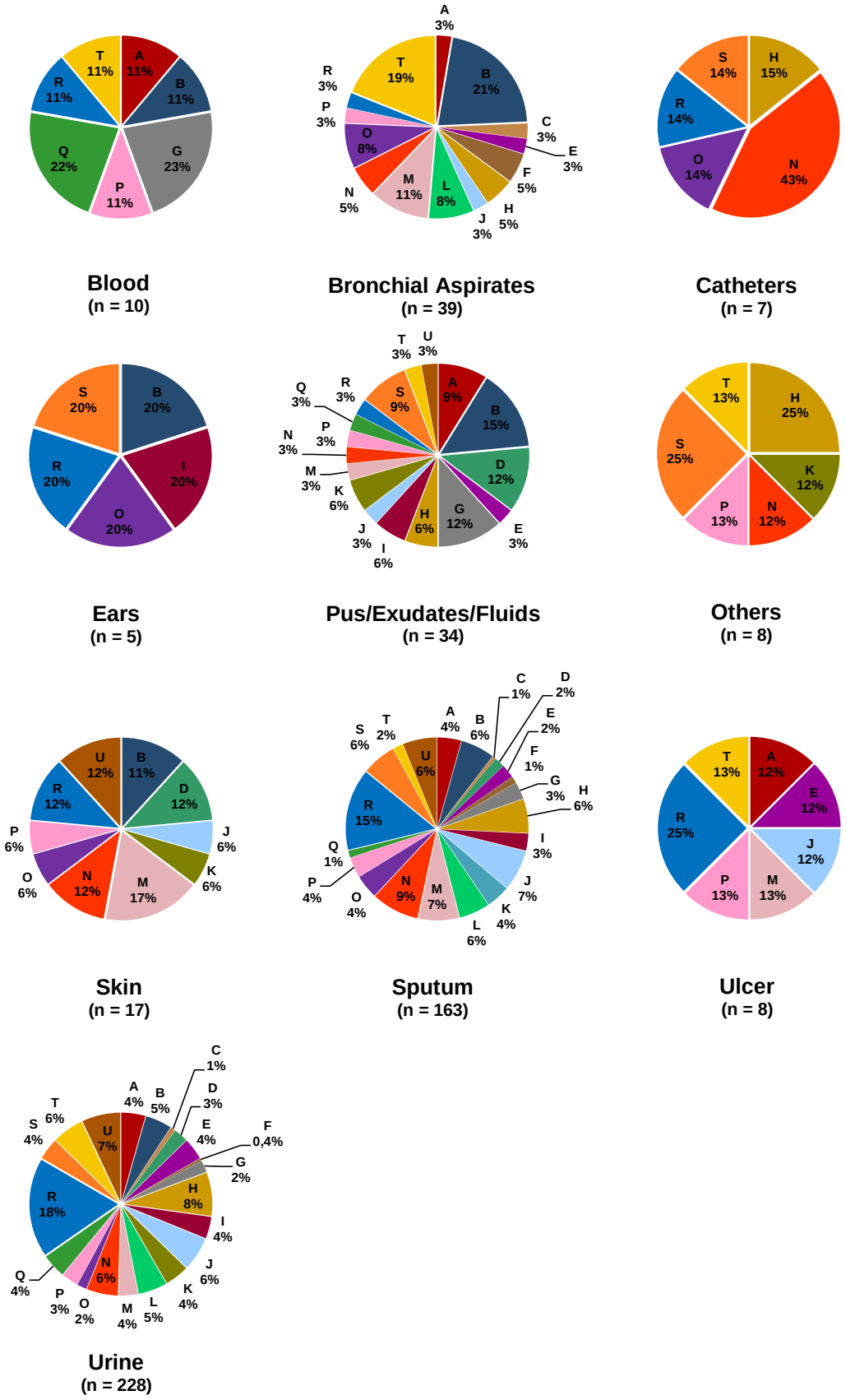

Figure D. Frequency of the *P. aeruginosa* genotypic groups according to isolation source. "n" represents the number of isolates.