Universidade do Minho
Escola de Engenharia

Ana Catarina Rodrigues

Study of the efficacy of combination therapy based on bacteriophages for the control of infectious biofilms

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Study of the efficacy of combination therapy based on bacteriophages for the control of infectious biofilms

Dissertation thesis for the Master degree in Biomedical Engineering

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Study of the efficacy of combination therapy based on bacteriophages for the control of infectious biofilms

Abstract

Pseudomonas aeruginosa is regarded as a "phenomenon of bacterial resistance". This gram negative bacterium is responsible for a high percentage of mortality in the hospitals all over the world and its prevalence can be a consequence of important reasons, such as: intrinsic resistance determined by virulence factors; acquired resistance mechanisms that lead to a low susceptibility to antimicrobial agents; and the ability of *P. aeruginosa* to grow on any natural and artificial surfaces leading to the development of biofilms. The emergence of new strategies to control *P. aeruginosa* biofilms is becoming more evident due to their resistance to traditional treatments, and (bacterio)phages have been recognized as an attractive alternative for this problem. Nevertheless, despite the potential of phages as antimicrobial agents, it is well known that bacteria can quickly adapt and create new survival strategies and the emergence of phage-resistant phenotypes is inevitable. Thus, the combination of phage and antibiotic therapies could have potentially more benefits than just using phages and antibiotics alone.

This work describes the combined effect of phages and antibiotics against planktonic cultures and biofilms of *P. aeruginosa*. The antimicrobial susceptibility of three reference strains of *P. aeruginosa* towards antibiotics belonging to four different action groups was initially evaluated and for this purpose, two important parameters were determined: Minimal Inhibitory Concentration (MIC) for planktonic cultures and Minimal Biofilm Eradication Concentration (MBEC) for biofilms. After that, phage infection assays were performed against cultures and biofilms of *P. aeruginosa* using four different phages from the Bacteriophage Biotechnology Group of the University of Minho. The efficacies of antibiotics and phages or both combined were evaluated in biofilms by viable cell enumeration and in planktonic cultures by measuring the absorbance (OD600nm). In general, all antibiotics tested showed little efficacy against biofilms which were also very tolerant to phage infection. The presence of degradative enzymes, such as beta-lactamase, and the survival of tolerant cells (persister cells) can explain the failure of antibiotics in reducing the cell numbers present in biofilms. In the

case of phage infection, the emergence of resistant phenotypes defective in LPS mutants can lead to long-term failure of these agents.

In combined treatments, phages used together with ciprofloxacin caused total biofilm eradication. Also, other combinations resulted in interesting results. For example, the combination therapy of both phage phiIBB-PAP21 and amikacin resulted in approximately 3.66 log reduction of viable cells while individually, phage and amikacin only caused a 1.3 and 1.76 log reduction, respectively. The effectiveness of combined phages-antibiotic treatments can be due to a higher burst size, as observed with phage phiIBB-PAP21, when their host cells were exposed to antibiotics. Also, the higher biomass reductions observed when biofilms are exposed to a combined treatment suggest that phages can enhance the antibiotic penetration through matrix disruption, rendering cells freely available to be killed with antibiotics.

Overall, the combination of phage and antibiotic enhances biofilm control; however the complex universe behind this synergistic interaction suggests that this is not always a linear process. Further studies should be conducted to complement and disclose this synergistic behaviour observed in the work described herein.

Avaliação da eficácia de terapias combinadas á base bacteriófagos no controlo de biofilmes infeciosos

Sumário

Pseudomonas aeruginosa é considerada um "fenómeno da resistência bacteriana". Esta bactéria gram-negativa é responsável por uma elevada percentagem de mortalidade nos hospitais de todo o mundo e a sua prevalência pode ser consequência de razões importantes como: resistência intrínseca determinada por vários fatores de virulência; mecanismos de resistência adquiridos que conduzem a uma baixa suscetibilidade aos agentes antimicrobianos e a capacidade de crescimento de *P. aeruginosa* em todas as superfícies naturais e artificiais que favorece ao desenvolvimento de biofilmes. A necessidade de novas estratégias de controlo de biofilmes de *P. aeruginosa* tem-se tornado mais evidente devido à sua resistência aos tratamentos tradicionais, e os bacteriófagos têm sido reconhecidos como uma alternativa atrativa para este problema. No entanto, apesar do potencial dos fagos como agentes antimicrobianos, sabe-se que as bactérias podem adaptar-se rapidamente e criar novas estratégias de sobrevivência e o aparecimento de fenótipos resistentes aos fagos é inevitável. Assim, a combinação de fagos com as terapias antibióticas pode ter potencialmente mais benefícios em relação á utilização isolada de fagos e antibióticos.

Este trabalho descreve o efeito combinado de fagos e antibióticos contra culturas planctónicas e biofilmes de *P. aeruginosa*. A suscetibilidade antimicrobiana de três estirpes de referência de *P. aeruginosa* contra antibióticos pertencentes a quatro grupos de ação diferentes foi inicialmente avaliada. Para este fim, dois parâmetros importantes foram determinados: Concentração Mínima Inibitória (CIM) para culturas planctónicas e Concentração Mínima de Erradicação do Biofilme (CMEB) para os biofilmes. Depois desta etapa realizaram-se ensaios de infeção fágica contra as culturas e biofilmes de *P. aeruginosa* utilizando quatro fagos diferentes do Grupo de Biotecnologia de Bacteriófagos da Universidade do Minho. A eficácia dos antibióticos e fagos ou dos dois agentes combinados foi avaliada nos biofilmes pela enumeração de células viáveis, e nas culturas planctónicas pela medição da absorvência (OD600nm). Em geral, todos os antibióticos testados apresentaram pouca eficácia contra os biofilmes, os quais também foram muito tolerantes à infeção fágica. A presença de enzimas de degradação,

tais como a beta-lactamase, e a sobrevivência de células tolerantes (células *persister*) pode explicar a falha dos antibióticos sobre a redução do número de células presentes nos biofilmes. No caso da infeção fágica, o aparecimento de fenótipos deficientes na produção de LPS pode conduzir ao insucesso destes agentes a longo prazo.

Nos tratamentos combinados, os fagos utilizados juntamente com ciprofloxacina causaram a erradicação total do biofilme. Também outras combinações demonstraram resultados interessantes. Por exemplo, a combinação de ambos fago phiIBB-PAP21 e amicacina resultou numa redução de células viáveis de cerca de 3,66 log, enquanto individualmente, o fago e amicacina só provocou uma redução de 1,3log e 1,76log, respetivamente. A eficácia da combinação fago-antibiótico pode ser devido a elevados *burst size*, tal como observado com o fago phiIBB-PAP21, quando o respetivo hospedeiro foi exposto a antibióticos. Além disso, as maiores reduções da biomassa observadas quando os biofilmes são expostos a um tratamento combinado sugerem que os fagos podem melhorar a penetração de antibióticos através de rutura da matriz, permitindo uma libertação das células para a ação dos antibióticos.

Em geral, a combinação de fagos e antibióticos permite melhorar o controlo dos biofilmes; no entanto o universo complexo por trás desta interação de sinergia sugere que esta não é sempre um processo linear. Novos estudos devem ser realizados para complementar e divulgar este comportamento sinergístico observado neste trabalho.

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Abbreviations

ATCC - American Type Culture Collection

CECT - Colleción Española de Cultivos Tipo

CFU - Colony Forming Unit

DNA - Deoxyribonucleic acid

h - hours

MIC - Minimum Inhibitory Concentration

MBEC - Minimal Biofilm Eradication Concentration

min - minutes

MOI - Multiplicity of Infection

MTA - Molten Top-Agar

OD - Optical Density

OSGC - One Step Growth Curve

PEG - Polyethylene glycol

PFU - Plaque Forming Unit

RNA - Ribonucleic acid

rpm - revolutions per minute

s - seconds

TSA - Tryptic Soy Agar

TSB - Tryptic Soy Broth

Motivation and aim of the project

Pseudomonas aeruginosa is a gram-negative bacterium that can be found in two distinctive forms: planktonic form as unicellular organism; or sessile form, when attached to a substrate or a surface, leading to a biofilm formation. In the last years, *P. aeruginosa* has received a special attention because this microorganism has been described as a "phenomenon of bacterial resistance" and is responsible for a range of nosocomial infections or hospital–acquired infections. Actually, about 15% of the nosocomial infections worldwide, for example respiratory tract (in patients with cystic fibrosis), blood, urinary tract, ear, skin and soft tissue infections, are caused by this microorganism.

The prevalence of *P. aeruginosa* in the hospital environment is becoming a critical issue, and for this reason, it is important to know what are the main factors involved. The emergence of these bacteria in clinical areas is a result of many factors, such as: intrinsic resistance determined by several virulence factors, including the presence of efflux systems, restricted membrane permeability and antibiotic degrading enzymes. Also, the acquired resistance mechanisms and the ability of *P. aeruginosa* to grow in any natural and artificial surfaces, like medical devices, leading to the development of biofilms, play an important role in this context. *P aeruginosa* is known to be tolerant to a variety group of antimicrobial agents, including *b-lactams*, *aminoglycosides* and *fluoroquinolone* antibiotics. Actually, infections caused by this microorganism become even more critical since the conventional treatments with antibiotics are failing because of an increasing resistance of the bacteria to many of the agents available on the market, and its broad spectrum of virulence factors. This unpleasant reality has led to an interest in using alternative strategies to combat infections caused by *P. aeruginosa*, one of which is the use of (bacterio)phages. Although there are few studies on biofilms, phages have shown significant potential in controlling this protective life form of bacteria. In fact, the use of phages presents important advantages over the use of antibiotics, in particular in clinical areas, because they don't have lethal effects on eukaryotic cells. However, the emergence of bacterial resistance to phages is inevitable leading to a rapid conversion on resistant phenotypes.

For these reasons, the aim of the work presented in this thesis consists on developing a strategy to control biofilms formed by *P. aeruginosa* based in a combination of antibiotics and phages in the same antimicrobial solution. To accomplish this goal, the following approach was performed both for *P. aeruginosa* planktonic cultures and biofilms:

1. Evaluation of the antimicrobial susceptibility using a set of antibiotics with distinct mechanisms of action;
2. Phage efficacy studies to compare the two therapies and highlight some differences between them;
3. Finally, study the synergy efficacy using the antibiotic-phage combined treatment.

Chapter 1: Review Literature

1.1 *Pseudomonas aeruginosa* characteristics

P. aeruginosa is a gram-negative and rod-shaped bacterium belonging to the class of Gamma Proteobacteria. It measures 0.5 to 0.8 µm in width by 1.5 to 3.0 µm in length, and almost all strains possess a single polar flagellum that allows motility. In terms of metabolism, it is an obligatory aerobe, because it prefers oxygen as the terminal electron acceptor, but in certain situations it can grow under anaerobic conditions using nitrate as the terminal electron acceptor [1,2].

This bacterium is ubiquitous in soil and water and can be found in two distinctive forms: planktonic form, as a unicellular organism; and biofilm, when attached to abiotic surfaces or substrates. In terms of nutrition, *P. aeruginosa* has very simple requirements, and for this reason, growth factors and other complements are not necessary for its growth. The optimal temperature for growth is 37 degrees at a neutral pH. Nevertheless, it should be noted that *P. aeruginosa* is tolerant and resistant to a wide variety of physical conditions, like temperature, high concentrations of salts, disinfectants and antibiotics [1–3].

1.2 *Pseudomonas aeruginosa* virulence factors

Additional to the highest versatility in terms of nutrition and metabolism, *P. aeruginosa* is an opportunistic pathogen in humans and animals, displaying a variety of virulence mechanisms, derived from its intrinsic resistance which allow the establishment of the infection. These factors can be group in three main stages: adhesion, colonization and dissemination [3–5].

For the development of these processes, the role of certain components is crucial. The most important one's refer to enzymes such as *proteases*, *elastases*, and *phenazine* pigments such as *pyocyanin* which interferes with the tissue damage and destruction of certain immune cells [1,6]. Also, the motility, the presence of pili, flagella,

lipopolysaccharides (LPS) and extracellular polymeric substances (EPS) constitute virulence mechanisms. For example, the LPS molecules (Figure 1.1) that are present on the outer membrane of this Gram-negative bacterium contribute to their pathogenicity, because they allow bacterial adhesion mediating the entry of bacteria into eukaryotic cells. In terms of structure and composition, the LPS of *P. aeruginosa* is very specific being composed of a lipid A, a core oligosaccharide divided in outer and inner cores and O-antigens that involve the A and B bands [7,8–11]. Additionally, *P. aeruginosa* possesses several different export systems that are involved in the secretion of virulence factors, such as the injection of effector proteins directly into the cytoplasm of host cells that will interfere with the functioning of macrophages and neutrophils. Another factor that could be indirectly related to this virulence is the large and complexity genome of *P. aeruginosa*, which allows this microorganism to adapt and survive to different ecological niches. This could happen either through mutation of an existing gene product, or through the acquisition of a drug resistance plasmid [7,12].

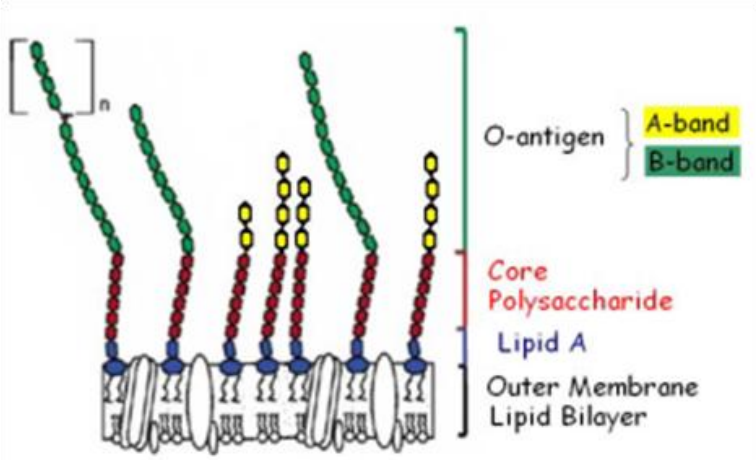

Figure 1.1: LPS structure of *P. aeruginosa*. Adapted from Atabek et al [7].

1.3 Clinical impact of *Pseudomonas aeruginosa*

Biofilms are sessile microbial communities attached to a solid surface, in which the organisms produce a hydrated matrix of extracellular polymeric substances (EPS)

consisting of polysaccharides, proteins, nucleic acids, and lipids [13]. Biofilms constitute an essential and protective lifestyle for bacteria in many different hostile and man-made environments, including dental plaques, water pipes, medical devices, and industrial systems [14,15]. When the biofilm formation occur on indwelling medical devices (Figure 1.2), this can lead to infection processes by detachment and dispersion of individual cells or aggregates of cells, resulting in serious damages on human tissues and organs. Additionally, the populations of bacteria within biofilms exhibit differences in the expression of surface molecules, antibiotic resistance, nutrient condition and virulence factors, and for these reasons, antimicrobial treatment that is sufficient to inactivate planktonic organisms is generally inadequate to inactivate biofilm organisms [13,15–18].

In the hospital and community environments, the development of antimicrobial resistance plays a very important role in infections caused by *P. aeruginosa* biofilms. This bacterium is ubiquitous in clinical settings, it can be isolated from respiratory therapy equipment (endotracheal tubes used in mechanical ventilation), sinks and physiotherapy and even hydrotherapy pools. According to surveillance data collected by the European Centre for Disease prevention and Control (ECDC) organization, *P. aeruginosa* is considered the second most frequently isolated organism in cases of intensive care unit nosocomial infections. Additionally, according to ECDC, this bacterium was responsible for 30% of pneumonias and chronic lung infection in cystic fibrosis patients, 19% of urinary tract infections, and 10% of bloodstream infections, mainly in severe burn victims. In particular, in cases of nosocomial pneumonia there is a high rate of mortality and the treatment is difficult due the long-term of antibiotic treatment [5,19–21].

Outside of the hospital environment, this microorganism can be found in swimming pools, whirlpools, hot tubs, contact lens solutions, home humidifiers, soil and vegetables [5,12,19,20].

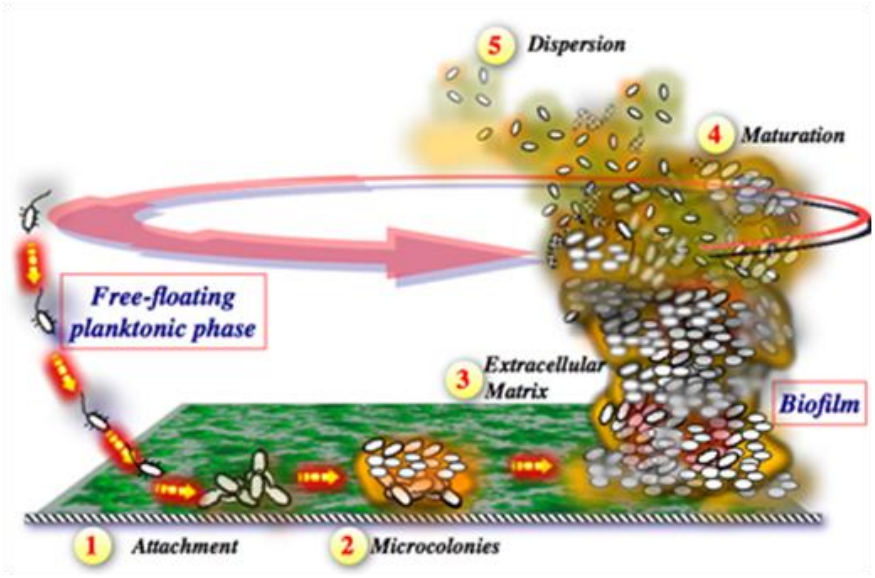

Figure 1.2: Schematic representation of stages of bacterial biofilm development. At stage 1, the bacterial cells attach reversibly to the surface, where weak forces prevalence. Next, at stage 2, the cells attach irreversibly, due mainly by exopolymeric substances, and as a consequence the cells lose their flagella-driven motility. At the next stages 3 and 4 the maturation of the biofilm occur, with the increment of biofilm architecture with formation of extracelular matrix. At stage 5 the dispersion of biofilm cells happen where single motile cells diffuse from the microcolonies. Adapted from ***http://www.pasteur.fr/recherche/RAR/RAR2006/Ggb-en.html.***

1.1.1 Antimicrobial resistance of *Pseudomonas aeruginosa*

Biofilm formation can be considered as a potential factor of antimicrobial resistance of *P. aeruginosa*, and there are important aspects that contribute for this situation. For instance, the mucoid exopolysaccharide matrix, that surrounds the biofilm cells, can act as an effective barrier that limits penetration of antimicrobial compounds [1,19,22]. However, there are differences between certain classes of antibiotics in terms of biofilm penetration, because antibiotics like **fluoroquinolones** (such as, ciprofloxacin) have better penetration into biofilms, in opposite to aminoglycosides, which penetrate more difficultly [19,20]. This can be related to the differences observed in delivery and transport of these molecules through the outer membrane of cells and since fluoroquinolones are small hydrophilic molecules, they pass easily through the aqueous channels on the membrane provided by porin proteins. On the other hand, the aminoglycosides (cationic antibiotics) promote their own uptake by binding to the negatively charged LPS on the outer surface of the membrane [23].

Furthermore, the biofilm population of *P. aeruginosa* is heterogeneous, which means that there are fast- and slow-growing cells. This happens because of gradients of nutrients and oxygen availability into the biofilm structure [19,23]. The divergence of the cell population will create a fraction that is resistant to a range of mechanisms leading to a decrease in antimicrobial susceptibility of *P. aeruginosa* biofilms. This resistant subpopulation, also called persister cells, is a major source of reoccurring infections (Figure 1.3) [17,24]. Also, the architecture and complexity of biofilms provides an appropriately environment for gene transfer and cell-to-cell signaling. This mechanism is called *quorum sensing*, and has been shown to play an important role in the expression of virulence factors, biofilm differentiation, cell attachment and detachment, through the activation of certain genes [7,23,25].

In addition to biofilm formation, there are other factors which increase the resistance of *P. aeruginosa* to multiple antimicrobial agents. For example, the efflux pumps are responsible for extruding drugs, like antibiotic molecules, toxic metal ions, organic solvents and other ligands from inside the cell. In *P. aeruginosa* there are some important efflux pumps, such as the system MexAB-oprM that is responsible for extrusion of *b-lactams*, *quinolones* and a range of disinfectants; MexXY-oprM that extrudes *aminoglycosides* and MexEF-oprN that is responsible to extrudes *carbapenems* and *quinolones* [19,23].

Other important mechanism of resistance of this bacterium is the modification of the primary target for antibiotics. In fluoroquinolones, one possible situation is the mutation in the gyrA gene that encodes the A subunit of the target enzyme, DNA gyrase, of this class of antibiotics [23,25].

In terms of antibiotic uptake, the intrinsic resistance of *P. aeruginosa* plays also a central role in the rejection of some of these molecules. The own bacterial outer membrane, that possesses water filled channels, designated the transmembrane porins, constitutes a semi-permeable barrier to the uptake of antibiotics into the cell. For example, the OprF porin has a large exclusion limit and only allows small portions of *Beta-lactams* molecules moving inside the cells [8,9,23]. Other examples include an expression of inactivating enzymes, like β-lactamases that will act on *β-lactam* antibiotics (for example piperacillin), degrading the β-lactamic ring present in these antibiotics and leading in this way to their inactivation [1,19,23].

On the other hand, LPS, as described above, can interfere with antimicrobial resistance of *P. aeruginosa,* because when bacteria present a mutation in this structure,

(absence of O-specific chain for example) this can affect the antibiotic uptake, in terms of acceptance or rejection. In addition, there are some studies that reveal that the oligosaccharide portion of LPS structure may play an important position in the gating mechanism of porin proteins, such as OprF [7–9,26].

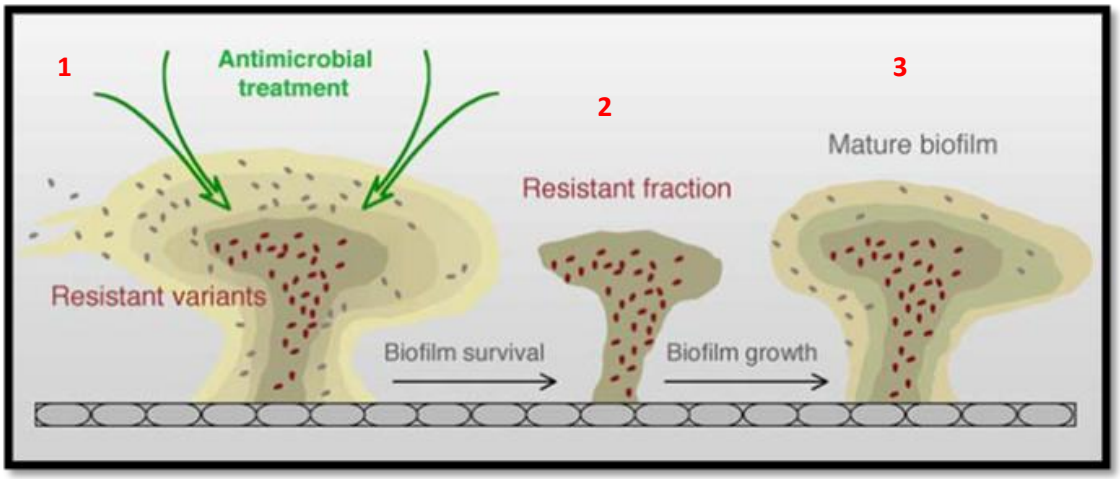

Figure 1.3: Resistance mechanism mediated by phenotypic/persister variants in the biofilm. In the first part, antimicrobial treatment of bacterial biofilms leads to the eradication of part of the biofilm susceptible population (1). A small portion of phenotypic/a persister variant (represented as maroon bacteria) survive to the treatment (2) and are able to establish biofilm development after antimicrobial therapy is suspended (3). Adapted from Drenkard et al [19].

1.4 Control strategies for *Pseudomonas aeruginosa* biofilms

This section will briefly review two strategies that can be used to control *P. aeruginosa* biofilms: antibiotics and bacteriophages.

1.4.1 Antibiotics

Antibiotics are chemical, natural or even synthetic substances that have been used for the last 70 years to treat patients who have infectious diseases, by preventing the growth of bacteria or their destruction. One important concept is that antibiotics are not active against viruses [27,28]. Since the 1940s, the use of antimicrobial agents has been beneficial, because when prescribed and taken correctly they significantly reduce illness and death. However, often, these drugs have been used inappropriately and

therefore, the antibiotics that are designed to kill infectious organisms become less effective causing emergence and selection of resistant and multiresistant bacteria [27,29,30]. Antimicrobial resistance is nowadays one of the major threats to public health, mainly in health care settings. Every year, in the European Union, it is estimated that 25.000 patients die because of serious resistant bacterial infections acquired in hospitals. Additionally, antibiotic resistance causes a direct impact to hospital and to the patient's family: longer hospital stays increase of treatment costs; and frequently more expensive antibiotic drugs are used in treatments. There are also indirect factors that significantly affect the respective families and the society, such as: greater absenteeism at work, lower economic yield, decrease productivity by sequels and psychological and emotional changes [27,29,30].

1.4.1.1 Categories of Antibiotics and mode of action

Antibiotics can be categorised according to their action spectrum and mode of action. Regarding the first criteria, there are two types of antibiotics: narrow spectrum antibiotics and broad spectrum antibiotics. The first ones are specific for a specific bacterium and only prescribed when it is known which bacterium is causing the infection, and the second type refers to antibiotics that are used in case it is unclear which bacterium or bacteria is/are causing the infection. Contrarily to the first type, this group of antibiotics also kill harmless bacteria due to their non-specificity [31].

In terms of mode of action, antibiotics can be classified as bactericidal if they kill the susceptible bacteria; or bacteriostatic if they reversibly inhibit the growth of bacteria. In spite of bactericidal antibiotics being preferred, this does not mean that they are more effective than bacteriostatic [32]. In this context, antibiotics can be classified in five major groups (Table 1.1), according to the physiological and metabolic functions in bacterial cell [33,34] .

Table 1.1: Antibiotic families and mechanisms of action. Adapted from Levy et al [34]

Mechanism of action	Antibiotic families
Inhibition of cell wall synthesis	Penicillins; cephalosporins; carbapenems; daptomycin; monobactams; glycopeptides
Inhibition of protein synthesis	Tetracyclines; aminoglycosides; oxazolidonones; streptogramins; ketolids; macrolides; lincosamides
Inhibition of DNA synthesis	Fluoroquinolones
Competitive inhibition of folic acid synthesis	Sulfonamides; trimethoprim
Inhibition of RNA synthesis	Rifampin
Other	Metronizadole

1.4.2 Bacteriophages

Bacteriophages, also called phages, are viruses that kill bacteria and like other viruses they are obligate parasites, so they use the bacteria host to multiply and spread. Phages are ten times more numerous in the environment than bacteria, making them the most abundant 'life' forms on earth, with an estimated 10^{32} on the planet [35–37]. Bacteriophages cannot infect mammalian cells, but only specifically target bacteria.

There are a variety of families of bacteriophage and according to the International Committee on Taxonomy of Viruses (ICTV) they are classified in terms of morphology and nucleic acid type (Table 1.2). A significant percentage of bacteriophages, about 96%, are distributed over three families that belong to the *Caudovirales* order: *Myoviridae*, *Podoviridae* and *Siphoviridae* (Figure 1.4) [1,36,38].

Table 1.2: Bacteriophages families. Adapted from Hanlon [36].

Family	Morphology	Genome
Corticoviridae	Icosahedral capsid with lipid layer	dsDNA
Cystoviridae	Enveloped, icosahedral capsids, lipids	dsRNA
Fuselloviridae	Pleomorphic, envelope, lipids, no capsids	dsDNA
Inoviridae	Rod-shaped with helical symmetry	ssDNA
Leviviridae	Quasi-icosahedral capsids	ssRNA
Lipothrixviridae	Enveloped filaments, lipids	dsDNA
Microviridae	Icosahedral capsids	ssDNA
Myoviridae	Non-enveloped , Contractile tail	dsDNA
Plasmaviridae	Pleomorphic, envelope, lipids, no capsids	dsDNA
Podoviridae	Non-enveloped , short non contractile tail	dsDNA
Rudiviridae	Non enveloped, helical rods	dsDNA
Siphoviridae	Non-enveloped , long non contractile tail	dsDNA
Tectiviridae	Icosahedral capsid with inner lipoprotein vesicle	dsDNA

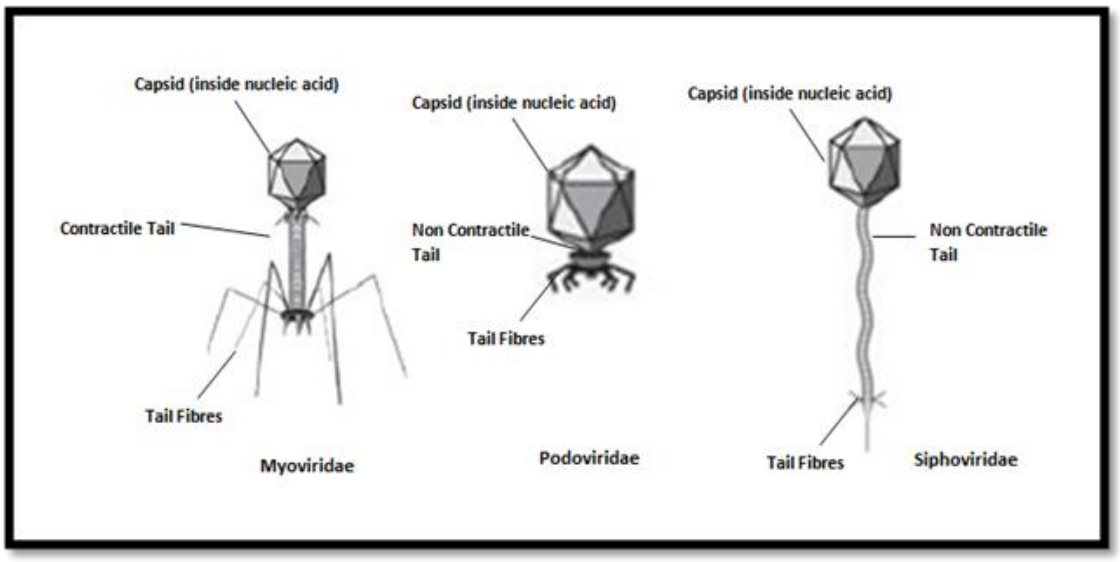

Figure 1.4: Diagrammatic representation of a typical bacteriophage structure. Adapted from Harper et al [37].

Bacteriophages can exhibit one of two types of life cycle: virulent (lytic phages) or temperate (lysogenic phages). Virulent phages, the only ones allowed to be used in phage therapy, cause a rapid lysis and death of the host bacterial cell leading lead to a release of a hundreds of viral particles (Figure 1.5). The infection process starts from recognition and binding of the phage to the host by a specific receptor. This binding constitutes a critical stage, and when phage are irreversible connected to the host, the injection of genetic material of the phage occurs. Then, through the metabolism of the host, the DNA replication of the phage takes place, culminating in the formation of new phage particles. Subsequently, there is the packaging of the genome and recovery of phage particles. In the case of dsDNA phages, after the assembly, the phages present into the cytoplasm of bacterial host can produce enzymes that attack the bacterial peptidoglycan, leading to the instability of cell wall. These enzymes are designed lytic enzymes or endolysins and they have a therapeutic activity, mainly against gram positive bacteria. All of this process ends in lysis of the host bacteria and release of new phages that are able to infect other bacteria [36,39] .

On the other hand, temperate phages spend part of their life cycle in a quiescent state called prophage, where their DNA is integrated into the host chromosome. This latent stage can be activated by specific stimuli, and then the phage can initiate a lytic mode infection [36,37]. Phages with a lysogenic phage cycle can transfer fragments of host bacterial DNA, such as toxin-encoding or antibiotic resistance-mediating genes into other bacterial species by a mechanism of transduction producing new virulent strains [35,36] and therefore are not to be used therapeutically.

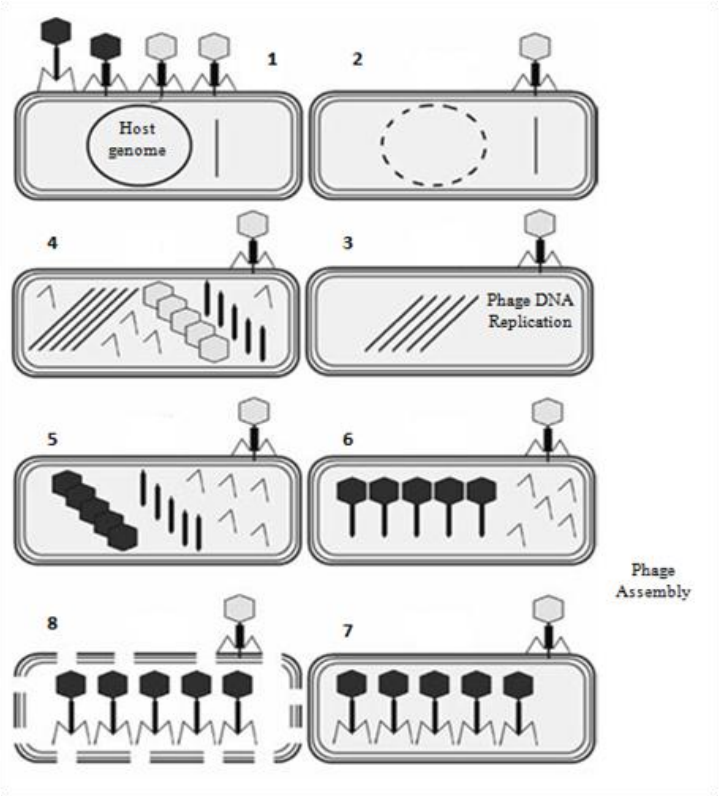

Figure 1.5: The life cycle of lytic bacteriophages: 1 – phage adsorption and DNA injection; 2 – host genome degradation; 3 - phage DNA replication; 4 – appearance of morphogenesis intermediates, including empty heads (proheads); 5 – packaging of phage DNA into capsids; 6 – phage assembly; 7 – lysis and release of progeny phage. Adapted from Kropinski **[**[39]**].**

In addition, there are filamentous bacteriophages that have a different life cycle in which the infection is persistent, that is, there is no death of the host bacteria, but there is a continued production of viral particles [3,36,37].

In a general context, phage therapy could be a potential approach in three different applications: 1) using phages as direct antibacterial agents, which, biotechnologically, could be fairly rapidly adapted for clinical applications; 2) isolating phage-encoded lytic enzymes that can be used as antibacterial agents alone or incorporated in the phage genome to be expressed during production of new phages by replication in their host cells. One example described in literature is the incorporation of Dispersin B (dspB) in phage T7 [14] . Furthermore, phage polysaccharide depolymerases, specific enzymes found in the tail spikes of the phage baseplate, have been isolated and used to degrade the polysaccharide matrix, helping in this way phages in getting access to the biofilm cells [14,40]. Other alternative, the most long-term application, but still very

promising is based in the study of determinants of phage lytic mechanisms to identify novel drug targets [41,42].

Currently, applications of phage therapy cover two main areas: clinical targeting their use to treat wounds, burns, chronic ulcers and respiratory tract infections, and food industry [43–45].

1.4.3 Antibiotics vs Bacteriophages

As any antimicrobial agents, antibiotics and bacteriophages have similarities, but also significant differences. First, phages only affect the target bacteria, while antibiotics act both on microorganisms and on the normal microflora of the patients and subsequently, at high concentrations, they may raise the risk of side effects. Phages have also a self-reproducing capability as long as their respective host is present, in opposite to antibiotics which require an administration of several doses since they are metabolized and eliminated from the body.

Many authors have emphasized phage therapy as an alternative or adjuvant approach to antibiotics mainly due to the fact that their isolation, selection and production is relatively rapid contrarily to the time-consuming development of new antibiotics which involve several critical steps. However, this doesn't necessarily mean that it is simple to isolate a highly virulent, lytic, broad-spectrum and non-transducing phage appropriate for effective therapy [36,45–47].

A critical point that is inherent to both therapeutics is the appearance of bacterial resistance described already in section 1.3.1 for antibiotics. The development of bacterial resistance to phages happens due to a diversity of factors, such as: modification by mutation of the structure or exposure of host receptor molecules where phages adsorb; degradation of phage genome by DNAses after injection of the phage DNA into bacteria; inactivation of phages by the action of proteolytic enzymes present in biofilm matrix; entrapment of released phage particles in biofilm matrix [16,43,47,48].

The combination of phages and antibiotics is of great interest and, a few studies have shown the potential of this combined treatment for the control of bacterial biofilms [49–52]. One the few studies reported, describes that a phage-antibiotic combination resulted in a weaker biofilm matrix, mainly through the ability of phages to create pores

and channels to enter into the biofilms leading to a dispersion of a great part of biofilm cells which becoming more available to the action of the antimicrobial agent [50].

On the other hand, it has also been described that combined treatments can decrease the mutation rate of the bacterial population, because a cell population hardly has, in the same genome, resistance mechanisms to survive both phage and antibiotic attack [49]. Nevertheless, it can be considered that the mechanisms behind phage-antibiotic synergistic action are still poorly explored.

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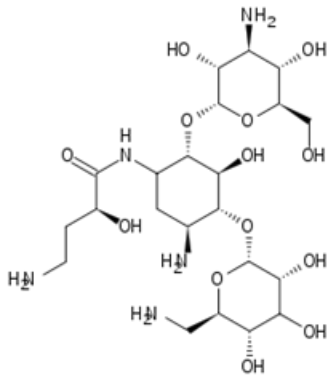
Chapter 2: Antimicrobial Susceptibility of *P. aeruginosa*

2.1 Background

The selection of an antimicrobial agent and its dosage used in the treatment of *P. aeruginosa* is very important to prevent the growth of resistant bacteria, and this choice is crucial for the efficacy of process. Certain classes of antipseudomonal drugs that are frequently administered [1] and their mechanisms of action are described below:

Amikacin

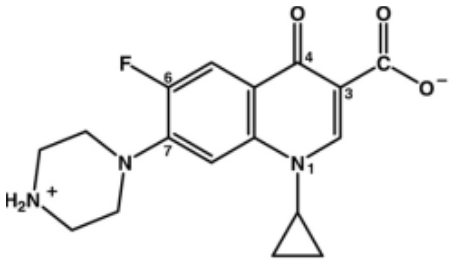
Amikacin is an antibiotic of aminoglycosides family. In general, this antibiotic is administered to treat infections caused by gram-negative bacteria, such as *Pseudomonas, Acinetobacter,* and *Enterobacter*. In case of *P. aeruginosa* infections, due to its cationic nature, this antibiotic has the capacity to bind to LPS molecules (negative charged) and other anionic molecules such as DNA, RNA and phospholipids to access the interior of the cells. Inside the cells, amikacin acts on the 30S ribosomal subunit, preventing, in this way, the formation of an initiation complex with messenger RNA. Thus, the bacterium becomes unable to synthesize proteins that are vital to its growth. Also, amikacin can interfere with the cell membrane integrity [2–4].



Amikacin

Ciprofloxacin

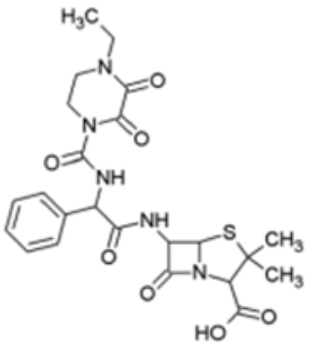
Ciprofloxacin is an antibacterial agent of the fluoroquinolones class. This antibiotic has a broad spectrum activity, contrarily to amikacin, because it can act on gram-negative and gram-positive microorganisms. The main consequence of the bactericidal action of ciprofloxacin is the inhibition of the enzymes topoisomerase II (also called *DNA gyrase*) and topoisomerase IV. These enzymes are necessary for DNA replication, transcription, repair and recombination to bacterial cell. In *P. aeruginosa*, ciprofloxacin enters into the cell using porin protein OprF, and the positively charged piperazine ring at the C-7 position of the quinolone allows this molecule to interact with negatively charged phosphate groups of the phospholipid bilayer [2,5,6].



Ciprofloxacin

Piperacillin

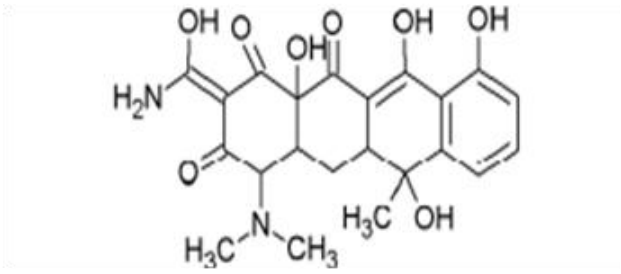
Piperacillin is a *penicillin* beta-lactam antibiotic. The antibacterial action of this antibiotic result from the binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall. Subsequently, piperacillin inhibits the last point of bacterial cell wall synthesis. Alike ciprofloxacin, piperacillin has activity on both gram-positive and gram-negative bacteria. However, it is mainly indicated to treat pseudomonal infections. In these bacteria, the diffusion of this anionic antibiotic is achieved by OprF protein in the outer membrane of the cell [2,7].



Piperacillin

Tetracycline

This antibiotic has a bacteriostatic action against bacteria. Tetracycline belongs to tetracyclines family, and has a short action on the bacterial cell. In gram-negative bacteria, its mechanism of action allows the inhibition of bacterial growth, because when it diffuses through the porin channels in the bacterial outer membrane as a positively charged molecule, it reversibly binds to the 30S ribosomal subunit and prevents the binding of tRNA to the mRNA-ribosome complex. As a consequence, the protein synthesis is affected [2,8].



Tetracycline

The entry pathway (porin pathway or self-promoted pathway), the molecular characteristics of each antibiotic, and the diversity of intrinsic resistance factors of *P. aeruginosa* may dictate some important differences between antibiotics molecules, before the culmination of their action inside the cells. Figure 2.1, describes

schematically a process for the entry of antibiotics in *P. aeruginosa* cells in order to realize the major differences in the mechanism of reception of these molecules.

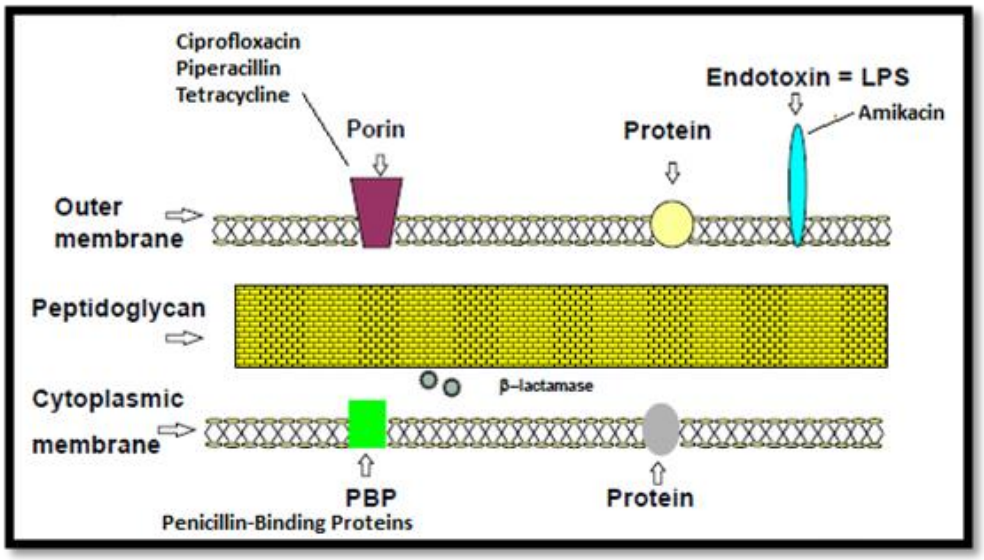


Figure 2.1: Schematic representation of the arrangement of components in the cell wall of *P. aeruginosa* and access of antibiotics. Adapted from [4]

There is a diversity of laboratory methods that can be applied *in vitro* to determine the susceptibility of bacteria to antimicrobial agents: broth microdilution test; disk diffusion test; antimicrobial gradient method (also called E-test) and automated instruments systems. In general, the majority of the methods available provide quantitative results. The Minimum Inhibitory Concentration (MIC), is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after an appropriate period of incubation [9].

This chapter focuses on the main results obtained with the antimicrobial susceptibility tests against planktonic cultures (section 2.3) and biofilms (section 2.4).

2.2 Materials and methods

2.2.1 Bacteria and growth conditions

The bacterial strains that were used in this work were: PA01, CECT 111 and ATCC 10145. All strains were grown in Tryptic Soy Broth (TSB) medium. The solid medium was Tryptic Soy Agar (TSA): TSB + 1.2% w/v of agar. Both media were prepared according to the manufacturer's instructions. Then, the media were sterilized by autoclaving at 121 °C for 15 minutes.

2.2.2 Preparation of Antibiotics

The standard powders of antibiotics were obtained from Sigma - Aldrich. Stock solutions were prepared and diluted according manufacturer's recommendations and stored at -20 °C after filtration. During manipulation of antibiotics, these were always protected from light.

2.2.3 Determination of Antimicrobial susceptibility

2.2.3.1 MIC determination

To determine the MIC of the three *P. aeruginosa* strains (strains ATCC 10145, PAO1 and CECT 111) against the four antibiotics (amikacin, ciprofloxacin, piperacillin and tetracycline) the micro-broth dilution method was used. The experiment was performed according to the Clinical and Laboratory Standards Institute guidelines [10,11].

Briefly, for each strain of *P. aeruginosa* used, two colonies were transferred from an overnight grown plate (from the first sub-culture, a second sub-culture on an appropriate agar plate was made) into 1 ml of TSB to approximate the density to 0.5 of the McFarland standard. This suspension, with an inoculum concentration of 10^8 colony forming units per ml (CFU/ml) was then diluted to 10^6 CFU/ml with the TSB medium. Serial two-fold dilutions of all the antimicrobial agents with the following concentrations (mg/l): ciprofloxacin (0.03125 - 1), amikacin (0.5 - 256), piperacillin (0.5 - 256), tetracycline (0.5 -256) were prepared with NaCl (0.9%) and then 100 µl of

each concentration were placed in 96-well microtiter plates. After, 100 µl of the suspension were added to the respective wells. TSB (100 µl) and 100 µl of 0.9% NaCl were used for the control experiments. Finally, the lowest concentration inhibiting visible growth after 20-24 h at 37 °C and 120 rpm was recorded as the MIC and the density (600 nm) was also measured. For these experiments three independent assays (each one in duplicate) were performed.

2.2.3.2 MBEC determination

Minimal Biofilm Eradication Concentration (MBEC) is defined as the lowest concentration of an antimicrobial agent that will inhibit the growth of a biofilm. Briefly, for each strain of *P. aeruginosa*, two colonies were transferred from an overnight grown plate (a second sub-culture on an appropriate agar plate was made from a first sub-culture) into 1 ml of TSB to obtain a density of 1.0 in the McFarland standard (approx. 3×10^8 CFU/ml) and then diluted to obtain an inoculum of 10^7 CFU/ml in TSB medium. After, 200 µl of *P. aeruginosa* cultures grown were added to 96-well microplates and incubated overnight at 37 °C and 120 rpm. After 24 h of biofilm formation, all medium was removed and the wells were washed with fresh TSB medium. Following that, 100 µl of fresh TSB and 100 µl of antimicrobial solutions with different concentrations (256-64 mg/l) were added to the wells and control experiments were performed with 100 µl of TSB and 100 µl of NaCl. The duration of MBEC assays was 24 h and after the microplates were washed twice with saline solution (0.9% NaCl) to remove all unattached bacteria. Fresh saline solution (200 µl) was added to each well, the biofilm scraped and the microplates were put in a water bath sonicator for 30 min. The number of viable cells present in biofilms before and after the treatment was determined by colony-forming unit (CFU) counts using the microdrop technique. For these experiments three independent assays (each one in duplicate) were performed.

2.2.4 Biomass Quantification

To quantify the total biomass attached to each well of 96-well microtiter plate, the crystal violet assay was used. This procedure consists of washing twice the biofilms

with a saline solution (0.9% NaCl), fixing with 200 µl of methanol (100%) (Merck) for 15 min and after this period, the methanol was removed and the microplate allowed to dry at room temperature. Following that, 200 µl of crystal violet (1% v/v, Merck) is added to each well and the plate is let to stand still for 5 min. After, the wells were washed with water and allowed to dry at room temperature. Finally, 200 µl of acetic acid (33% v/v, Merck) was added to dissolve the stain attached to the biofilm, and the absorbance was read at 570 nm. For these experiments three independent assays (each one in duplicate) were performed.

2.2.5 Microscopy analysis after BacLight™ staining

P. aeruginosa cells were stained LIVE/DEAD® BacLight™ (Invitrogen Bacterial Viability Kit) [12] to access their viability according to the manufacturers' specification. Briefly, an overnight culture was adjusted to an OD600 of 0.4 – 0.5, and after that the cultures were added to a microplate and incubated for 2 h at 37 °C and 120 rpm. After this incubation period the antibiotic solutions were added and allowed to act during 2 h at the same conditions as described above. The adhered cells were scrapped from the wells and put 20 µl in a microscope slide with 5 µl of LIVE/DEAD® BacLight™ stain. The solution was mixed thoroughly and incubated at room temperature in the dark for 15 min. The fluorescence from both live and dead bacteria were observed simultaneously using a fluorescence microscope with a longpass 485 nm filter.

2.2.6 Adhesion assays - Flow cell

The cell adhesion assays were carried out using a laminar flow cell apparatus mounted on an inverted optical microscope (Nikon, Diaphot 300) coupled with a digital camera (CCD camera - Sony, AVC-D5CE) to capture images over time (Figure 2.2) [13]. Before each assay, the entire system was washed with sterile water for 30 min and without recirculation. Then, in the same condition, TSB medium was passed through the system.

Cultures of *P. aeruginosa* were grown overnight at 37 ° C, centrifuged (7,000 ×g, 10 min, 4 ° C) and the pellet resuspended in TSB to an OD of about 0.5. After this, the suspension of *P. aeruginosa* was placed in the balloon of the system. During the feeding of the flow cell with bacterial cells, the system operated with recirculation of the suspension. When the whole surface was coated with cells, the feeding of cells was stopped and the washing with TSB medium was started without recirculation, to remove non adhered cells. After 30 min of washing, the antimicrobial agent was placed on the other balloon of the system, and the feeding started again. At the end of the tests, the entire system was washed. It is important to refer that the flow used was 0.124 ml/min.

Subsequently, for image processing, a treatment using the software developed by Sigma (Sigma Scan Pro 5) was applied.

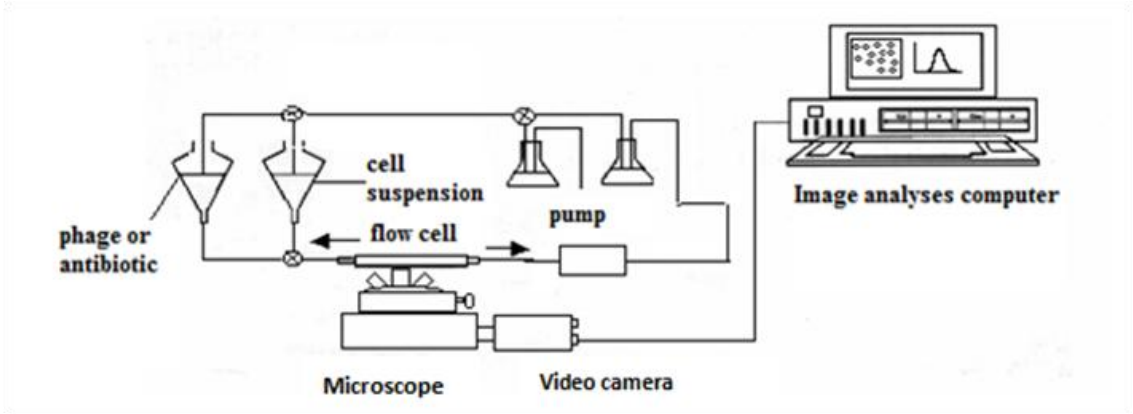


Figure 2.2: Assembly used in adhesion assays. Adapted from Azeredo et al [13].

2.3 Results and discussion

2.3.1 Determination of the minimum inhibitory concentration (MIC)

The micro-broth dilution method was used to determine the susceptibility of *P. aeruginosa* cells to specific antibiotics. The choice of this method is based in two main characteristics: it is a practical and reproducible method [9]. Table 2.1 presents the MIC values obtained for each antibiotic tested and for each of the *P. aeruginosa* strains investigated.

Table 2.1: Values of Minimum Inhibitory Concentration (MIC) of antibiotics for the three strains of *P. aeruginosa* tested

Antibiotic/strain	MIC Range tested (mg per l)	CECT 111	PA01	ATCC 10145
Amikacin	0.5-256	4	4	4
Ciprofloxacin	0.03125-1	0.125	0.125	0.125
Piperacillin	0.5-256	8	16	8
Tetracycline	0.5-256	8	8	16

According to the results, it was possible to determine a MIC value to all antibiotics tested against the different bacterial strains studied. The results were very similar among the strains tested and showed that ciprofloxacin was the most effective antibiotic in eradicating bacteria in suspension, even at low concentrations. On the other hand, the strains were more resistant to tetracycline and piperacillin. In the case of tetracycline, its bacteriostatic action could explain the higher MIC values, because its binding to the ribosomal complex is only reversible, which makes its action very short [8]. In the case of piperacillin, the higher MIC values can be due to the presence of inactivating enzymes, common in *P. aeruginosa*, such as beta-lactamases which disrupt the amide bond of the b-lactam ring present in piperacillin [14].

Overall, it is important to note that the results are consistent with clinical breakpoints defined by EUCAST for *P. aeruginosa* (Appendix 1 Figures A.1 and A.2) and all strains are present in the respective range of susceptibility described in literature [10,15–18].

2.3.2 Determination of the minimum biofilm eradication concentration (MBEC)

The ability of antibiotics to disrupt the formation of biofilms (formed in 96-well microplates for 24 h) of the three strains of *P. aeruginosa* was explored (Figure 2.3).

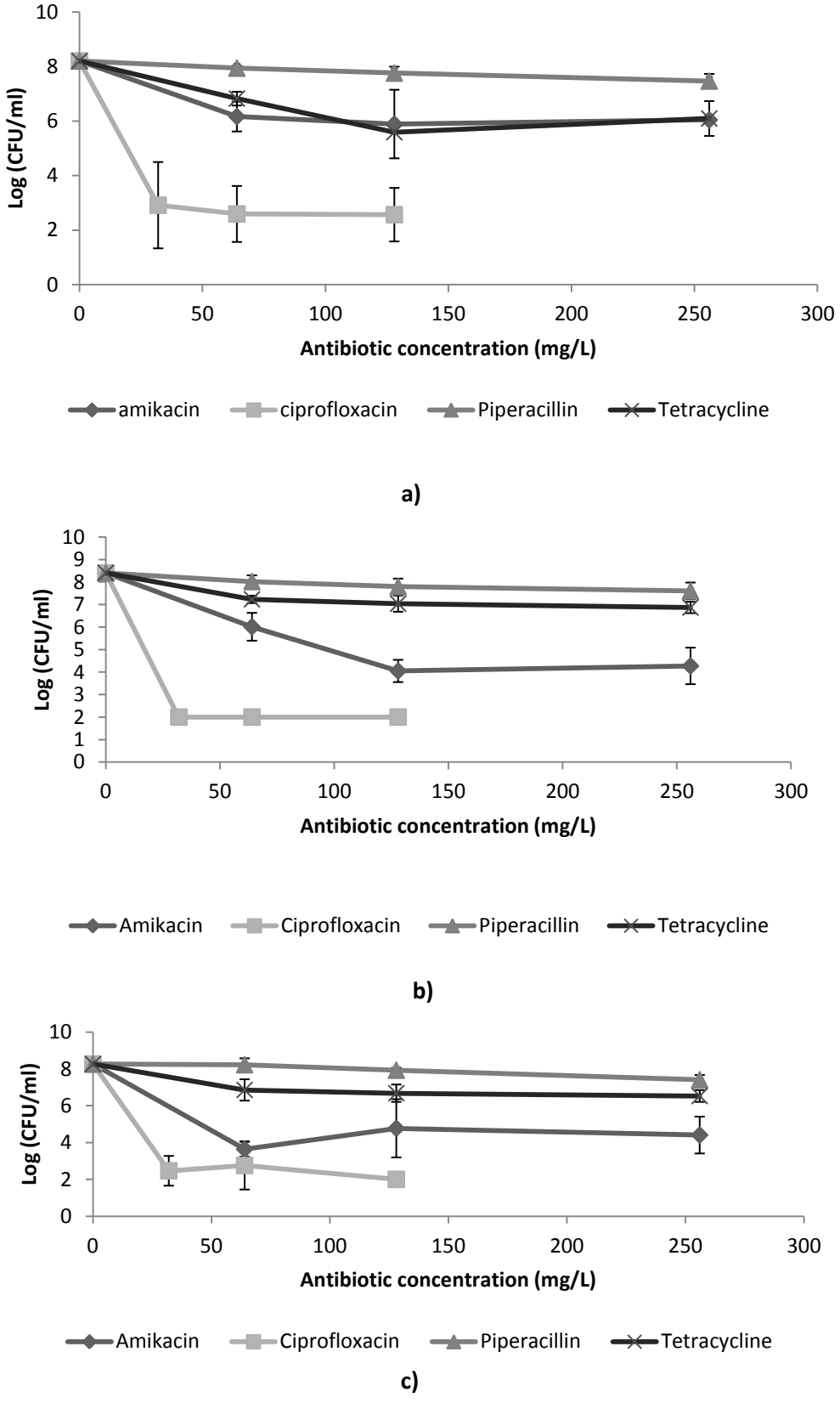


Figure 2.3: Minimum Biofilm Eradication Concentration of antibiotics against *P. aeruginosa* strains: a) PA01; b) CECT 111; c) ATCC 10145.

Table 2.2: Values of Minimum Biofilm Eradication Concentration (MBEC) of antibiotics for the three strains of *P. aeruginosa* tested

Antibiotic/strain	MBEC Range tested (mg per l)	CECT 111	PA01	ATCC 10145
Amikacin	64-256	> 300	> 300	> 300
Ciprofloxacin	32-128	>150	>150	>150
Piperacillin	64-256	> 300	> 300	> 300
Tetracycline	64-256	> 300	> 300	> 300

The results confirm that biofilms can be up to 100 times more resistant to antibiotics than planktonic bacteria (compare Table 2.1, Table 2.2 and Figure 2.3). Furthermore, the results show that there are no significant differences between the susceptibility of the biofilms formed by the three strains to the tested antibiotics and that none allowed a total eradication of the established biofilms. Nevertheless, ciprofloxacin seems to be the most efficient antibiotic, reducing by approximately 6 logs the number of viable cells present in biofilms. These results are in agreement with those obtained by Abdi Ali and colleagues (2006) whom observed that the permeation of ciprofloxacin through alginate (component present in biofilm matrix of *P. aeruginosa*) was higher and had higher bactericidal activity in relation to aminoglycosides and beta-lactams antibiotics against *P. aeruginosa* biofilms [19]. Furthermore, it is known that ciprofloxacin kills both rapidly dividing and slow-growing cells [20,21].

Nevertheless, it is important to refer that even increasing ciprofloxacin concentration, there is always a 2 log of viable cells that remain on the biofilms, which probably correspond to a portion of persister cells that are tolerant to antibiotic action [20].

In spite of significant structural differences between planktonic cells and biofilms, the behavior and efficiency of antibiotics was very similar, and again piperacillin was the less effective, with negligible effect on biofilms. Piperacillin is described to be only active on dividing cells, leaving intact all cells at the stationary phase of growth [21]. To confirm, *P. aeruginosa* biofilms were stained with LIVE/DEAD® Baclight™ (Figure 2.4).

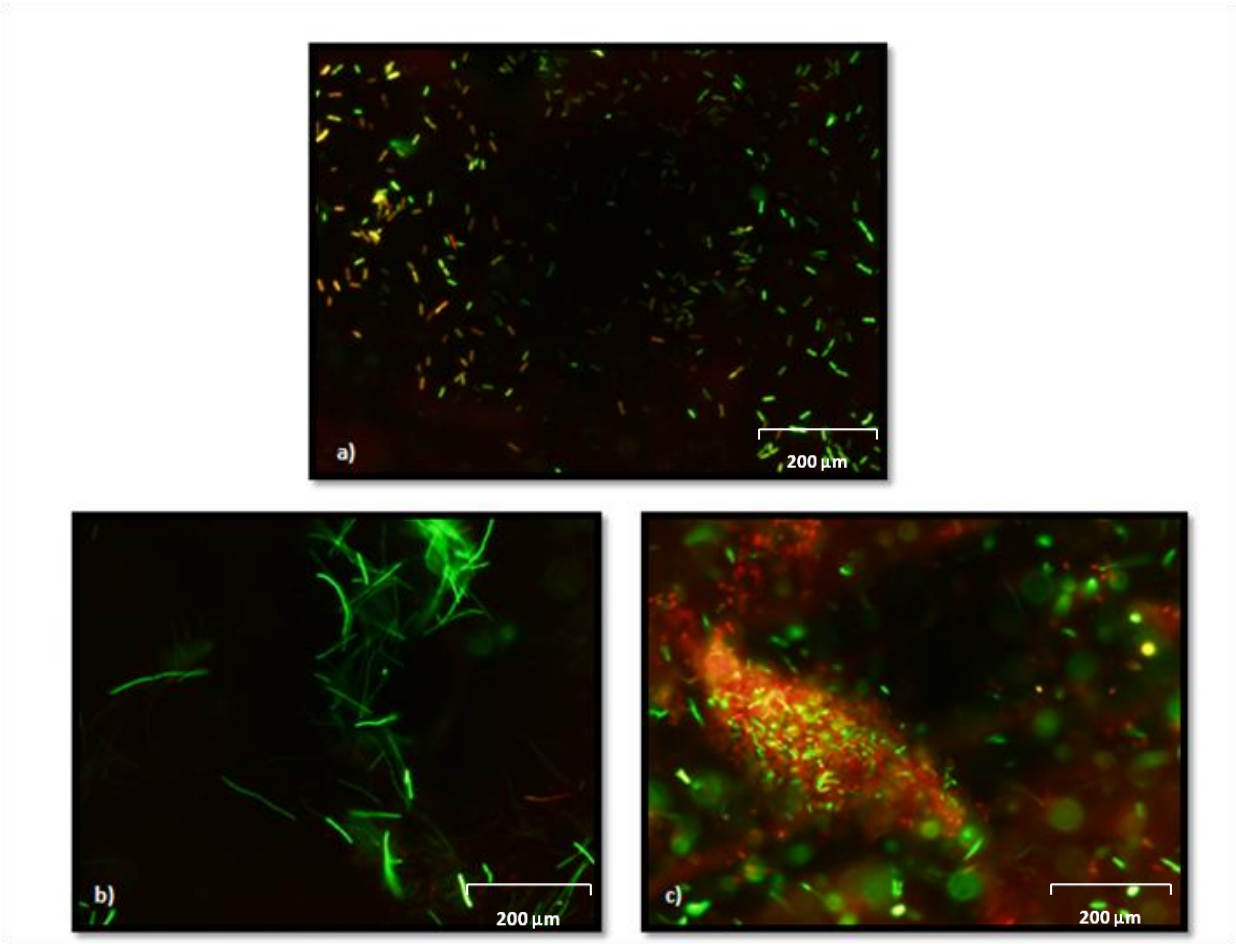


Figure 2.4: LIVE/DEAD Baclight™ staining of *P. aeruginosa* CECT 111a) bacteria without antibiotic (control); b) treatment of bacteria with piperacillin; c) treatment of bacteria with ciprofloxacin (Live cells – stained in green; dead cells – stained in red).

Staining with LIVE/DEAD BacLight™ shows that ciprofloxacin treatments result in both dead (red) and live (green) cells, while piperacillin causes an inhibition of cell wall synthesis leading to an elongation of the cells, and has a poor bactericidal effect. All other strains were also subjected to piperacillin treatment (Figure 2.5).

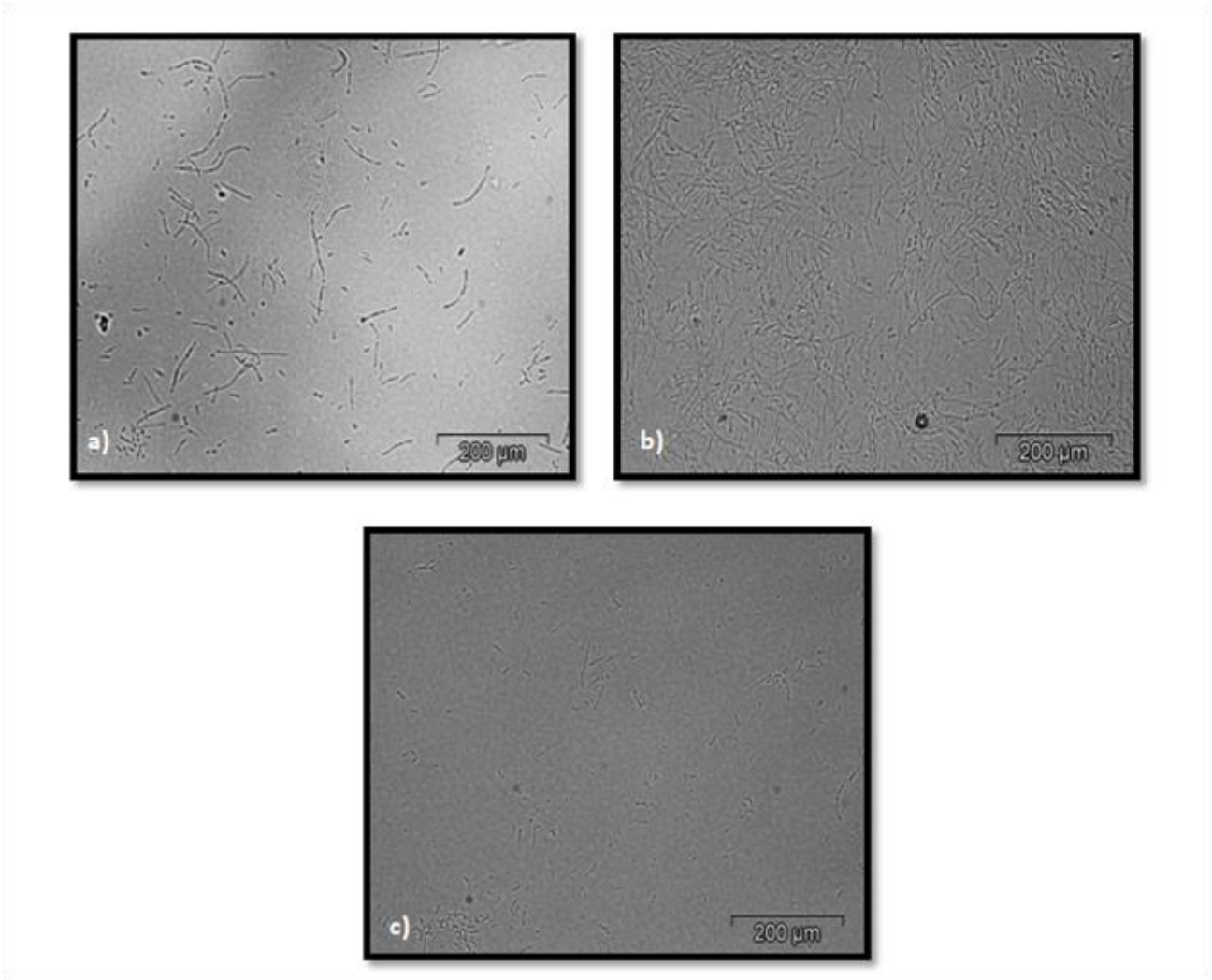


Figure 2.5: Visualization by electron microscopy of the elongation of *P. aeruginosa* cells in the presence of piperacillin. a) ATCC 10145, b) CECT 111, c) PA01.

As observed for CECT 111 (Figure 2.4), piperacillin caused an elongation of all other strains used in this work. This can be a survival strategy developed by bacteria against antibiotic molecules, because the cells stop dividing, but they still growth. Furthermore, it was also observed that the elongation effect increased with the concentration and contact time with the antibiotic (data not presented).

Although after 24 h the viable biofilm bacteria decreased in number, more than 10^4 CFU/ml still remained after the treatment with amikacin at a concentration of 256 mg/l. These results are contradictory to those reported by Gotto et al, where *P. aeruginosa* biofilms growing on Teflon catheters were eradicated already with a 64 mg/l concentration [22].

Overall, there are several possible factors that can be behind the reduced efficacy of antibiotics towards *P. aeruginosa* biofilms, like the presence of a polymer matrix that act a a barrier for the penetration of antibiotics molecules; the presence of inactivation enzymes; the different microenvironment inside biofilm (metabolic heterogeneity) and the presence of dormant cells (also called persister cells) that are tolerant to antibiotic treatment and so, responsible for the survival of the biofilm population [21]. In the work presented herein, in order to eradicate the biofilms formed it would be necessary to use antibiotic concentrations that exceed the peak serum concentrations.

2.3.3 Evaluation of ciprofloxacin against adhered cells

In order to study bacterial adhesion and the role of antibiotics as removal agents, the ability of antibiotics against adhered cells was evaluated using a parallel plate flow chamber with controlled hydrodynamic conditions and real time monitoring of the number of cells adhered over time [13,23]. For this purpose, the adhesion assays were carried out with the strain CECT 111, using the most effective antibiotic – ciprofloxacin. In these assays, a supra-inhibitory concentration of ciprofloxacin was used (4mg/l - 32×MIC).

Figure 2.6 presents images taken from the same sampling spot after 30 and 120 min of adhesion of *P. aeruginosa* and after 40 min of treatment with ciprofloxacin.

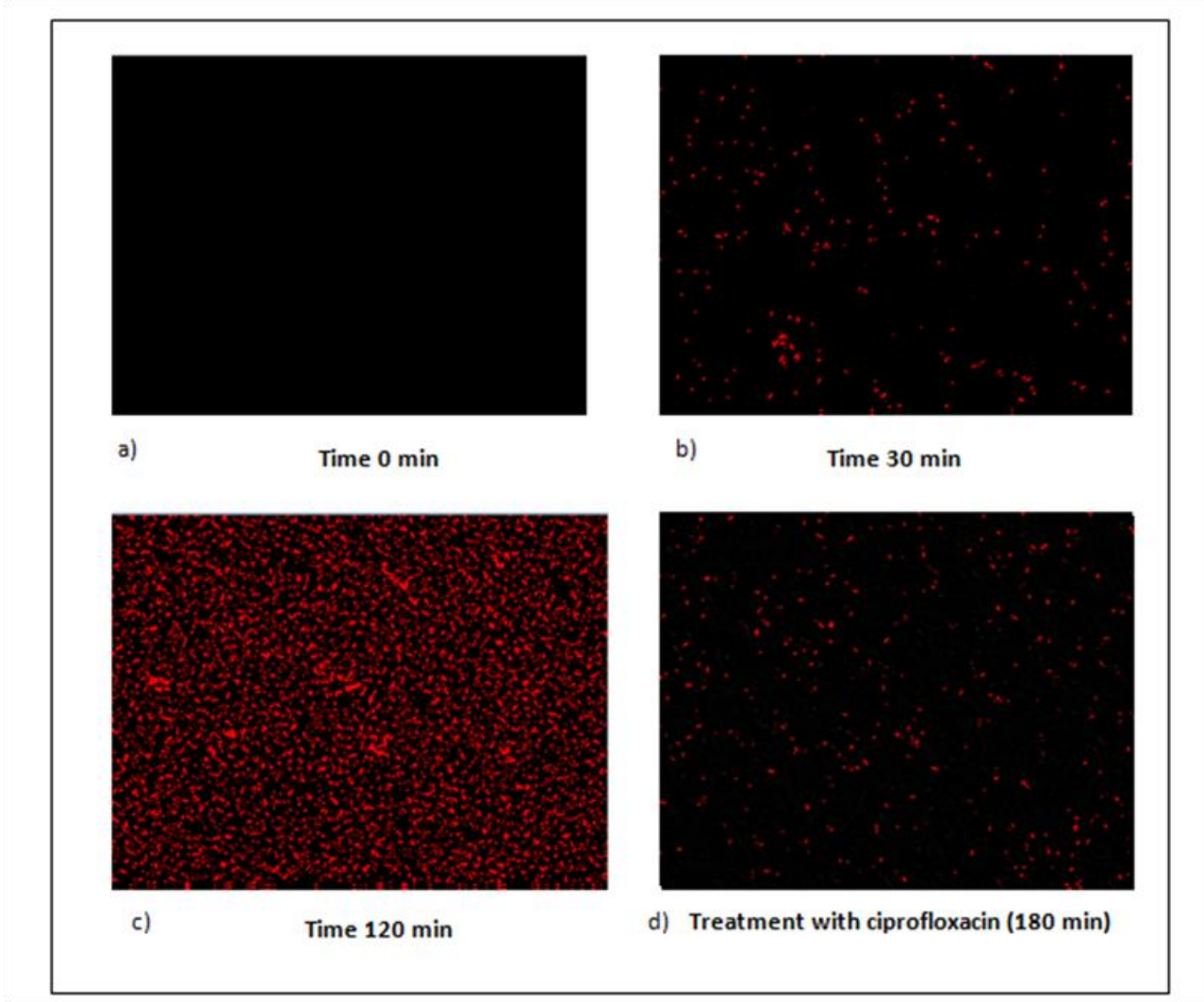


Figure 2.6: Images of *P. aeruginosa* cells adhered over time to a glass surface after image processing by Sigma Scan Pro 5: a), b) and c) adhesion of cells; d) treatment with antibiotic ciprofloxacin.

Ciprofloxacin treatment caused a significant reduction in the number of cells present in the glass surface, suggesting that it might have a positive role in the primary stage of biofilm formation. In spite of not having supportive data, it is suggested that the efficacy of ciprofloxacin in removing attached cells is related with modification of the cell surface properties stimulated by the antibiotic. This phenomenon could be confirmed by studies of the hydrophobicity and surface charge before and after exposure to the antibiotic.

In clinical applications, these results can be very interesting, because the coating of medical devices with ciprofloxacin can contribute to a partial inhibition of biofilm formation.

Nevertheless, it is important to conduct additional studies, particularly to determine if a recolonization of the surface takes place.

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Chapter 3: Bacteriophage Treatment

3.1 Background

P. aeruginosa have the ability to form dense and consistent biofilms with a low antimicrobial susceptibility. The emergence of pathogenic bacteria resistant to most currently available antimicrobial agents has become a critical problem in modern medicine. Thus, the development of alternative modalities has become one of the highest priorities in biomedical area to deal with infectious biofilms [1,2]. The use of bacteriophages could be one of these modalities [3-5]. The phage infection of biofilm cells is still not well understood and various variables involved in phage-host system can be determinant for its success, such as for instance, susceptibility of the biofilm cells to the phages, the availability of receptor sites and the production of polysaccharide-degrading enzymes by phages [6–8]. Nevertheless, some phages are highly active towards biofilms, even mature ones [9].

Similarly to the structure used in the previous section, this section will describe the main results obtained with the application of phages to cultures and biofilms of *P. aeruginosa* whereas the main objective is to evaluate the efficacy of phage therapy and compare it with the antibiotic therapy.

3.2 Materials and methods

3.2.1 Bacteriophages

All phages used in this work have been previously isolated and belong to the bacteriophage biotechnology group collection [10,11]. Table 3.1 presents their main characteristics.

Table 3.1: Characteristics of the phages

Phage phiIBB- PA...	Host bacteria	Phage family and genus	Phage genome data
P21	CECT 111	*Siphoviridae* *MP22like*	
P1	CECT 111	Unclassified *Myoviridae*	66.2 kb 96 ORFs 56% GC
A2	ATCC 10145	*Podoviridae* *LUZ24like*	45,1 kb 69 ORFs 52% GC
C23	PAO1	*Podoviridae* *LUZ24like*	45,1 kb 68 ORFs 52% GC

3.2.2 Bacteriophage propagation, concentration and purification

The plate lysis and elution method was used for phage production [12]. Briefly, the method consists of spreading phage lysate, using a sterile paper strip, on a Petri dish containing a top-agar layer (3 ml *Molten Top-Agar* (MTA) - 30 g/l of TSB and 0.6% of agar) with 100 µl of the respective host bacteria. After overnight incubation at 37 °C, 3 ml of SM buffer (5.8 g/l NaCl, 2 g/l MgSO4 × 7 H2O, 50 ml/l 1 M Tris, pH 7.5) was added to each dish and the plates were incubated during another 16-18 h stirring at 4 ° C. After this, the liquid and the MTA were placed on Erlenymer flasks to which 5.84 % (wt/vol) of NaCl was added, and after dissolved the flasks were placed under stirring during 1 h at 4 ° C, centrifuged (10 min, 10.000 ×g, 4 ° C), and 10% (wt/vol) of PEG 8000 was added to the supernatant. This solution was incubated under agitation at 5 ° C for 16-18 h, centrifuged (10 min, 10.000 ×g, 4 ° C), and the supernatant discarded. The pellet was resuspended in 5-6 ml of SM buffer, chloroform 1:4 (vol/vol) was added, the tubes vigorously mixed during 30 s, and then centrifuged (15 min, 3500 ×g, 4 ° C). The phage, present in the upper aqueous phase, was carefully removed into new tubes, filtered (0.22 mm) and stored at 4° C.

3.2.3 Phage titration

Phage titer was determined using either the small scale drop method described by Mazzoco et al with some modifications or the double agar overlay plaque assay

formalized by Adams M [13,14]. The first method consists of adding 20 µl of serially diluted phage to 20 µl of the overnight grown host, followed by 15 min incubation at 37 °C (120 rpm) and plating of 20 µl of sample on TSA agar plate. This method was only applied to phages phiIBB-PAP1 and phiIBB-PAP21 due to their plaque turbidity and size characteristics. The double agar overlay plaque method consists in adding 100 µl of serially diluted phage, 100 µl of an overnight grown bacteria and 3 ml of MTA onto a TSA Petri dish. For both enumeration methods used, plates were incubated for 18 h at 37 °C and after plaque forming units (PFU) were determined.

3.2.4 Infection of planktonic cells with phages phiIBB-PAP1, phiIBB-PAP21, phiIBB-PAA2, phiIBB-PAC23

The procedure followed in this experience was similar to the protocol of MIC determination. Thus, after adjusting the cellular suspension to 0.5 (McFarland) with TSB, 100 µl of *P. aeruginosa* cultures (ATCC 10145, PA01, CECT 111), were put on the wells of a 96-well microplate. After, phage solutions (100 µl) with different concentrations (10^3 – 10^8 PFU/ml) prepared in SM buffer, were added to the wells. Control experiments were performed with 100 µl of suspension and 100 µl of SM buffer. The duration of phage infection was 24 h. After this period, the OD (600nm) was measured. For these experiments three independent assays (each one in duplicate) were performed.

3.2.5 Infection of biofilm cells with phages phiIBB-PAP1, phiIBB-PAP21, phiIBB-PAA2, phiIBB-PAC23

Phage assays were performed similarly to the MBEC protocol. Briefly, after adjusting the OD600 to 1.0 (McFarland) with TSB, 200 µl of *P. aeruginosa* cultures (ATCC 10145, PA01, CECT 111), grown overnight at 37 °C and 120 rpm, were added to the wells of a 96-well microplate and incubated during 24 h. After 24 h of biofilm formation, the wells were washed with fresh TSB medium. Following that, 100 µl of fresh TSB and phage solutions (100 µl) with different concentrations (10^3 – 10^8PFU/ml) were added to the wells, or 100 µl of TSB and 100 µl of SM buffer in the case of control experiments. The duration of the phage infection was 24 h. Viable bacteria and phages

present in biofilms were determined by CFU and PFU enumeration. For these experiments three independent assays (each one in duplicate) were performed.

3.2.6 Statistical Analysis

In order to compare the amount of viable cells present in biofilms, it was performed an analysis of data variance (ANOVA single factor MS Office). This analysis was performed for a confidence interval of 95%.

3.3 Results and discussion

3.3.1 Infection of planktonic cultures

The infection of *P. aeruginosa* cultures with different concentrations of phages was evaluated (Figure 3.1).

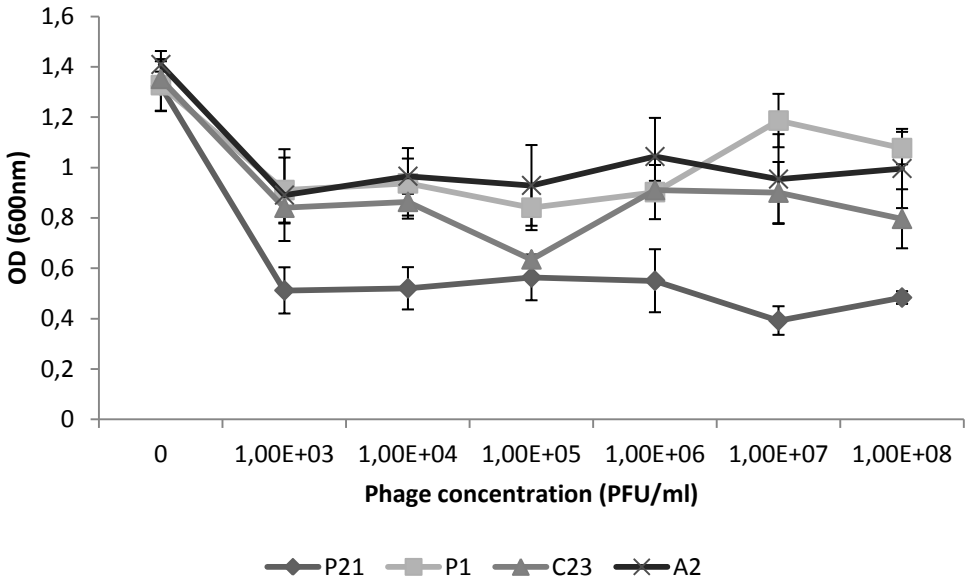

Figure 3.1: Phage infection of planktonic cells of *P. aeruginosa* strains PA01, CECT 111 and ATCC 10145 with different concentrations of the respective phages.

The results revealed that none of the phage concentrations used decreased enough the density of the cultures. Furthermore, after 24 h of assay, it is well evident that the increase of phage solution does grant a higher density reduction. Of all phages tested, phage phiIBB-PAP21 was the most effective, reducing by approximately 50 percent the initial density of the culture. This density reduction by phage phiIBB-PAP21 was significantly different ($p<0.05$) from all other phages tested.

Commonly, phage infection results in a density decrease which reaches a maximum after 5 to 7 h of infection. After this, the density rises mainly due to an emergence of resistant phenotypes [11]. This may explain the poor phage efficacies observed since these experiments are performed during 24 h using the same conditions as for the MIC assays. In same line and with the same conclusion, other study made by Kay M. (2010), reveal that after 24 h of phage infection planktonic culture of *P. aeruginosa* were barely affected by phage PB-1 [15].

3.3.2 Infection of biofilms

Biofilms of *P. aeruginosa* were challenged with phage (Figure 3.2) and accordingly, phage phiIBB-PAC23 appears to be the least effective phage, with a negligible reduction in the number of viable biofilm cells ($p> 0.05$). This result suggests that the complex structure of biofilm can affect and limit the penetration of the phage to the cells. On the other hand, phage phiIBB-PAP21 causes a significant reduction ($p <0.05$) of viable cells present in the biofilm (about of 1.3 log). In spite of different conditions, Priscila (2011) observed similar rate of reduction with phage phiIBB-PAP21 [12]. Surprisingly, the phage phiIBB-PAP1 showed greater efficacy in the control of biofilm cells compared to planktonic cells, with a 1.3 log reduction of cells present in biofilm of *P. aeruginosa* CECT 111.

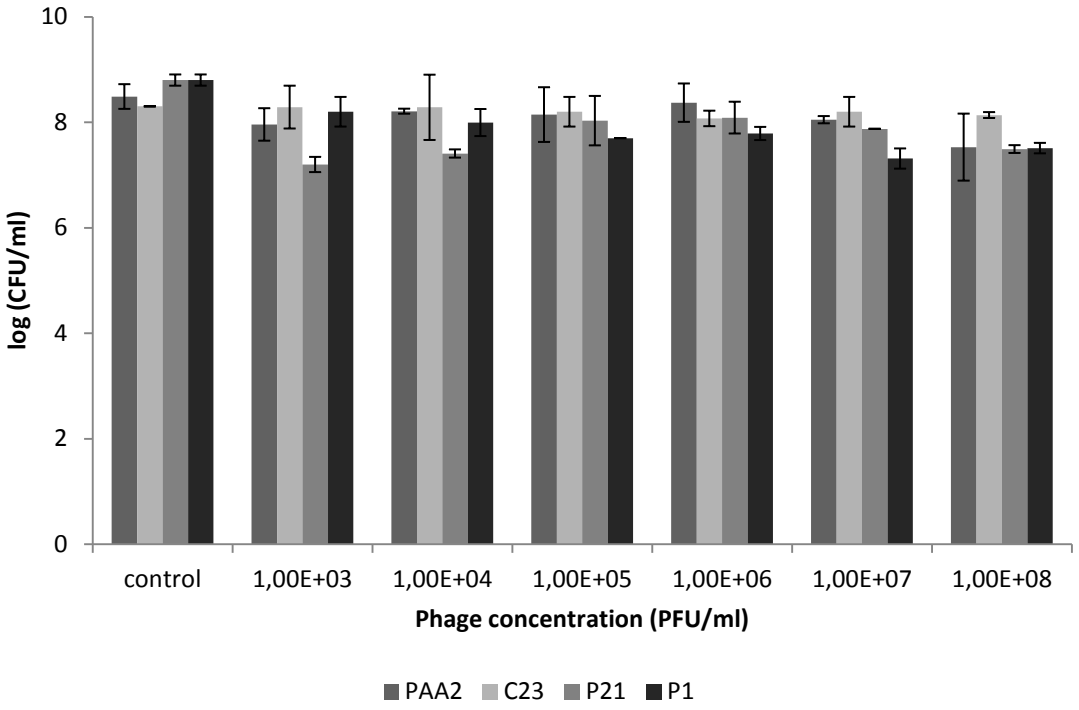

Figure 3.2: Number of viable cells present in biofilms of *P. aeruginosa* strains PA01, CECT 111 and ATCC 10145 after exposure to different concentrations of the respective phages.

Compared to antibiotics, it can be affirmed that phages had a lower efficacy in controlling biofilms of *P. aeruginosa* after 24 h of infection. Also, as it is possible observe in Figure 3.2, the viable cell numbers remained practically unchanged regardless of the different concentrations used. Contrarily to what was observed herein, Carson et al (2010) achieved a 4 log reduction in viable cells number of *E. coli* biofilms, after an infection of 24 h with T4 bacteriophage [16]. Therefore, as with planktonic cells, one possible reason for this poor efficacy may be related with the quick emergence of LPS mutants phenotypes during a long period of phage infection since lack or loss of O-antigen side-chains or any other conformational change can seriously affect phage adsorption to the bacterial surface [17,18].

Additionally to these factors, with the exception of phage phiIBB-PAP1, all other phages lack exopolysaccharide (EPS) depolymerases which may contribute to an inefficient diffusion through the biofilm. The presence of depolymerases in phage phiIBB-PAP1 was confirmed by measuring over time the diameter of the halo around a phage plaque which is an indicator for the presence of phage-associated EPS

depolymerases. Since there was no halo around all other phages, it can be assumed that these phages lack depolymerases (data not shown).

Verma et al (2010) has demonstrated the important role of phage associated depolymerase enzyme in the biofilm eradication of *Klebsiella pneumonia* [19]. He observed that within a short period of time, the combination of depolymerase with a non-depolymerase phage caused a drastically reduction of cells in biofilms, compared to the individual application of the non-depolymerase phage. It is not very common to obtain isolate phages that express this type of enzymes. To overcome this difficulty many researchers have engineering commonly used cloning phages such as the circularized T7 and the filamentous phage M13 [6,20,21] . The engineered phages reported by Lu and Collins (2007) allowed a reduction of bacterial biofilm cells of *E. coli* by two orders of magnitude more than the non-enzymatic phage [6].

Furthermore, it phage replication occurred within the biofilms, as expected, with phage plaque forming units increasing by more than 1 log at the end of the assay (data not shown).

In general, phages, as well antibiotics, proved to have a minor role in controlling biofilms of *P. aeruginosa*. During the period of 24 h, the emergence of resistant phenotypes seems to be the main cause in its poor efficiency.

3.3.3 Evaluation of phages against adhered cells

Similarly to the tests performed with antibiotics, also the adhesion of cells and their infection with phage was evaluated. In this case, the phage phiIBB-PAP21 (10^8 PFU/ml) was used for these tests (Figure 3.3).

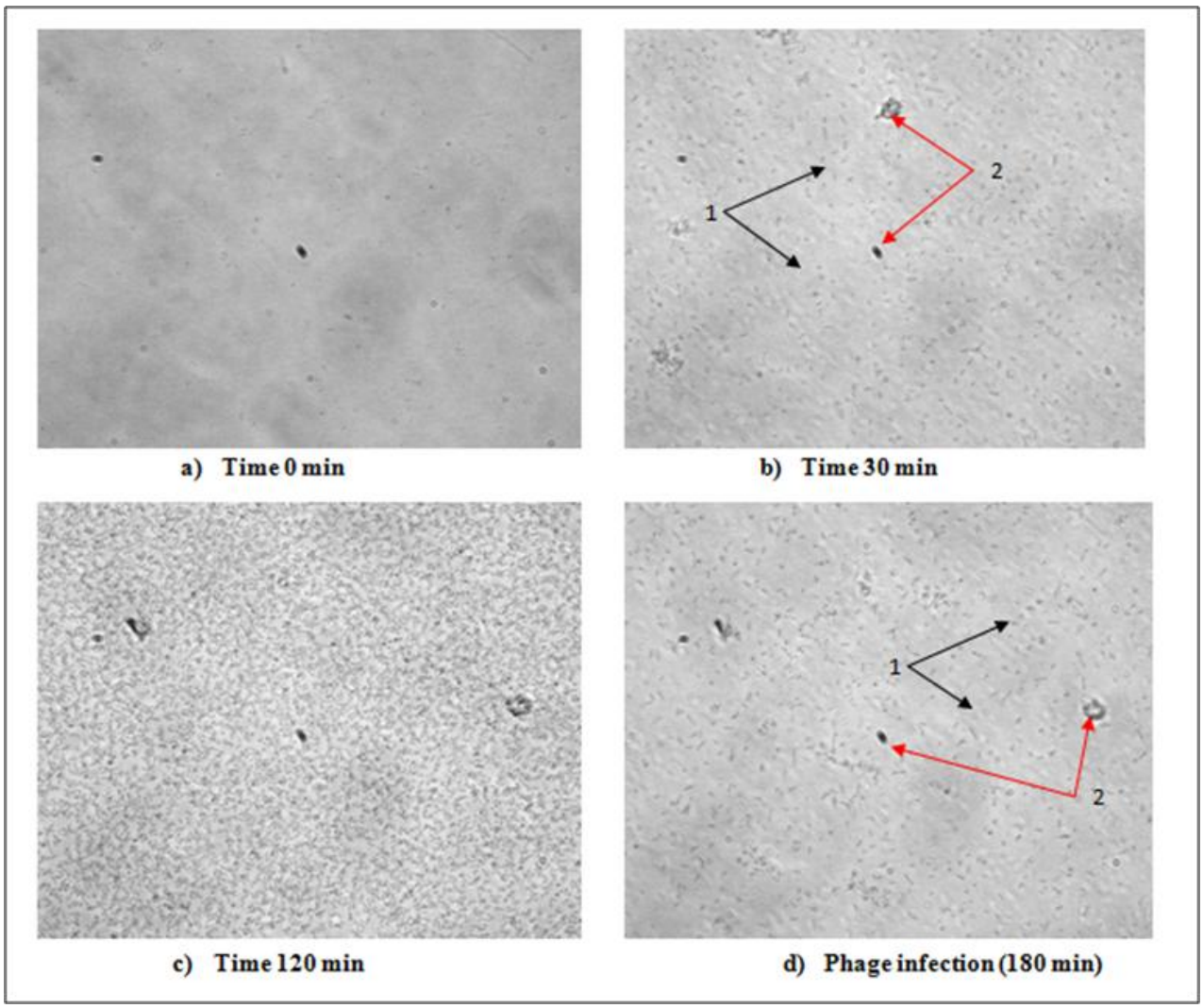

Figure 3.3: Images of P. aeruginosa cells adhered over time to a glass surface: a), b) and c) adhesion of cells; d) Infection of bacterial cells with phage phiIBB-PAP21. 1 – Bacterial cells; 2 – artefacts.

In order to compare the two agents (phage and antibiotic), the same periods of time were analysed. Likewise ciprofloxacin, also phage phiIBB-PAP21 removed cells from the surfaces analysed. However, a higher amount of cellular debris was observable at the end of the phage treatment compared to antibiotics. Despite not having quantitative results, the removal rate of the cells by phages seems to be very close to the rate obtained with the antibiotic. It has been previously reported that a phage, specific to *P. fluorescens*, at a similar concentration had a reduction rate of adhered cells of *P. fluorescens* above 90% [9].

These results suggest, once more, that phages and antibiotics can be controlling agents of the initial stage of biofilm formation, controlling biofilm formation on surfaces.

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Chapter 4: Combined Treatment

4.1 Background

The combination of phages and antibiotics has taken the first steps in clinical research as a strategy for combating infectious biofilms. In spite of very recent, some studies have shown that the use of phages and antibiotics as adjuvants of each other is more effective in control biofilms, compared to their independent application [1]. A research developed by Coulter L. (2012) demonstrated a greater efficacy of a combination of T4 phage and antibiotic trombamycin in the reduction of both biofilm mass and cells, compared to the use of phage and antibiotic alone [2].

Nevertheless, there are still many questions about how this process is developed, particularly about the relationship that exists between the SOS response developed by bacteria against antibiotics and phage action, and how this affects the resistance evolution of bacteria when exposed simultaneously to both modalities agents (phage and antibiotic) - Figure 4.1 [3–5].

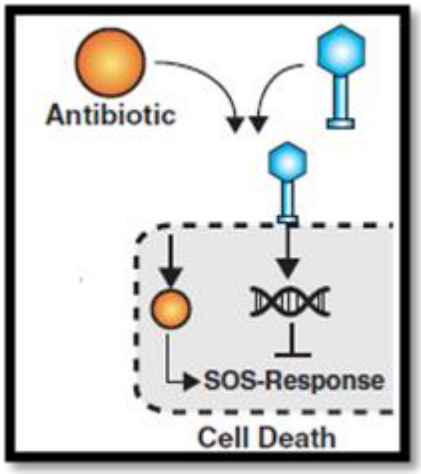

Figure 4.1: Schematic representation of a possible synergy process between phage and antibiotic. Adapted from Lu et al [5].

With the aim of improving the efficacy of phage and antibiotic therapy against *P. aeruginosa* biofilms, different phages were combined with different antibiotics and tested against planktonic cultures and biofilms.

4.2 Materials and methods

4.2.1 *P. aeruginosa* control by combined therapies using phages and antibiotics

In combined therapy, all the structure and procedure of the treatments, based on the exposure of planktonic cells or biofilms to phage or antimicrobial agent alone, was followed. Antibiotics solutions were prepared on a 10^8 PFU/ml phage solution, creating in this way a combined solution, in which each phage was combined with each antibiotic used. In planktonic cultures, all the antimicrobial agents were tested with the following concentrations (mg/l): ciprofloxacin (0.03125 - 1), amikacin (0.5 - 256), piperacillin (0.5 - 256), tetracycline (0.5 -256). In the case of biofilm treatment, the antibiotic's concentrations prepared in 10^8 PFU/ml phage solutions were: amikacin – 64mg/l; ciprofloxacin – 4mg/l; piperacillin and tetracycline – 256 mg/l. The choice of this range of concentrations is based in the preliminary assays that were made. Combined experiments were also performed with antibiotics and endolysin 68gpLys, provided by Hugo Oliveira that is efficient against *P. aeruginosa* (unpublished data).

The efficacy of the combined treatments was evaluated by enumeration of viable cells (CFU's) and the determination of biofilm biomass by crystal violet assay, or by the measurement of absorbance (OD600nm) in the case of planktonic cultures. For these experiments three independent assays (each one in duplicate) were performed.

4.2.2 Zeta potential measurement of wild type strain and LPS mutants

Measurements of zeta potential were performed in order to obtain the surface charge characteristics of wild type and LPS mutant strains. For this, several LPS mutant phenotypes were provided by Prof. Joseph Lam (University of Guelph, Canada) and by Diana Priscila Pires (CEB, University of Minho). The susceptibility of these strains to the phages used in this work has already been evaluated (Diana P. P. Pires, unpublished data) and is presented below.

Table 4.1: Lytic spectra of phages against different *P. aeruginosa* wild type strains and mutant phenotypes

		***P. aeruginosa* ATCC 10145**					***P. aeruginosa* PAO1**				
		wildtype	M2	B2	B3	P2	wildtype	wbpL	rmlC	wzy	Rmd
Phage phiIBB-PA(…)	A2	+	-	-	-	-	+	-	-	+	+
	P21	+	-	+	+	+	+	+	+	+	+
	C23	+	-	+	+	-	+	-	-	+	+

To perform zeta potential measurements, bacterial cells were grown overnight in TSB, centrifuged at 10000 ×g for 10 minutes, and the cells were washed once with PBS buffer (pH of 7.0) and resuspended in PBS buffer. The bacterial suspension was introduced into Zetasizer Nano ZS (Malvern Instruments) - Figure 4.3 - and disposable folded capillary cells were used to measure the zeta potentials of the bacterial suspensions. The measurements were performed at 37 °C five times per strain, to ensure reproducibility. Finally, electrophoretic mobility was converted to zeta potential through the *Smoluchowski equation* [6].

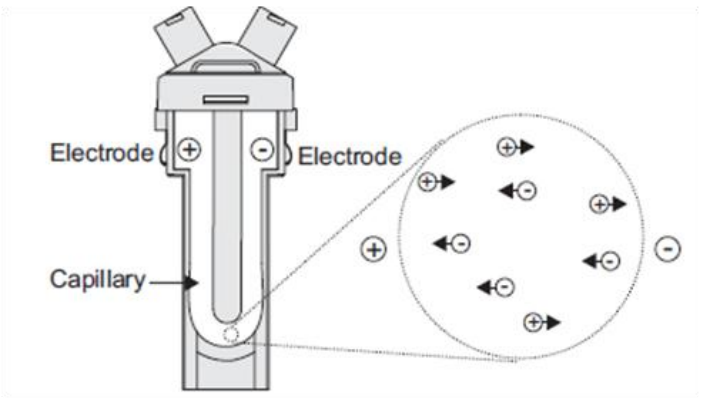

Figure 4.2: Disposable folded capillary cells used in zeta potential measurements. Adapted from [6]

Equation 4.1: $U_E = \frac{2\varepsilon z \times 1.5}{3h}$

z : Zeta potential;$\mathbf{U_E}$: Electrophoretic mobility; ε : Dielectric constant. η : Viscosity.

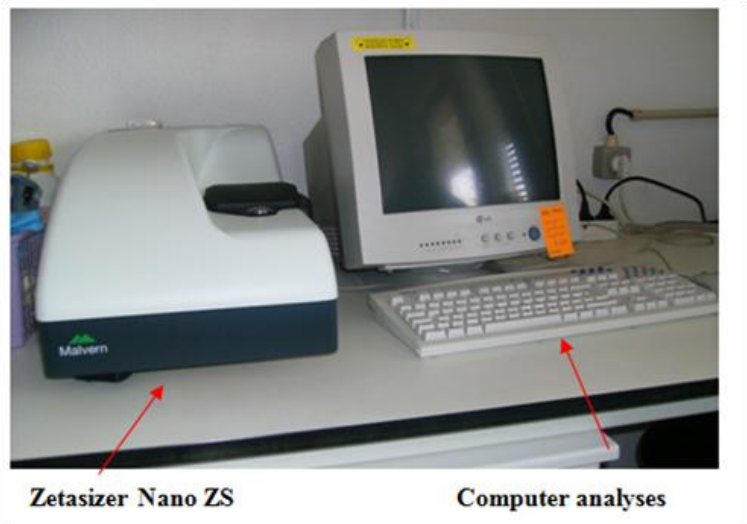

Figure 4.3: Zetasizer Nano ZS used in zeta potential measurements.

4.2.3 One-step growth curve

In this work, OSGCs were performed as described by Rahman M. *et al.* with some modifications [7]. In brief, 10 ml of a mid-exponential-phase culture of bacteria (OD 600nm: 0.35) was harvested by centrifugation (7,000 ×g, 5 min, 4 ºC) and resuspended in 5 ml TSB medium. To this suspension, 5 ml of phage solution was added in order to have a multiplicity of infection (MOI) of 0.01 and phage was allowed to adsorb for 5 min at 37 °C and 120 rpm. The mixture was then centrifuged as described above, the pellet was resuspended in 10 ml of fresh TSB medium and the mixture was incubated at 37 ºC under agitation (120 rpm) . Two samples were taken every 5 min over a period of 30 min and every 10 min until the end of the assay (120 min).

In relation to the experiments with cells infected with antibiotic, the method was slightly different in the first part. That is, firstly cells were incubated overnight with antibiotic at a sub-MIC concentration (4 mg/L) at 37 °C and 120 rpm and in the

following day, cells were spun down (7,000xg, 10 min, 4 ºC), the antibiotic (supernatant) discarded, and the pellet washed twice with TSB. A fresh inoculum was incubated for 3 h and after that, the number of cells was counted using a neubauer chamber. After, the phage solution was prepared in order to have a MOI of 0.01, and from this point the rest of the method was followed as describe above.

4.3 Results and discussion

4.3.1 Combined therapy in planktonic cultures

The effect of combined therapy in planktonic culture of PA01, CECT 111 and ATCC 10145 strains is presented in Table 4.2.

Table 4.2: MIC values for the combined treatment phage + antibiotic on planktonic cultures

Antibiotic	Strain + phage			
	CECT + phiIBB-PAP1	CECT + phiIBB-PAP21	PA01 + phiIBB-PAC23	ATCC + phiIBB-PAA2
Amikacin	1	1	4	16
Ciprofloxacin	0,0625	0,0625	0,125	0,25
Piperacillin	2	2	4	8
Tetracycline	16	4	32	32

The combination of phage phiIBB-PAP21 and all antibiotics show a strong enhancement of antibiotic killing leading to lower MIC values than when antibiotics alone were used (see Table 2.1). With phage phiIBB-PAP1 the reduction of antibiotic susceptibility was observed for all antibiotics with the exception of tetracycline. Conversely, phage phiIBB-PAC23 only enhanced antibiotic killing with piperacillin and combined phage-antibiotic assays with phage phiIBB-PAA2 did not result in any MIC reduction compared to antibiotics alone.

Nevertheless, contrary to the results obtained in the phage infection, with combined treatment is already possible to get MIC values within the susceptible range defined by EUCAST clinical breakpoints, which means that all strains are susceptible to

the combined phage-antibiotic treatment. Additional, since it was possible define a MIC value for all strains, it is possible to conclude that after 24 h there is no development of resistance by the cells, which proves to be an innovative result in relation to the application of phages alone. Zhang et al (2011) also prove that the combination of phage and antibiotic potentiate the reduction in the survival of *P. fluorescens* populations [8].

On the other hand, these results demonstrate in some way that the synergy between phage and antibiotics is not a simple phenomenon, since significant differences were found between the strains.

Overall, some of these tested combinations proved to be good candidates for further testing for control of infectious biofilms.

4.3.2 Combined therapy in biofilms

In biofilm assays, the number of cells present in biofilms was evaluated in two distinct moments after the exposure to the combined treatment: 6 h after infection and 24 h after infection (Figures 4.4 to 4.7). The choice of these two periods was to determine if the simultaneously action of phage and antibiotic allows the reduction of bacterial resistance after 6 h of treatment. In previous studies it was demonstrated that phage resistance appears after 6 h of infection [9].

It is important to refer that since the antibiotics amikacin and ciprofloxacin showed the lowest MBEC values in MBEC assays, for these combined experiments the concentrations of these antibiotics used were inferior (see Materials and Methods) . The goal was to see if with the combined treatment maintains or exceeds the rate of efficacy while the antibiotic concentration is reduced.

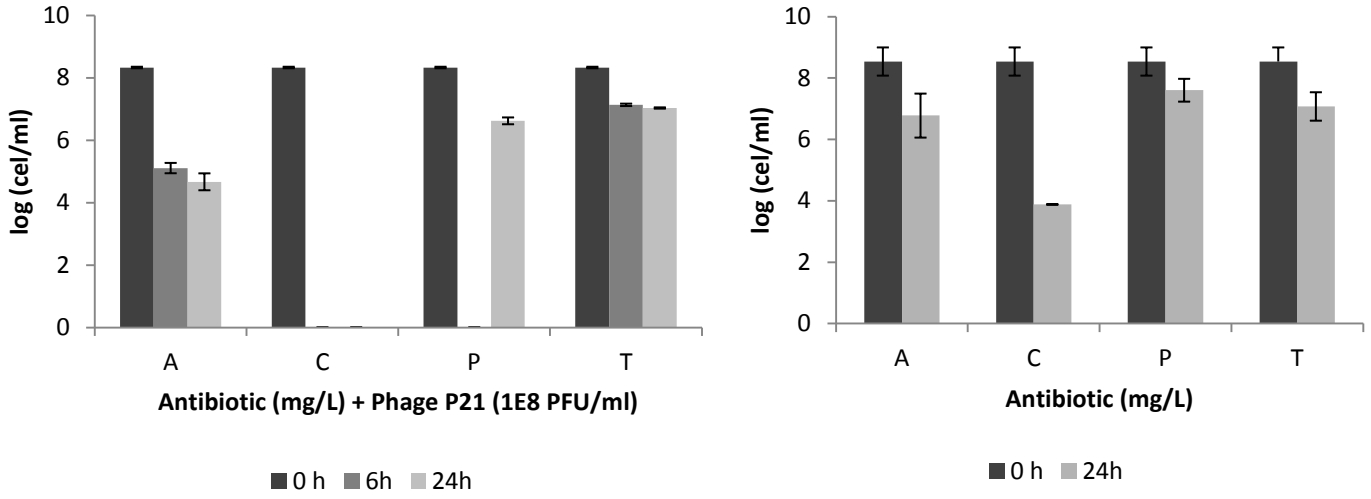

Figure 4.4: Number of cells present in biofilm after exposure to a) Combined treatment phage-antibiotic during 6h and 24 h; b) Antibiotic treatment during 24 h for CECT 111 strain A - amikacin 64mg/l; C – ciprofloxacin 4mg/l; P – piperacillin and T- tetracycline 256mg/l.

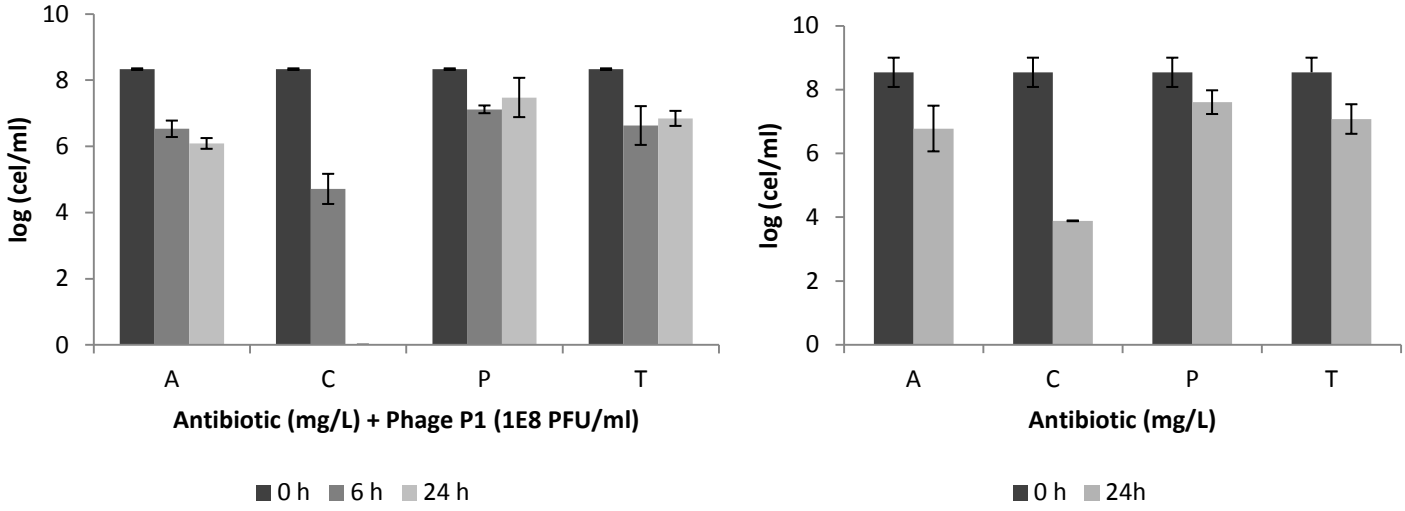

Figure 4.5: Number of cells present in biofilm after exposure to a) Combined treatment phage-antibiotic during 6h and 24 h; b) Antibiotic treatment during 24 h for CECT 111 strain A - amikacin 64mg/l; C – ciprofloxacin 4mg/l; P – piperacillin and T- tetracycline 256mg/l.

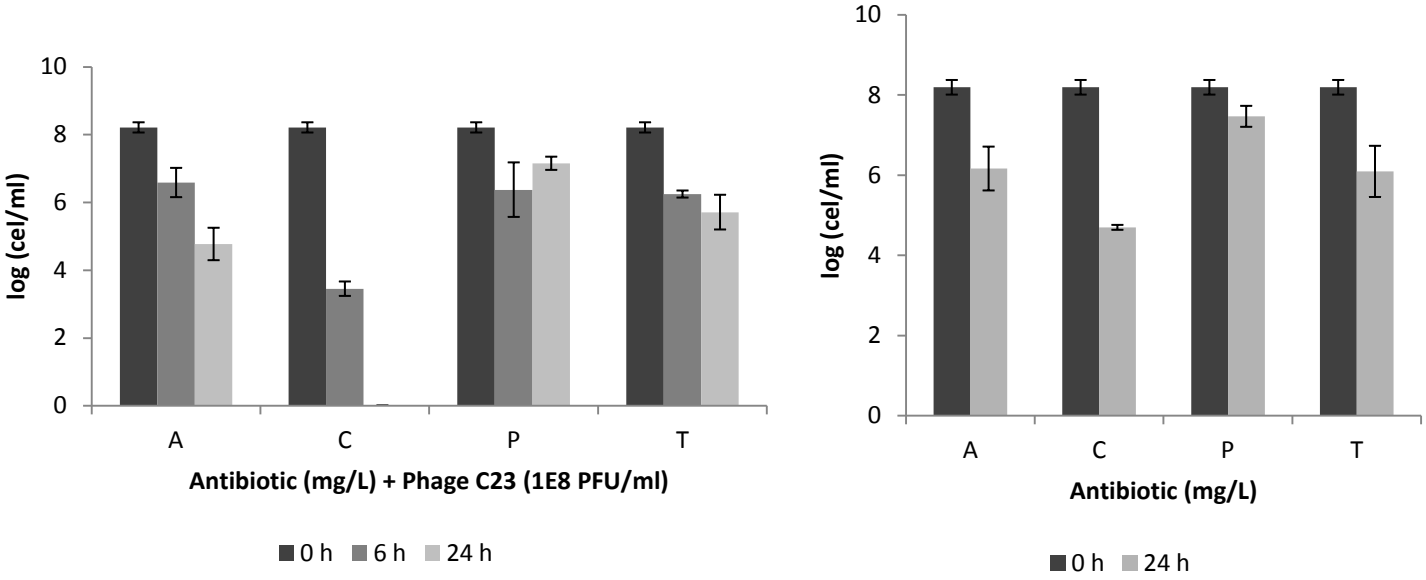

Figure 4.6: Number of cells present in biofilm after exposure to a) Combined treatment phage-antibiotic during 6 h and 24 h; b) Antibiotic treatment during 24 h for PA01 strain A - amikacin 64mg/l; C – ciprofloxacin 4mg/l; P – piperacillin and T- tetracycline 256mg/l.

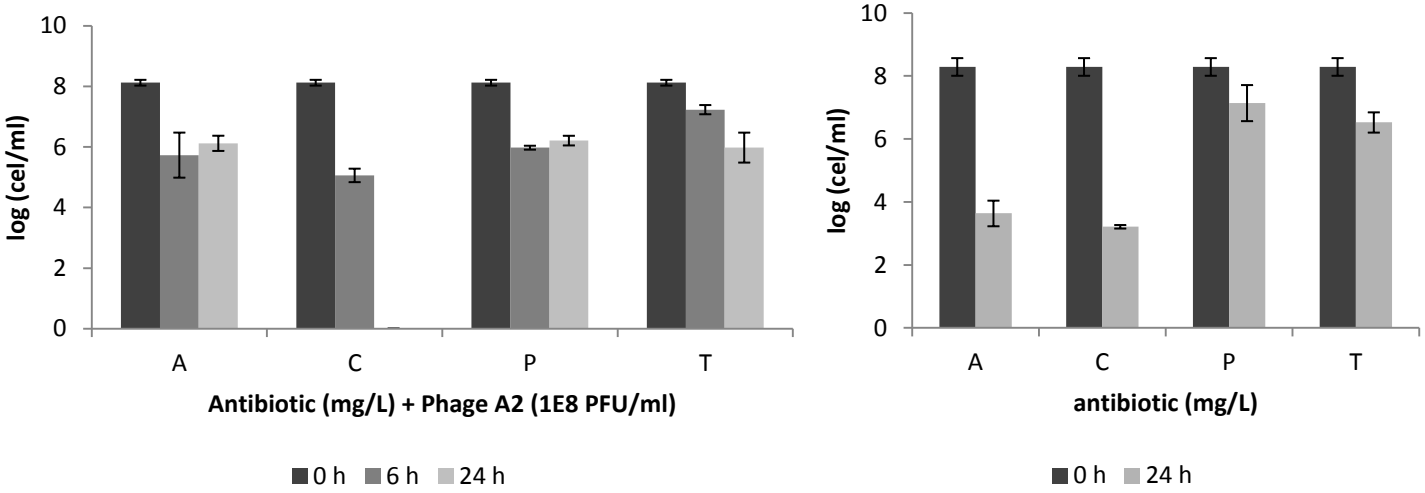

Figure 4.7: Number of cells present in biofilm after exposure to a) Combined treatment phage-antibiotic during 6 h and 24 h; b) Antibiotic treatment during 24 h for ATCC 10145 strain A - amikacin 64mg/l; C – ciprofloxacin 4mg/l; P – piperacillin and T- tetracycline 256mg/l.

As it is possible observe in Figures 4.4 to 4.7, between 6 h and 24 h of treatment there was, with the exception of the combination phage and piperacillin, no viable cell increase and under certain circumstances there was even a further decrease.

An interesting and very promising result is related with the complete eradication of the biofilm, after 24 h when combining ciprofloxacin with the different phages. Furthermore, total eradication was already observed after 6 h of combined treatment between phage phiIBB-PAP21 and ciprofloxacin. Also, with the combined treatment the residual cells observed after ciprofloxacin treatment alone are eliminated. One of the possible explanations for this event is the action of phages on persister cells. This was observed by Pearl et al (2008), under a study about host-phage interaction, whom demonstrated that with lytic infection, persister cells from biofilms can be eliminated when they are submitted to their normal growth state [10]. Possibly, the combination of phage and antibiotic can be, in a certain way, a stimulus for the reactivation of persister cells to normal growth. Furthermore, in this work the combination solution was performed using a very low concentration of the antibiotic ciprofloxacin (4 mg/l), which means that with this phage, the concentration of antibiotic needed to control biofilms of *P. aeruginosa* is much lower than when the antibiotic is used alone and for this reason the toxicity of the antibiotic can be reduced. This result can also have important effects in studies *in vivo*, because the maximum achievable concentration for ciprofloxacin in serum is in the range of 2-4mg/L [11]. Moreover, these results demonstrate that there is a potential synergy between phage phiIBB-PAP21 and ciprofloxacin, and the value of the MBEC can be roughly reduced by using ciprofloxacin and phage at the same solution. In spite of using different bacteria, Ryan et al (2012) also observed an enhancement effect on biofilm eradication of *E. coli* combining cefotaxime with phage T4, observing a reduction from 256 to 32 mg/L of MBEC values [12].

Regarding now the other combinations, the enhancement killing seems to be also significant ($p<0.05$) between phage phiIBB-PAP21 and amikacin, with more 2 log reduction of cells number, respectively compared to antibiotic treatment. Also, the phage phiIBB-PAA2 together with the antibiotic piperacillin can be a good choice to get a significant decrease of cells number ($p<0.05$). Nevertheless, with amikacin this phage does not appear to act synergistically because a greater number of cells were observed after 24 h, compared to treatment with antibiotic alone. On the other hand, the combination of any of the phages with tetracycline has not a significant effect on the reduction of viable cell numbers, presenting only a negligible effect on biofilms. In a

certain way, this result was expected, due the previous results obtained with antimicrobial susceptibility. The short action that this antibiotic has on bacterial cells can explain this scenario.

Also, an important and unexpected result is related to the increased efficiency of phage phiIBB-PAC23 when used in combination with antibiotics. As in the previous results, the evaluation of the effectiveness of phage infection in biofilms by phage phiIBB-PAC23 has dictated poor results after 24 h of treatment. The combination of this phage with amikacin revealed interesting results with more than 1.4 log reduction of viable cells counts in relation to the application of these agents alone.

Additionally, to test the combined therapy in other ratios, two antibiotics and phage were combined in the same solution. In literature, there are studies that show that the combination of beta-lactams and aminoglycosides may result in good synergy [13] and therefore a combination of amikacin, piperacillin and phage phiIBB-PAP1 was used. However, after 24 h, better results have not been achieved in relation to what had been obtained (data not presented).

Another important point is related with the stability of the phage in the presence of antibiotics (Figure 4.8).

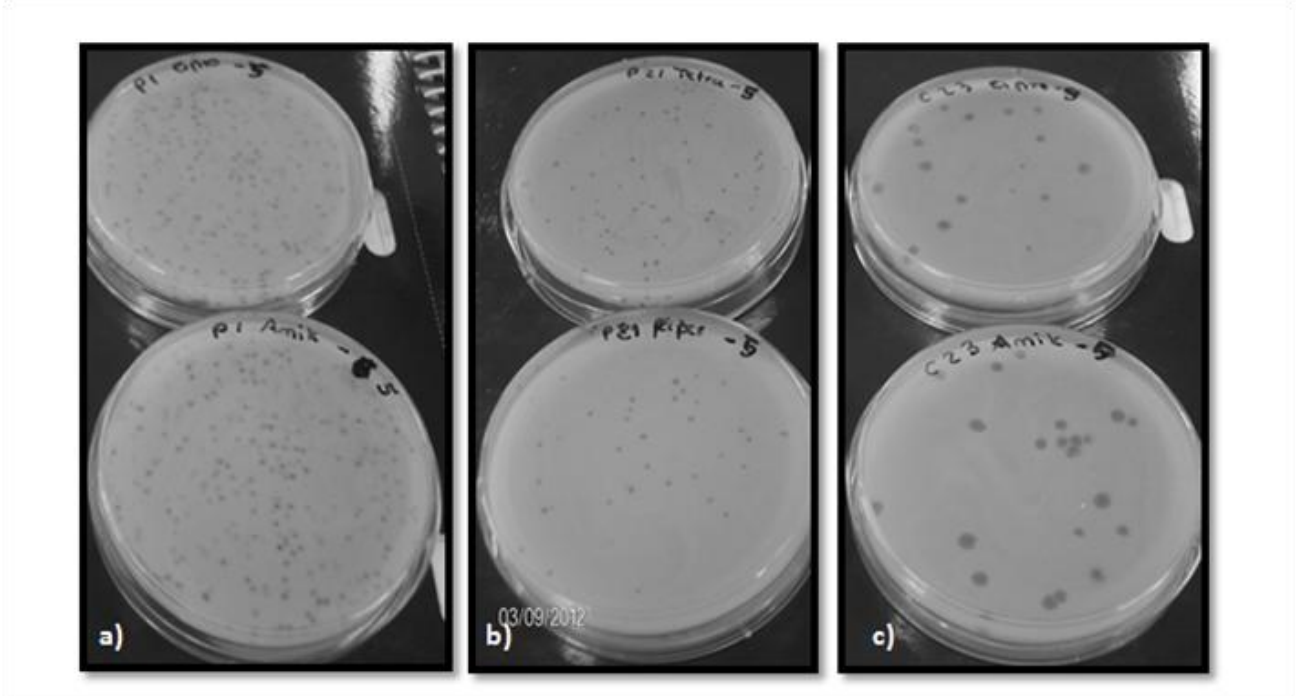

Figure 4.8: Analysis of phage viability during synergy treatment. a) phage P1 with ciprofloxacin and amikacin; b) phage P21 with tetracycline and piperacillin; c) phage C23 with ciprofloxacin and amikacin.

Counting PFU's during the incubation period of a combined solution shows that phages did not lose their viability during the 24 h of contact with the antibiotic solution. In general, after 24 h, the phage titre continued at 10^8 PFU/ml.

In general, using phage and antibiotics simultaneously not only against *P. aeruginosa* but also other pathogens is an emerging and promising alternative for the control of biofilms [1,14]. Studies by Rahman M. et al (2011) showed that total eradication was not achieved, however the co-treatment with phage and rifampicin reduced the viable cells of *S. aureus* in biofilms by 5 log [7]. Similarly, Verma et al (2009) demonstrated that the combination of a lytic phage and amoxicillin can be an alternative instead use the two therapies alone to reduce *Klebsiella pneumonia* viable cells in biofilms [15].

On the other hand, the use of phage-antibiotic combinations can be an attractive option to deal with chronic infections, instead use multiple combinations of antibiotics, which in turn allows to an increase in multidrug resistance of bacteria and toxicity in the body [13].

Nevertheless, the synergy between phage and antibiotics may be a very complex process since all this mechanism is not uniform, and according to the obtained results, it seems to be dependent of the phage and antibiotic used and also of the phage-host system. Furthermore, it is not easy to understand and associate which are in fact the variables involved in the combined effect of phage and antibiotic, since the behaviour against the combined treatment was not always linear between planktonic cultures and biofilms using exactly the same biological and chemical agents. Furthermore, synergy is not observed with all phages and antibiotics. Therefore, in order to understand what possible mechanisms are behind the synergy between phage and antibiotics, several factors were analysed, such as: the antibiotic susceptibility and zeta potential of LPS mutant phenotypes, the effect of the EPS matrix, the effect of phage growth cycle characteristics after exposure to antibiotics and susceptibility of antibiotic challenged cells to the action of endolysins.

4.3.3 Antibiotic susceptibility and zeta potential of LPS mutant phenotypes

In the combined assays, resistant phenotypes were found and according to previous results (Diana P. P. Pires, unpublished data) it is expected that this phenotypes

are LPS defective strains, consequently it is hypothesise that these mutants can have altered susceptibilities to both phages-antibiotics compared with the wild type strains. To confirm this hypothesis, MIC assays and potential zeta experiments were carried out (Table 4.3) to some LPS mutants (see description in materials and methods).

Table 4.3: Zeta potential measurements for wild-type strains and its LPS derivatives mutants

		MIC (mg/l)				
Strain	Potential zeta (mv)±SD	**Amik**	**Cipro**	**Piper**	**Tetra**	**LPS property**
Wzy PA01	-8,27 ± 0.193	4	0,125	4	16	**A+ B-**
Rmd PA01	-14,9 ± 0.961	4	0,0625	8	8	**A- B+**
Rmlc PA01	-21,5 ± 0.757	2	1	4	16	**A- B-**
Wbpl PA01	-14,8 ± 0.173	4	0,0625	16	4	**A- B-**
M2 ATCC	-17,7 ± 0.964	2	0,125	8	8	**A- B-**
PA01 – wild type	-12.6 ± 0.7	4	0,125	16	8	Wild type; 05 (B band), A band LPS
ATCC – wild type	-4.43 ± 0.364	4	0,125	8	16	Wild type; 06 (B band), A band LPS

The results reveal that differences in LPS structures result in different antibiotic uptakes by bacterial cells compared to their parental strains (Table 4.3). For example *Rmlc* and *M2* are the most electronegative mutant strains and both have lower MIC values than the wild type strain when they were exposed to amikacin. This happens due to better interaction between more negative LPS and cationic antibiotics. In relation to piperacillin, an unexpected result was observed for strain *Rmlc*, because a more electronegative surface would dictate a higher repulsion between the cell surface and piperacillin that has an anionic structure. Probably these differences are due to complex interactions between cell surface and the surface charge of the antibiotic. Computational simulations have shown that electrostatics interactions between the phosphate groups and cations present in the oligosaccharide portion of the LPS can influence the conformation of the pore in the OprF protein. For example, in the absence of the oligosaccharide chains and cations, which happens with mutants that have both A- and B-bands affected, the extra-cellular loops of OprF porin will adopt a closed conformation, restricting the entrance to the pore [16,17]. From another perspective,

Godfrey et al (1984) showed also that changes in the structure of LPS molecules lead to increases of MIC values for some Beta-lactam antibiotics [18]. On the other hand, *Wzy*, one of the less electronegative strain, presents a lower MIC value in relation to wild type after the exposure of piperacillin. *Wbpl* and *Rmd* strains, with an intermediate value of potential zeta, present a lower MIC value, in relation to wild type, when expose to ciprofloxacin. These results suggest that the type of LPS can significantly affect the interaction between antibiotic and cell surface, and in some cases this can reflect the synergism between phage and antibiotic.

Another factor that can influence the adsorption of antibiotics and phage molecules can be related with the type of serotype (O-antigen in LPS structure) present in the strains - see Table 4.3 for wild type strain. In the analysis made by Lam et al (1994), the B-band LPS molecules present in O5 strain are more filled in relation to the O6 strain, that only show a patchy B-band [19]. The zeta potential measurements performed in this work show that the strain PA01 is more electronegative than the ATCC 10145 strain and this is in agreement with the work of Lam et al (1994). Possibly, in ATCC 10145 strain there is a limitation of the number of sites for amikacin and phage interaction, which in turn can cause co-competition between phage and amikacin for adsorption sites on LPS. Note that previously (Figure 4.7) it was observe that amikacin and phage phiIBB-PAA2 not acted synergistically.

In general, these results suggest that the presence of LPS mutants may contribute to a positive configuration on the phage-antibiotic synergy.

4.3.4 Biofilm biomass

Another possible mechanism that can explain the perfect dualism between phage and antibiotic is the impact of combined treatment in the total biomass of biofilm. The total biomass present after antibiotic and combined treatment was measured by violet crystal assays (Figure 4.9).

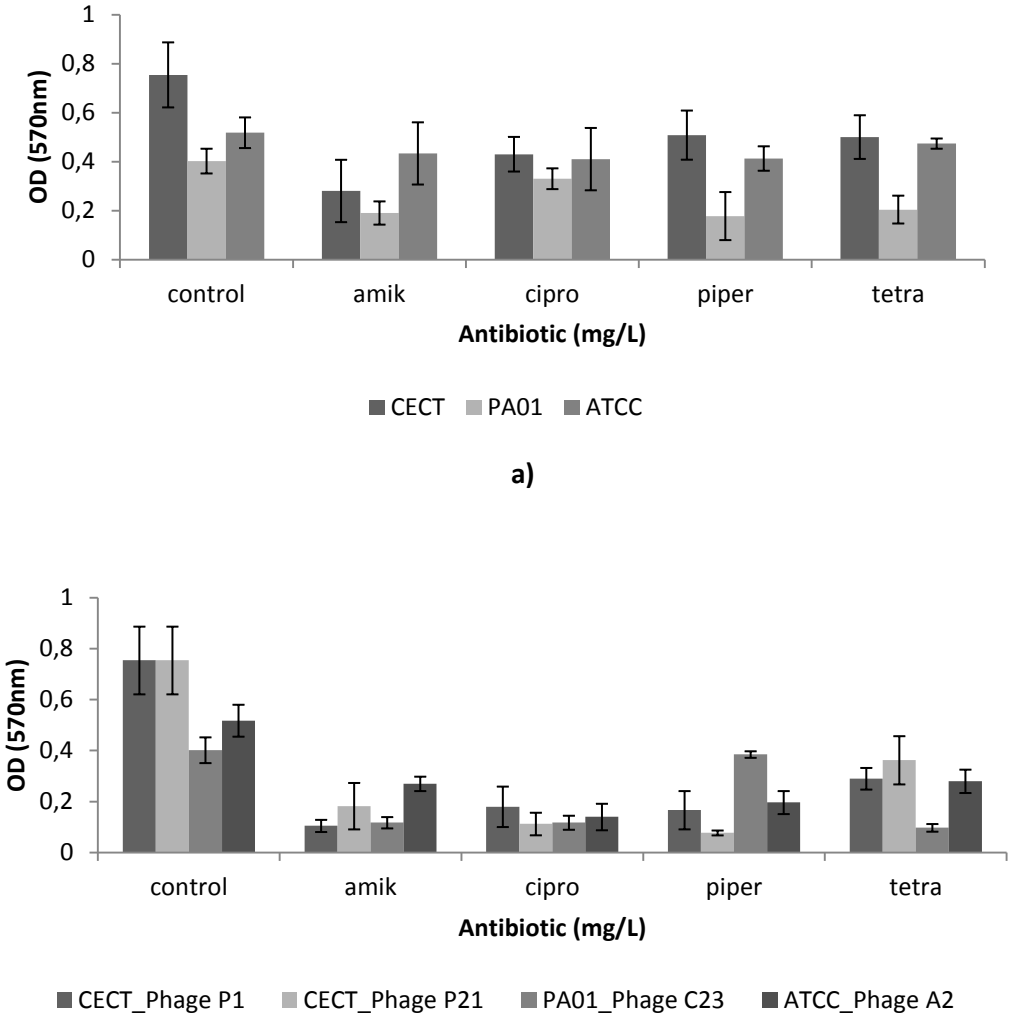

Figura 4.9: Biomass quantification using Crystal Violet Staining: a) Treatment with antibiotic; b) combined treatment phage-antibiotic.

According to the results in Figure 4.9, in general, the total biomass is significantly reduced ($p<0.05$) after the exposure to all phage-antibiotic combinations tested, suggesting that this treatment option potentiates biofilm matrix disruption rendering cells without a matrix shield become more vulnerable to antibiotics and phages action. In this context, it is suggested that the phages create some opening channels along the biofilm matrix, allowing the cells present in the structure of the biofilm stay in suspension, allowing a more effective action of antibiotics and phage themselves.

4.3.5 Effect of antibiotics on the phage growth cycle

It has been described that any stimuli such as temperature, agitation or cell filamentation can have an important effect in the increase of protein synthesizing system, and consequently, in higher phage production [20]. Therefore, it was important to realize if the presence of antibiotics could lead to changes in the parameters of the phage infection process. For this purpose bacteria were exposed to piperacillin to determine if the cell elongation, previously observed in Chapter 2, could enhance the phage phiIBB-PAP21s' growth cycle characteristics (Figure 4.10). In Table 4.4 are presented the life cycle parameters that were obtained by performing phage OSGC. With these experiments it was possible compare the latency period and the period of increase in the average number of released phages per infected cell (burst size).

Table 4.4: Life cycle parameters determined for phages used in this project

Phage phiIBB-PA...	Life cycle parameters
P21	Latent period – 50 min Rise period – 40 min Burst size – 20 PFU per infected cell
P1	Latent period – 40 min Rise period – 30 min Burst size – 72 PFU per infected cell
A2	Latent period – 40 min Rise period – 20 min Burst size – 37 PFU per infected cell
C23	Latent period – 10 min Rise period – 10 min Burst size – 4 PFU per infected cell

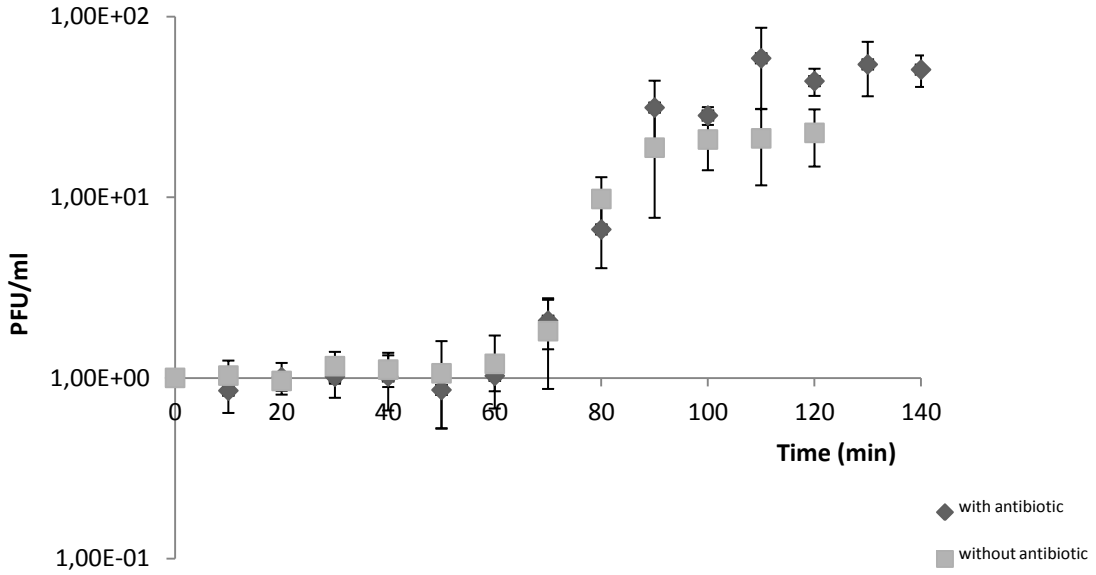

Figure 4.10: One-Step-Growth curve for phage P21 with antibiotic and without antibiotic.

The presence of sub-lethal piperacillin concentrations resulted in a 2.5 fold greater burst size (Figure 4.10 and Table 4.4) which further helps to understand the synergy observed when combined with phage phiIBB-PAP21. These observations are in agreement with those by *Ryan et al* (2012) in which the burst size of coliphage T4 increase when *E. coli* was exposed to sub-inhibitory concentrations of cefotaxime. Furthermore, Ryan also reported a decrease of 6 minutes on latent period [12]. Although in this work there was an increase of phage burst size in cells treated with piperacillin, unexpectedly the latent period remained similar to the experiments performed in non-challenged host cells.

Overall, it seems that the bacterial SOS response to β-lactam antibiotics which results in cell filamentation [3], is an advantageous mechanism since it enhances phage production[20,21]. This happens because larger cells have a proportional increase of the protein synthesizing system [20].

4.3.6 Efficacy of combined antibiotic-endolysins treatment

To close the cycle of synergy between phage and antibiotics, there were also performed tests with (endo)lysins, enzymes that are encoded by a majority of dsDNA bacteriophages during the last stage of lytic cycle [22,23], to verify if this combination

could enhance the penetration of both agents on biofilms of *P. aeruginosa* compared in this case to antibiotic or lysin alone (Figure 4.11).

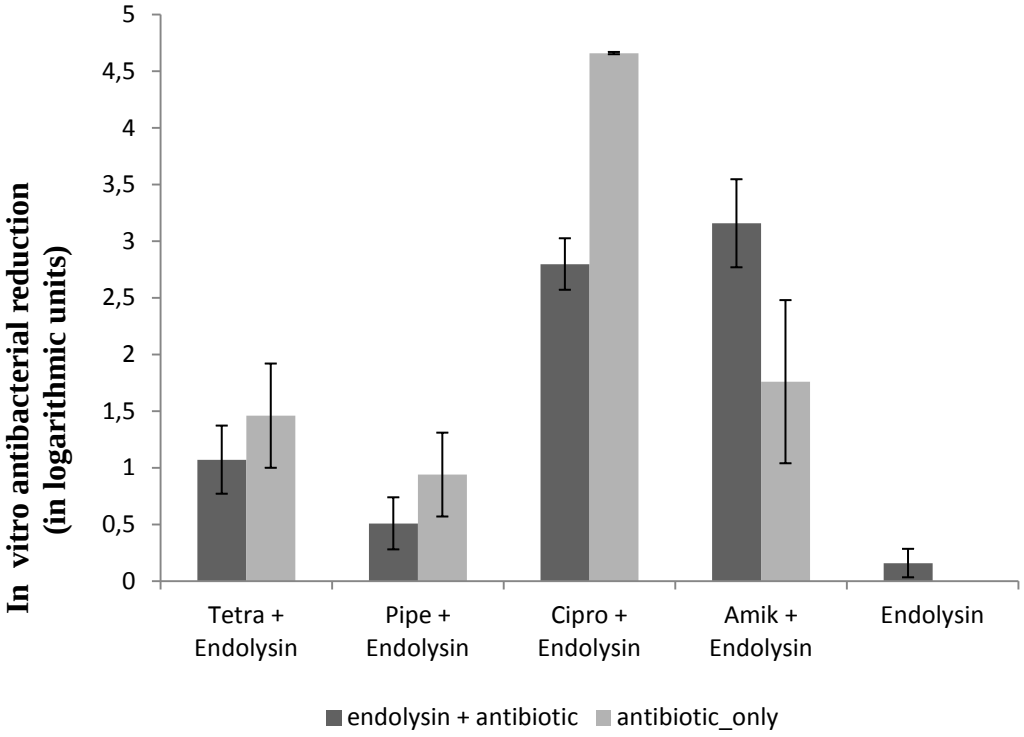

Figure 4.11: Reduction of cells number presented in biofilms of *P. aeruginosa* strain PA01 after the exposure to antibiotic, endolysin and endolysin in combination with antibiotics.

With exception of amikacin, lysins alone and in combination with antibiotics seem to be ineffective against biofilms. One possible explanation can be related with the entry of antibiotics into the cells. Amikacin is the only of the studied antibiotics that enter into the bacterial cell by binding to the LPS structure and causing the rupture of magnesium bridges adjacent to LPS. This situation can enable the creation of a channel that can promote the entry of lysins to the cell, thereby alleviating the effect of the outer membrane. In the other combinations, an antagonistic effect was observed possibly due to a blocking of the porins channel by the endolysins itself, similarly to what happens with polyamines [24]. Coupling a green fluorescent protein (GFP) to the endolysin 68gpLys and observing through the microscope, could be a further step to understand the interaction of endolysins in the cells.

Contrarily to these results, Meng et al (2011) showed that the application of a lysin acted synergistically with several antibiotics and the final result was a maximum dispersal of the *Streptococcus suis* (gram positive) biofilm [25]. Actually, these results prove to be in agreement with the bibliographic data, since the action of endolisins has been mainly directed to gram positive bacteria, due to the presence of a thick peptidoglycan layer.

Overall, these results allow to conclude that the synergy action between phage and antibiotic can be affected by several factors and can be a very complex process. Furthermore, this analysis constitutes a great opportunity to developing additional studies in this area to better realize the interaction between these two agents.

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Chapter 5: Main conclusions and Suggestions for forthcoming work

The main objective of the present work was to analyze the process of synergy between phage and antibiotics against cultures and biofilms of *P. aeruginosa*. For this purpose, the isolated action of antibiotics and phages was first evaluated and after their combination evaluated.

For antimicrobial susceptibility experiments, four antibiotics were used. In general, in spite of having determined a MIC within reference values for all antibiotics, in 24 h biofilms none of antibiotics led to its eradication. Ciprofloxacin was the most effective antibiotic in reducing the number of cells present in the biofilm and piperacillin showed to have a stressful effect on cells causing their elongation, and above all the later antibiotic had a poor bactericidal activity.

Regarding the experiments of phage infection, the reduction of OD in planktonic cultures was not significantly affected by any phage concentration. In biofilms experiments, phages phiIBB-PAP1 and phiIBB-PAP21 appeared to be the most effective, with a reduction of 15-20% of the total biomass of biofilm. Nevertheless, during the infection process phage resistant mutants emerged leading to a lower efficiency in controlling biofilms of *P. aeruginosa*.

In general, it can be affirmed that the individual application of phages and antibiotics, although disrupting the early stages of biofilm formation, do not achieve successful reductions of viable cell numbers in mature biofilms of *P. aeruginosa*.

In the last part of the work, the combination of phages and antibiotics was adopted as a strategy to improve the control of *P. aeruginosa* biofilms. In planktonic cultures, the combined action resulted in MIC values within the reference range. Surprisingly, with the combination of phage phiIBB-PAP21 with antibiotics, the MIC values were significantly lower in relation to the application of antibiotics alone. In biofilms, the association of phage and ciprofloxacin caused a total biofilm removal and the concentration of ciprofloxacin needed to this eradication was drastically reduced, which can be an important step for *in vivo* assays. In addition, other interesting results were obtained for other combinations. For instance, although phage phiIBB-PAC23 had

a poor activity when applied alone, it proved to have potential effect when combined with all tested antibiotics, resulting in significant reductions in the number of viable cells present in biofilms.

On the other hand, the synergy process that develops between phage and antibiotics is not linear, and it seems that several factors are involved. In this work it was possible to conclude that the effectiveness of combined treatments can be due to a higher phage burst sizes in cells exposed to antibiotics (ex. piperacillin), disruption of biofilm matrix by mutual action of phages and antibiotics. Besides, it was found that phage resistant phenotypes have in general lower MIC values and altered surface charges.

In general, the application of combined treatments of phage and antibiotics for the control and elimination of both planktonic cells and biofilms is an successful approach that must be considered in the future as an alternative and a good option in relation to the use of phages and antibiotics alone. However, the knowledge of the possible variables involved in the process of synergy is a fundamental step that should be explored to disclose the interaction of these two agents.

For future studies, and in order to optimize the work performed during this project, the following experiments are suggested:

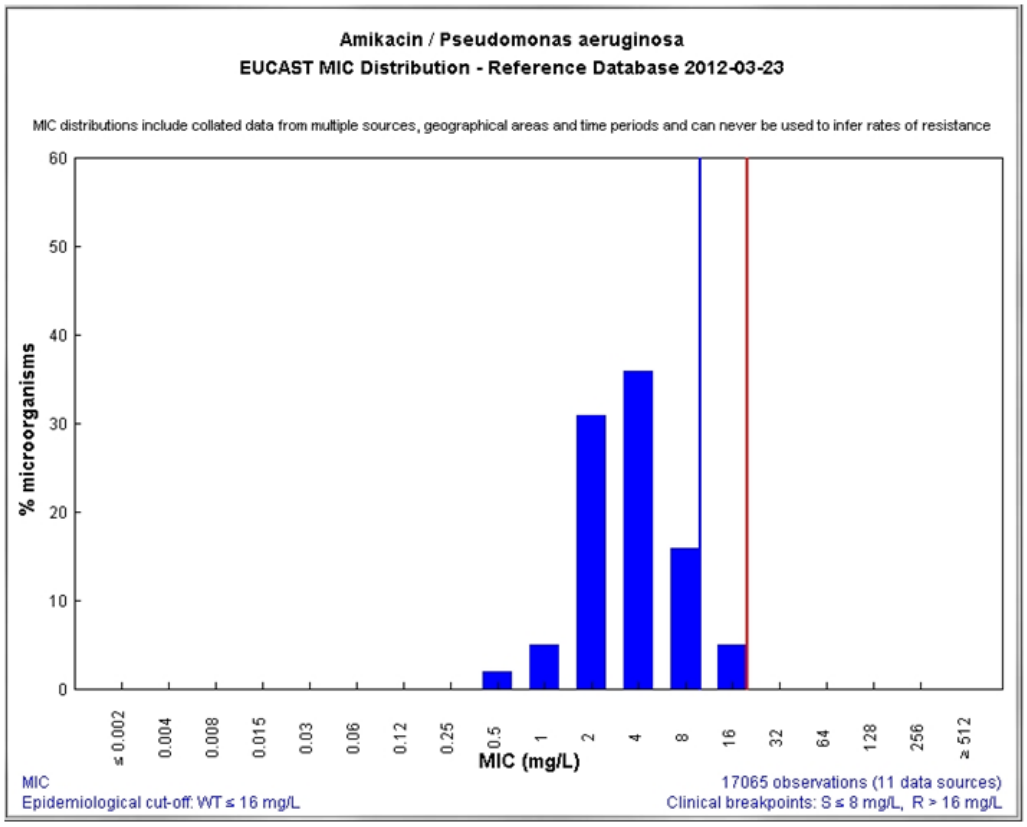
- Additional experiments with a sequential addition of phage and antibiotic. The objective is to compare with the simultaneous treatments performed in the present work and evaluate how the genetic diversity of a bacteria population evolve during the process of adaptation when exposed to phage and antibiotic.
- Application of a cocktail composed of phages, lysins, depolymerases and antibiotics against *P. aeruginosa* biofilms. With this, the objective is to obtain a solution with a greater range of action and which is able to minimize the appearance of resistant phenotypes. The depolymerases would allow a greater diffusion of antibiotics, phage and lysins by destruction of the physical integrity of the biofilm matrix, exposing a higher number of cells to their action.
- Directing the research for biomolecular analysis to evaluate the possible interactions between phages and antibiotics in biofilms to further

understand if the combined action of the two agents results in inactivation or repression of resistance genes.

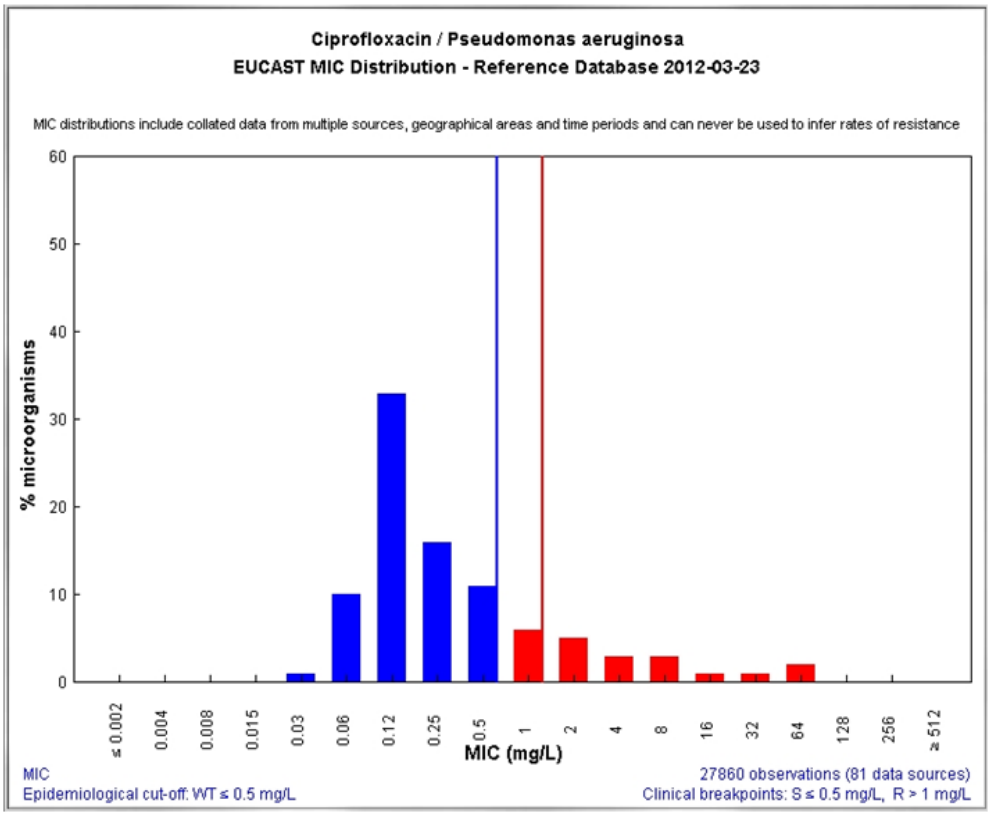
Chapter 6: Appendixes

Appendix 1: Clinical breakpoints defined by EUCAST

Figures A1 and A2 present the MIC distributions for P. aeruginosa.

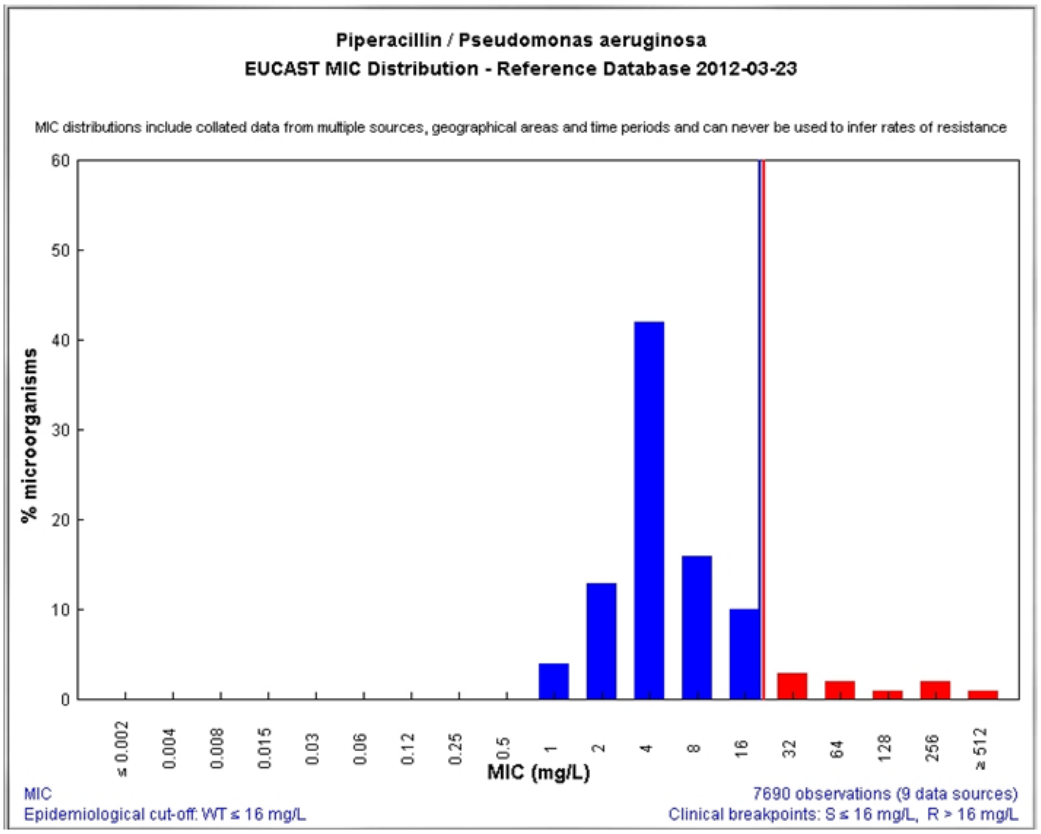


a)

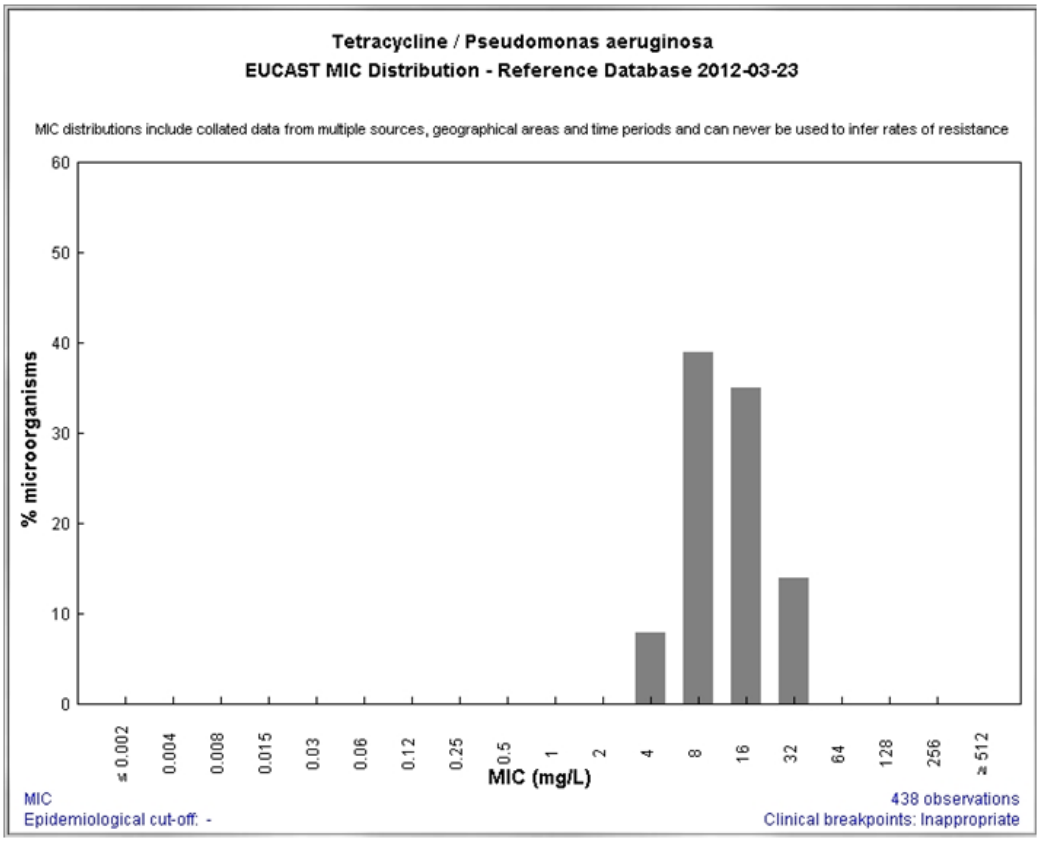


b)

Figure A.1: Clinical breakpoints EUCAST for a) amikacin and b) ciprofloxacin against *P. aeruginosa*.



a)



b)

Figure A.2: Clinical breakpoints EUCAST for a) piperacillin and b) tetracycline against *P. aeruginosa*.