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Phage therapy to inactivate multidrug-resistant *P. aeruginosa*

Terapia fágica para inactivar *P. aeruginosa* multi-resistente

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

o júri

Presidente do Júri

Prof. Doutora Maria Ângela Sousa Dias Alves Cunha
Professora Auxiliar
Departamento de Biologia da Universidade de Aveiro

Vogais

Prof. Doutora Maria Adelaide Pinho de Almeida (orientadora)
Professora Auxiliar
Departamento de Biologia da Universidade de Aveiro

Prof. Doutora Joana Cecília Valente Rodrigues Azeredo (arguente)
Professora Associada
Departamento de Engenharia Biológica da Universidade do Minho

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Anabela Vieira

keywords

Phage therapy, bacteriophage, *Pseudomonas aeruginosa*, multidrug resistant bacteria, human skin, wound infections

abstract

With the increase in antibiotic resistance and after several years of abandonment, the use of bacteriophages (phages), as antimicrobial agents, to destroy bacteria began to arouse interest in the scientific community. This has led to a huge phage research in different fields and currently several studies are ongoing with animals and humans. *Pseudomonas aeruginosa* is an opportunistic pathogen, which frequently colonizes wounds infections. It has been estimated that a high number of deaths caused by wound infections results of bacterial infection, often by antibiotic-resistant *P. aeruginosa.*

The main target of this work was to explore the potential of phages in controlling multidrug-resistant (MDR) *P. aeruginosa* strains *in vitro* and *ex vivo* (human skin).

A new bacteriophages (PA709) was isolated from sewage water samples collected from Hospital Universitário de Coimbra (HUC). A phage suspension (10^8 PFU mL^{-1}) was obtained using the clinical strain *P. aeruginosa* 709 as host. After the characterization of the phage candidate, their capacity to lyse other MDR *P. aeruginosa* clinical isolates from Aveiro, Matosinhos and Coimbra was investigated. The ability of the phage to cause inactivation of *P. aeruginosa* 709 was evaluated *in vitro* and *in ex vivo* (human skin), at 37°C, using a multiplicity of infection (MOI) of 0.5 to 50. In the *in vitro* assays, the effect of a second dose application, added after 4 hours of incubation, was also tested.

The lytic phage PA709 has an icosahedral head with a long contractile tail and a DNA molecule as nucleic acid, a morphology characteristic of members of the Myoviridae family. The phage PA709 show a relatively broad host range (infects 30% of the 51 MDR *P. aeruginosa* clinical isolates), infecting different genotypes isolated in the three hospitals (Matosinhos, Aveiro and Coimbra). For the best MOI, the number of MDR *P. aeruginosa* 709 in the human skin in the presence of the phage decreased 4 logs after 2 hours of incubation. The application of a second dose of phage did not increase the efficiency of the therapy. These results show that the phage PA709 was seen to have rapid lytic activity but the number of bacteria gradually increased after that. The occurrence of lysogeny and the development of resistance of the host to the phages could explain the bacterial re-growth. However, no evidence of lysogeny was observed after addition of mitomycin C and no resistant to PA709 phage was detected.

In conclusion, phage PA709 presents some interesting features, namely high efficiency in the inactivation of MDR *P. aeruginosa* , a broad host range and high stability in stock suspensions and in *ex vivo* human skin. All these attributes make this phage very promising for the treatment of *P. aeruginosa* skin wound infections. However, more phages should be isolated in the future, for the formulation of cocktails which might improve the inactivation efficiency against *P. aeruginosa* human skin infections.

Palavras-chave Terapia fágica, bacteriófagos, *Pseudomonas aeruginosa*, bactérias multi- resistentes, pele humana, infecções da pele

resumo

Com o aumento da resistência aos antibióticos e após vários anos de abandono, o uso de bacteriófagos (fagos), como agentes antimicrobianos, para destruir bactérias começou a despertar interesse na comunidade científica. Isto levou a uma enorme investigação dos fagos em diferentes áreas e actualmente muitos estudos estão em curso usando animais e humanos. *Pseudomonas aeruginosa* é um patogénico oportunista, que frequentemente coloniza infecções da pele. Foi estimado que o elevado número de mortes causado por infecções da pele resulta de infecções bacterianas, muitas vezes por *P. aeruginosa* com resistência aos antibióticos.

O principal objectivo deste trabalho foi explorar o potencial do fago em controlar estirpes de *P. aeruginosa* multi-resistentes (MR) *in vitro* e *ex vivo* (pele humana).

Um novo bacteriófago (PA709) foi isolado da água do esgoto do Hospital Universitário de Coimbra (HUC). A suspensão fágica (10^8 UFP mL^{-1}) foi obtida usando a estirpe clínica *P.* aeruginosa 709 como hospedeiro. Após a caracterização do fago candidato, a sua capacidade em lisar outros isolados clínicos MR de *P. aeruginosa* de Aveiro, Matosinhos e Coimbra foi investigada. A capacidade do fago causar inactivação da *P. aeruginosa* 709 foi avaliada *in vitro* e in *ex vivo* (pele humana), a 37ºC, usando uma multiplicidade de infecção (MOI) de 0,5 a 50. Em ensaios *in vitro*, o efeito da aplicação de uma segunda dose, adicionada após 4 horas de incubação, foi também testada.

O fago lítico PA709 tem uma cabeça icosaédrica com uma cauda longa e contráctil e molécula de DNA como ácido nucleico; morfologia característica dos membros da família *Myoviridae*. O fago PA709 infecta 30% dos 51 isolados clínicos MR de *P. aeruginosa,* indicando uma infecção relativamente ampla de hospedeiros. Para a melhor MOI, o número de *P. aeruginosa* 709 MR na pele humana, na presença de fago, diminuiu 4 logs após 2 horas de incubação. A aplicação de uma segunda dose do fago não aumentou a eficiência da terapia. Estes resultados confirmam que o fago PA709 parece ter uma rápida actividade lítica, mas o número de bactérias aumentou gradualmente depois disso. A ocorrência de lisogenia e o desenvolvimento de resistência do hospedeiro ao fago pode explicar o re-crescimento bacteriano. No entanto, não foi observada a presença de lisogenia após a adição de mitomicina C nem a resistência ao fago PA709 foi detectada.

Em conclusão, o fago PA709 apresenta algumas características interessantes, nomeadamente elevada eficiência em inactivar *P. aeruginosa* MR, uma infecção ampla de hospedeiros e elevada estabilidade na suspensão em stock e na pele humana. Todas estas características fazem este fago muito promissor para o tratamento de infecções na pele de *P. aeruginosa*. No entanto, no futuro mais fagos deverão ser isolados, para obter cocktails de fagos que podem melhorar eficientemente a inactivação contra infecções na pele humana de *P. aeruginosa.*

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List of acronyms and abbreviations

µl	Microliter
µM	Micromolar
CFU	Colonies forming units
DAO	Double Agar Overlay
DNA	Deoxyribonucleic acid
dsDNA	Double strain deoxyribonucleic acid
dsRNA	Double strain ribonucleic acid
FDA	Food and Drug Administration
HIDP	Hospital Infante D. Pedro
HUC	Hospital Universitário de Coimbra
i.m	Injection intramuscular
i.p	Injection intraperitoneal
ICU	Intensive care units
LPS	Lipopolysaccharide
M	Molar
MDR	Multidrug – resistant
mL	Milliter
MOI	Multiplicity of infection
Nm	Nanometre
OD	Optical Density
PBS	Phosphate buffer system
PFU	Plaque forming units
s.c	Injection subcutaneous
ssDNA	Single strain deoxyribonucleic acid
ssRNA	Single strain ribonucleic acid
TSA	Tryptic soy agar
TSB	Tryptic soy broth
ULSM	Unidade local de saúde de Matosinhos

Introduction

1. Introduction

1.1. Bacteriophages

1.1.1. Discovery of bacteriophages

The story of the discovery of bacteriophages or phages has been controversial and subject to many debates. In 1896, in India, Ernest Hankin observed in waters of two rivers the existence of high antibacterial activity against *Vibrio cholera* (Deresinski, 2009). He suggested that an unidentified substance was responsible for this phenomenon. Two years later, identical observation was made by Gameleya, while he worked with *Bacillus subtilis* (Sulakvelidze et al., 2001). These findings have not been explored and, only 20 years later, this topic has again been introduced (Sulakvelidze et al., 2001).

At the beginning of the twentieth century, Frederick Twort and Felix d'Herelle, independently, described entities that could destroy cultures of bacteria. D'Herelle named them bacteriophages. The name was formed from “bacteria” and “phagein” (to eat or devour, in Greek) (Sulakvelidze et al., 2001). In 1917, d'Hérelle published these observations, describing the general procedures for isolation bacterial viruses. The bacteriologist isolated phages for some pathogenic bacteria that caused diseases like cholera (Skurnik and Strauch, 2006). Moreover, d'Hérelle developed the method of quantification of viruses and other theories, including the replication cycle of the phage (Bratbak and Heldal, 1993).

1.1.2. Properties and classification of bacteriophages

Bacteriophages are viruses that infect bacterial cells. It has been estimated that phages are ten times more numerous in the environment than bacteria, making them the most abundant entities on Earth (Ackermann, 2007; Skurnik and Strauch, 2006).

Phages have two essential components, proteins and nucleic acids. Bacteriophage taxonomy is based on their shape, size, proteins as well as on their nucleic acid. Most phages have dsDNA, however, some have ssDNA, dsRNA or ssRNA (Matsuzaki et al., 2005). In total there are 17 families of phages (Figure 1.1) (Ackermann, 2001; Ackermann, 2007; Hanlon, 2007).

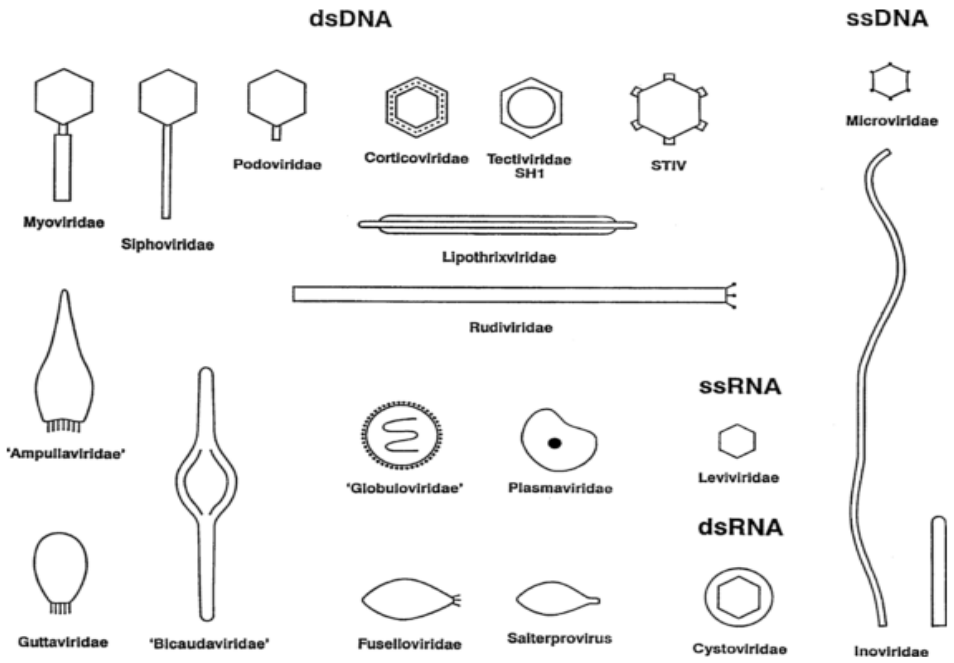

Figure 1.1: Schematic representation of the families described in the classification of bacteriophages (Ackermann, 2007).

Tailed phages are classified into three families and represent about 96% of the phages reported (Skurnik et al., 2007). These phages are composed of an icosahedral head and tail, and all of them have dsDNA (Table 1.1) (Ackermann, 2001; Ackermann, 2007). The Myoviridae family has got a contractile tail, the Siphoviridae family a long tail not

contractile and the Podoviridae family a very short tail. These three families comprise the order Caudovirales (Table 1.1) (Ackermann, 2001; Ackermann, 2007; Hanlon, 2007).

The other families, which only constitute 4% of reported phages, are cubic (polyhedral), filamentous or pleomorphic. They contain ds or ssDNA or RNA as the genome (Table 1.1) (Ackermann, 2001; Ackermann, 2007; Dabrowska et al., 2005).

Table 1.1: Main characteristics of bacteriophages and their classification (Ackermann, 2007).

Order	Family	Shape	Nucleid acid	Morphology
Caudovirales	Myoviridae	Tailed	ds DNA, linear	Tail contractile
	Siphoviridae			Tail long, non contractile
	Podoviridae			Tail short
	Microviridae	Cubic (polyhedral)	ss DNA, circular	Capsomers
	Corticoridae		ds DNA, circular superhelical	Complex capsid, lipids
	Tectiviridae		ds DNA, linear	Inner lipid vesicle, pseudotail
	Leviviridae		ss RNA, linear	Poliovirus-like
	Cystoviridae		ds RNA, linear segmented	Envelope, lipids
	Inoviridae	Filamentous	ss DNA, circular	Long filaments, short rods
	Lipothrixviridae		ds DNA, linear	Envelope, lipids
	Rudiviridae		ds DNA, linear	TMV-like
	Plasmaviridae	Pleomorphic	ds DNA, circular superhelical	Envelope, lipids, no capsid
	Fuselloviridae		ds DNA, circular superhelical	Lemon-shaped
	Salterprovirus		ds DNA, linear superhelical	Lemon-shaped
	Guttaviridae		ds DNA, circular superhelical	Droplet-shaped

1.1.3. Bacteriophage infection

The phages are metabolically inert in their extra cellular form. They are only able to self-reproduce as long as the host bacteria is present and their replication depends exclusively on the host intracellular machinery to translate their own genetic code (Dabrowska et al., 2005; Lorch, 1999).

Viruses can interact with their hosts in two major and distinctive ways, the lytic and lysogenic cycles of infection and more sporadically through pseudolysogeny. However, only lytic phages are suitable candidates for phage therapy since they may destroy bacteria (Almeida et al., 2009; Hanlon, 2007; Weinbauer, 2004).

In the lytic cycle, they multiply in the host cell and lyse the bacterial cell to release newly formed phage particles. Firstly, the phage binds to specific receptors of bacteria (Goodridge, 2010; Weinbauer, 2004). This phase is called adsorption. Phages can use different parts of lipopolysaccharide (LPS), flagella, fimbriae and many other surface proteins as receptors. Bacteriophages may also use enzymes to break down the bacterial surface (Skurnik and Strauch, 2006; Wróblewska, 2006). Then the phage genome is injected into the host bacterium and occurs early gene expression. Most of the proteins produced in this phase are involved in the shutting down of the host bacterium systems and phage genome replication. In some cases, the early proteins degrade the host DNA (Goodridge, 2010; Weinbauer, 2004). After replication of the phage genome, occurs the expression of the phage late proteins that are involved in the formation of new phage particles and lysis of host bacteria (Duckworth and Gulig, 2002). The phage head and tail are assembled and the phage genome is packaged. The bacteria are destroyed through lysis, resulting in an average release of 50 to 200 daughter particles (Huff et al., 2005) (Figure 1.2).

In lisogenic cycle, the phage genome is integrated into the host cell DNA. Prophage DNA will be replicated when the host cell genome replicates and so daughter cells will inherit the viral DNA (Figure 1.2). The prophage can stay in a dormant state for long periods of time and may become activated and turn on the lytic cycle. The lytic cycle is induced spontaneously by chemical or physical agents such as radiation, pollutantes, changes in temperature and nutrient concentrations (Almeida et al., 2009; Weinbauer, 2004). At the end the newly formed phage particles will lyse the host cell. Lysogeny might be a viral survival strategy to ensure periods of low host density during nutrient starvation (Weinbauer, 2004).

There is another phenomenon known as pseudolysogeny. However, unlike true lysogeny, the phage genome does not integrate into the host. Pseudolysogeny is a

condition in which the starved bacterial cell coexists in an unstable relationship with infecting viruses (Figure 1.2). In such host cells, there is insufficient energy available for the phage to initiate genetic expression leading to either a true temperate response or to the lytic response (Ripp and Miller, 1997). As nutrients are supplied to the bacterium, the pseudolysogens resolve into either true lysogeny or active production of virions (lytic cycle). The direct result of pseudolysogenic relationships is an extension of the effective phage half-lives in natural environments (Almeida et al., 2009; Ripp and Miller, 1997). The pseudolysogenic state was found to depend on the concentration of nutrients available to the host. As cells became more starved, the frequency of pseudolysogens increased (Ripp and Miller, 1997; Weinbauer, 2004).

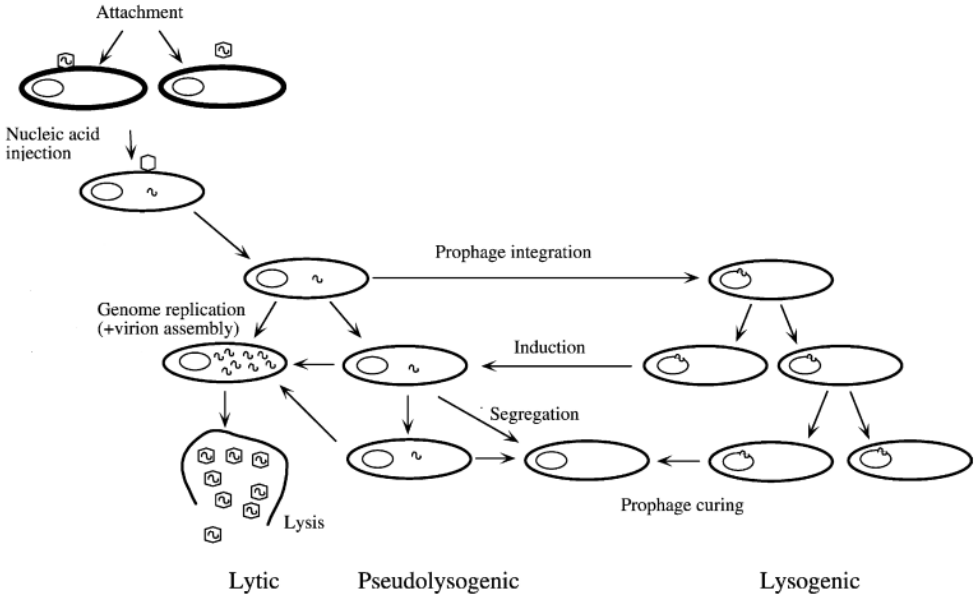

Figure 1.2: General phage life cycle. Adapted from Weinbauer (2004).

1.2. *Human skin* flora and wound infection

Human skin has intrinsic properties that are important to prevent infection and promoting healing in wounds (Church et al., 2006; Cunha, 1998). This organ provides sensation, thermoregulation, biochemical, metabolic, immune functions and physical protection and prevents infection caused by pathogenic microorganisms (Church et al., 2006).

The normal microflora of the skin includes fungi and bacteria. In 1938, Price reported that microorganisms found on the skin can be divided into resident flora, composed of commensals that rarely damage the host, or transient flora which do not grow on skin and reflects the host level of personal hygiene, lifestyle, personal activities and level of environmental contamination (Price, 1938).

The predominant bacterial resident flora of the skin is various species of coagulase-negative staphylococci (*Staphylococcus epidermidis*), *Corynebacterium* spp. and *Propionibacterium* spp. (Cunha, 1998). The Gram-negative bacteria often colonize healthy adult skin include *Proteus* sp., *Enterobacter* sp. and *Klebsiella* sp., *Acinetobacter* spp. and *Pseudomonas* spp., constituting about 25% of the adult skin microflora (Percival et al., 2010).

The bacteria become pathogenic soon they can adhere, grow and invade the host. Typically, soft tissue infections result from disruption of the skin by exogenous factor, extension from subjacent infection or disseminated through the blood stream from a distant site of infection. Most of skin and soft tissue infections are superficial, treated with local care and antimicrobial therapy (Cunha, 1998). Other factors predisposing to skin infections include vascular insufficiency, disrupted venous or lymphatic drainage, diabetes mellitus, previous cellulitis, foreign bodies, accidental or surgical trauma, burns, poor hygiene, obesity and immunodeficiencies (Cunha, 1998).

Pathogens causing initial infections are usually bacterial and the subsequent infections are caused usually by antibiotic-resistant bacteria. Antibiotics alter the balance of natural flora, leaving the surface vulnerable to colonization by exogenous gram-negative bacilli, yeasts and fungi which, usually occurs later due to the use of broad-spectrum antibiotic therapy (Church et al., 2006).

Colonization with organisms, such as Gram-negative bacilli, is not favored. Enzymes and other metabolic products produced by Gram negative bacteria, enhance the invasive potential and the rapid spread of these infections (Church et al., 2006). Moreover, many Gram negative organisms are resistant to antibiotics, which mean it becomes difficult to eradicate (Tredget et al., 2004). Some bacteria are often organized in biofilms. These bioflms can form within 10 - 72 hours and acts as an effective barrier against host defenses and antimicrobial agents. (Kutter et al., 2010; Rode, 2010). In addition, the immunosuppressive state of the patient and the immediate lack of antibodies, allow multiplication of potential pathogens in the wound (Edwards-Jones and Greenwood, 2003).

Infections by Pseudomonas aeruginosa

P. aeruginosa is a non-fermentative, Gram negative bacilli and oxidase-positive. These bacteria is the main pathogen to cause wound infections, remain a major cause of sepsis, morbidity and high mortality (Church et al., 2006). Cause other diseases such as, pneumonia, bacteremia, meningitis, urinary tract infection, skin and soft tissue infections in immunocompromised individuals and hospitalized patients (Wróblewska, 2006). Colonization is more common in the respiratory tract, gastrointestinal tract and skin (Church et al., 2006). It is an opportunist pathogen that is notoriously unresponsive to many antibiotics. *P. aeruginosa* have many virulence factors, including structural components, toxins and enzymes (Table 1.2).

Table 1.2: Virulence factors of *P. aeruginosa* and its biological effects.

Virulence factors	Biological efects	Reference
Capsule	growth as a biofilm; protection from innate and immune defenses	(Drenkard and Ausubel, 2002; Govan and Deretic, 1996)
Pili	adherence to host	(Govan and Deretic, 1996)
Adhesins		
Lipid A	Toxicity	(Govan and Deretic, 1996)
Lipopolysaccharide		
Exotoxins	inhibition of protein synthesis	(Edwards-Jones and Greenwood, 2003; Govan and Deretic, 1996)
Elastase	tissue damage	(Edwards-Jones and Greenwood, 2003; Govan and Deretic, 1996)
Protease		
Phospholipase C		

The capsule is composed by mucoid polysaccharides, which is important for growth as a biofilm in which bacterial cells are protected from innate and immune defenses, and become less susceptible to antimicrobials (Drenkard and Ausubel, 2002; Govan and Deretic, 1996). Its ability to form biofilm has been suggested to cause failure to heal in chronic wounds. The adherence to host is mediated by pili and adhesins (Govan and Deretic, 1996). The presence of lipid A and lipopolysaccharide (LPS) which is a component of the cell wall, enhances the toxicity of this microorganism (Govan and Deretic, 1996). Various toxins and enzymes are secreted, which causes inhibition of protein synthesis and cell death in the host. This causes local necrosis and can cause septicaemia (Edwards-Jones and Greenwood, 2003; Govan and Deretic, 1996).

1.3 Bacterial resistance to antibiotics

Chemotherapy has shown to be a rapid and effective method to treat or prevent microbial infections, but the regular use of antimicrobials has resulted in the development of drug resistance in common pathogenic microbial strains (Towner and Bergogne-Berezin, 1996). Even though novel classes of antibiotics may be developed, the prospect that

bacteria will eventually develop resistance to the new drugs (Lorch, 1999), emphasize that effective antibiotics may not be available to treat seriously ill patients in the near future.

Most antimicrobial agents used are categorized according to their principal mechanism of action. There are five major modes of action, disruption of bacterial membrane structure, interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis and inhibition of a metabolic pathway (Table 1.3) (Tenover, 2006).

Table 1.3: Mechanisms of action of antibacterial agents. Adapted from Tenover (2006).

Mechanisms of action	Antibacterial agents
Disruption of bacterial membrane structure Increase bacterial membrane permeability or membrane depolarization	polymyxins, daptomycin
Interference with cell wall synthesis Inhibit synthesis of the bacterial cell wall by interfering with the synthesis of the peptidoglycan layer	β-Lactams: penicillins, cephalosporins, carbapenems, monobactams Glycopeptides: vancomycin, teicoplanin
Protein synthesis inhibition Inhibit bacterial growth by binding to the 30S or 50S subunit of the ribosome	Macrolides, aminoglycosides, tetracyclines, chloramphenicol, streptogramins, and oxazolidinones
Interference with nucleic acid synthesis Inhibit DNA or RNA synthesis	Fuoroquinolones, rifampin
Inhibition of metabolic pathway Inhibit DNA synthesis	Sulfonamides, folic acid analogues

Multidrug-resistant (MDR) strains can be defined as resistance to at least three classes of the antibiotics used in the treatment of these infections (Wróblewska, 2006). The hospital environment is the main focus for the emergence and spread of MDR bacteria. The emergence of MDR strains, usually occurs due to the selective pressure of antimicrobial therapy, i.e., inappropriate or excessive prescription of these chemicals, the frequent transmission of microorganisms and the truly large variety of mechanisms adopted by microbial cells to increase their resistance (Wróblewska, 2006). The direct relationship

between use of antimicrobial agents and prevalence of resistant bacteria has been documented on several occasions, particularly in Intensive Care Units (ICUs) (Aarestrup, 1999).

Bacteria can adopt mechanisms conferring resistance to antibacterial drugs. Some species of bacteria are innately resistant to one or more class of antimicrobial agents and others become resistant to an antibacterial agent (Wróblewska, 2006). The organism may acquire genes encoding enzymes, such as β-lactamases, that destroy the antibacterial agent before it can have an effect; may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target; may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent; or may acquire mutations that limit access of antimicrobial agents to the intracellular target site (Tenover, 2006; Wróblewska, 2006).

P. aeruginosa are naturally resistant to a number of antimicrobials, such as ampicillin, amoxicillin, amoxicillin/clavulanate, cephalosporins of first and second generation, cefotaxime, ceftriaxone, nalidixic acid and trimethoprim. This intrinsic multidrug resistance occurs due to the synergy between broadly specific drug efflux pumps and the low degree of outer membrane permeability (Livermore, 2002; Pai et al., 2001; Wróblewska, 2006).

Pathogenic bacteria that express multiple mechanisms of antimicrobial resistance, are associated to high financial costs and high mortality and morbidity in humans (Tenover, 2006).

The rising prevalence of antibiotic resistance in wound bacterial pathogens represents a serious therapeutic challenge for clinicians. At the same time, the pace of development of new antibiotics has been inadequate, resulting in a shortage of novel classes of antibacterial agents to eliminate MDR pathogens. This dramatic situation has created an urgent need for developing alternative for controlling such infections, especially wound infections who do not respond to conventional antibiotic therapies. One approach is phage therapy, where the bacteriophages can be applied locally on wounds.

1.4 Phage therapy

Phage therapy is a non-antibiotic approach to inactivate microorganisms. It involves the application of bacteriophages, as antibacterial agents to combat bacterial infections (Duckworth and Gulig, 2002; Sulakvelidze et al., 2001).

1.4.1 Discovery and history of phage therapy

In 1919, the first time in France, d'Herelle applies the phage therapy in the treatment of cholera, obtaining therapeutic success (Lorch, 1999; Sulakvelidze et al., 2001). Phage therapy was vigorously investigated and numerous studies were undertaken to assess the potential of phage therapy for the treatment of bacterial infection in humans and animals (Lorch, 1999; Skurnik et al., 2007; Summers, 2001). Early success prompted the development of multiple commercial phage preparations. For example, in 1940 Eli Lilly Company produced seven phage products for human use (Housby and Mann, 2009). These preparations were used to treat infections that cause abscesses, purulent wounds, vaginitis, acute chronic upper-respiratory tract infections and mastoid infections (Fischetti et al., 2006; Housby and Mann, 2009; Sulakvelidze et al., 2001).

However, with the development of antibiotics in the 1940s, interest in phage-based therapeutics declined in the Western world (Lorch, 1999; Sulakvelidze et al., 2001). Besides antibiotics, the most important factors that contributed to this decline was the lack of standardized testing protocols and methods of production and the beginning of World War II (Górski and Weber-Dabrowska, 2005; Lorch, 1999). Nevertheless, in Eastern Europe and the former Soviet Union, in centers such as the Eliava Institute of Bacteriophage, Microbiology and Virology in Tbilisi, Georgia and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland, where access to antibiotics was limited, the development and use of phage therapy continued jointly with or in place of antibiotics

(Lorch, 1999; Summers, 2001). It is believed that the use of phages in these countries was due to two main reasons: phage therapy was used to treat the wounds of soldiers in World War II and the treatment was cheaper (Lorch, 1999). Much of the knowledge of the application of phage therapy is due to these research centers located in these eastern countries (Lorch, 1999; Summers, 2001).

1.4.2 Pre - requisites for phage therapy

The problems related to the production of phage complicated initial study and research. Diverse stabilizers and preservatives were initially used in attempts to increase the viability of the phage therapeutics (Summers, 2001). However, because the biology of both the phage and the various stabilizers were poorly understood, many of the ingredients added in an attempt to prolong the viability of phage preparations proved to be either toxic to humans (Summers, 2001).

Another problem related to phage production was the purity grade of the preparations of these viruses. At the time, phage therapy preparations generally consisted of lysates of host bacteria that had been treated with the phage of interest (Skurnik et al., 2007). Thus, many preparations contained bacterial components (endo-and exotoxins) and products of lysis of the host that can cause some allergies or toxic effects when applied in humans (Skurnik et al., 2007). Accordingly, adverse events were often associated with the preparations, particularly in patients receiving them intravenously (Lorch, 1999).

Today, microbiologists are aware of the need for advanced purification techniques to purify phages and to ensure that they are bacterium free. The viability and titer of phages should be determined before using them therapeutically (Skurnik et al., 2007; Sulakvelidze et al., 2001; Summers, 2001). The minimum requisites needed to use the phage in phage therapy, in order to minimize possible complications are summarized in the Table 1.4.

Table 1.4: Pre – requesites needed to use the phage in phage therapy.

Pre - requisites for phage therapy	
Free of products of lysis	techniques to purify phages
Well characterization phage	structure, behavior *in vitro* and *in vivo*
Lytic	lysogenic phages may carry genes that encode toxins or virulence factors
Broad host range	infecting members of the target species and/or genus
Complete genome sequences know	absence of any genes encoding pathogenicity associated or potentially allergenic proteins
Sufficiently stable over storage and application	determination of viability
Amenable to scale up for commercial production	efficacy against specific bacterial and no side-effects

The phages used in phage therapy should be characterized in detail. It is necessary to sequence the genome of the phage, to identify its structure, test its behavior *in vitro*, and especially to prove their efficiency *in vivo*. Ideally, in the first place, should be tested in an animal model (Skurnik and Strauch, 2006).

For phage therapy, lytic phages should be used and the development of lysogeny must be avoided. When lysogeny is established the host becomes immune to an infection caused by the same phage or phage related (Gill and Hyman, 2010). In addition, lysogenic phages may carry genes potentially dangerous from one host to another, such as genes that encode toxins or virulence factors, which may be toxic to humans (Alisky et al., 1998; Skurnik and Strauch, 2006; Sulakvelidze et al., 2001). For these reasons, we should sequence the whole genome of the phage, which will allow us to identify genes associated with presence of lysogenic cycle, such as the integrase and repressor gene (Skurnik et al., 2007).

1.4.3 Advantages and disadvantages of phage therapy

Advantages

There are several potential advantages of phage therapy over chemotherapy (Table 1.5).

Table 1.5: Main advantages of the phage against the antibiotics

Phages	Antibiotics
Very specific	Affects normal microflora
Low resistance	High resistance
Concentrated at the local of infection	May not concentrated at the local of infection
Low costs	High costs
No serious side effects	Multiple side effects
One single dose	Multiple doses

Phages are very specific to the target, while the antibiotics destroy pathogenic microorganisms and normal microflora. This affects the microbial balance in the patient, which may lead to serious secondary infections (Vinodkumar et al., 2008). The specificity of the host usually occurs at the level of strain, at the species level and rarely at the level of genus (Hagens and Loessner, 2010). The host range of phages is determined by receptors on the surface of the bacterium, allowing the binding of phage to bacteria (Skurnik and Strauch, 2006; Wróblewska, 2006). Therefore, first for an appropriate phage treatment, it will be necessary to identify the bacteria causing the infection and know which phages that infect bacterial strains. Secondly, it will be necessary to create databases with hundreds or thousands of phage preparations with different specificities (Balogh et al., 2010).

They have limited resistance development and selecting new phages is a relatively rapid process that can frequently be accomplished in days or weeks, while the antibiotics quickly become resistant to bacteria and the development of new antibiotics may take several years (Harcombe and Bull, 2005; Skurnik and Strauch, 2006; Sulakvelidze et al., 2001).

They are safe, no serious side effects have been described, because phages or their products (amino acids and nucleic acids) do not affect eukaryotic cells (Abedon and Thomas-Abedon, 2010; Gorski et al., 2003).

The phages have the capacity to self-multiply at the site of infection, while the antibiotics do not necessarily concentrate at the site of infection (Skurnik et al., 2007). Systemic antibiotic therapy has little utility in patients with extensive wounds, because of poor penetration of the antibiotic into the wound, being the infection difficult to eliminate (Kutter et al., 2010). The reproductive ability of bacteriophage, avoids this problem. This makes phages ideal for wound treatment, in contrast to antibiotics, whose concentration decays rapidly with distance from the source and are eliminated by metabolic degradation or excretion (Brussow, 2005). Due to self-replication of the phage, the pharmacokinetics are problematic. The *in vitro* growth data for a phage cannot be directly applied to the *in vivo* situation and the *in vivo* data for one phage cannot be transferred to another phage. The use of phages as drugs may differ from antibiotics due to differences in the phage pharmacokinetics, which becoming the great challenge of phage therapy (Payne and Jansen, 2003). In simulations of the population and evolutionary dynamics of the phage–bacteria interactions, the phage can eliminate all of the host bacteria in the culture. However, in reality, this cannot happen. There are, at least, three reasons for this not happen. First, the phages do not infect the host bacteria when their density is below the host cell threshold (Comeau et al., 2008). Second, the host may develop resistance to the phage (Levin and Bull, 2004). Third, the bacterial population might reach stationary phase and therefore might be physiologically refractory to the phage (Levin and Bull, 2004). However, *in vivo* the combination of phage and the host defenses are sufficient to keep the bacterial density below lethal threshold after phage therapy. Phage therapy only needs to

decrease the numbers of infecting bacteria to a level from which the host defenses can take care of the remaining bacteria (Levin and Bull, 2004).

Finally, phage therapy is a technology flexible, fast, cheap and efficient against MDR pathogens, since the mechanism used by phage to lyse the bacteria is different from those used by antibiotics (Matsuzaki et al., 2005; Sulakvelidze et al., 2001).

Disadvantages

One of the disadvantages of phage therapy is the possible development of bacterial resistance to the phages. In phage infection, one essential step is the attachment of the phage onto specific receptors of bacteria. By mutating in the gene that encodes a bacterial product essential for losing the phage receptor, bacteria become resistant to phages (Levin and Bull, 2004; Skurnik and Strauch, 2006). However, this resistance cannot be serious. If the receptor used by the phage is a virulence determinant, loss of the receptor would decrease the virulence of the bacterium, and then it would be easier for the host immune system to eliminate the pathogen (Levin and Bull, 2004; Skurnik and Strauch, 2006). Furthermore, even if the bacteria becoming resistant to a particular phage is easier to find a new phage that can infect the pathogen than a new antibiotic (Harcombe and Bull, 2005; Skurnik and Strauch, 2006). In addition, the rate of mutation and replication is higher in the phage, which can overcome the adaptation of bacteria (Deresinski, 2009). Finally, according to some authors, the rate of development of bacterial resistance to phage is 10 times less than the antibiotics (Carlton, 1999; Sulakvelidze et al., 2001). This rate may be much smaller if provided different phages in the same phage preparation. These cocktails of phages can be composed of two or more phages that use different receptors to infect bacteria of the same species or pathogenic bacteria more common for that particular infection (Goodridge, 2010).

The lysogenic conversion can be another problem when phages are used to infected bacteria. When lysogeny is established the phenotype of the host cell can be altered. The temperate phage (prophages) can express some genes that can result in the production of toxins and antibiotic resistance (Alisky et al., 1998; Skurnik and Strauch, 2006; Sulakvelidze

et al., 2001). In addition, this host becomes resistant to infection by the same or similar strains of phages (Gill and Hyman, 2010).

Another drawback is the possibility of phage particles were remove by the circulatory system of the host, i.e., phages can be neutralized by antibodies. However, first, the problem can be solved if it was prepared several phage strains with different antigens (James et al., 2004). Second, Duckworth and Gulig (2002) suggest that the kinetics of phage action is much faster than the production of antibodies by the host. Therefore, this neutralization is not significant during the initial treatment of infections. The phage therapy is complete before developing specific immunity (Duckworth and Gulig, 2002).

1.4.4 Studies and applications developed in phage therapy

1.4.4.1 Eastern Europe and the former Soviet Union

In 1923 was founded the first institute of research on phage therapy, the Institute of Bacteriophage, Microbiology and Virology in Tblisi. Since 1950, the problem of antibiotic resistance was also known in the Union Soviet. Most resistant bacteria samples isolated in the Soviet Union were sent to Tblish in order to find phages corresponding to these bacteria (Lorch, 1999). Thousands of monophages and cocktail of phages (pyophage and intestiphage) for pathogenic bacteria strains, such as *Staphylococcus*, *Streptococcus*, *Proteus*, *Pseudomonas aeruginosa* and *Clostrium* were prepared (Kutateladze and Adamia, 2008; Lorch, 1999).

Scientist of the Eliava Institute continually renewed the cocktail *pyophage* and *intestiphage* with new phages against the most frequent and virulent strains for the prevention and treatment of wound infection and enteric bacteria, respectively (Kutateladze and Adamia, 2008). For deeper wounds, phages embedded in polymer called *PhageBioderm* is often used in addition to *pyophage* wound irrigation. *PhageBioderm* is a biodegradable, non-toxic polymer developed by Georgian chemists and microbiologists

since 1995 and approved for commercial release in 2000 (Kutateladze and Adamia, 2008; Kutter et al., 2010). As a result, very broad-range and effective bacteriophage preparation were obtained and the phage sensitivity of the infections was more than 85%. These preparations were used immediately for empiric phage therapy even before the bacterial sensitivity of the phage had been tested (Kutter et al., 2010).

Research on bacteriophages was not limited to the Eliava Institute. For instance, one well-documented clinical phage therapy was carried out at the *Institute for Immunology and Experimental Medicine,* in Poland. While the western scientific community contributed to exchanging scientific results in English, the scientists of the Soviet Union were not included in the scientific community (Gorski et al., 2003; Lorch, 1999). However, some of these studies and their applications are being translated and provided to English-speaking scientists.

This institute, in Poland, has administrated phages against a variety of target microorganisms responsible for a number of diseases. They have a *phage-bank*, where they can choose one or more phages from their collections, which are active against a given bacterial isolate. Reportedly the Institute *phage-bank* presents over 300 specific bacteriophage strains against staphylococci, enterococci, *Escherichia* sp., *Klebsiella* sp., *Salmonella* sp., *Shigella* sp., *Enterobacter* sp., *Proteus* sp., *Serratia* sp., *Acinetobacter* sp. and *Pseudomonas* sp. (Kutter et al., 2010).

In the past, phage were administered orally, topically or systemically to treat a wide variety of infections, such as suppurative wound, gastroenteritis, sepsis, osteomyelitis, dermatitis, emphysemas and pneumonia (Alisky et al., 1998; Sulakvelidze et al., 2001).

Some of the clinical applications carried out in the Eastern Europe and former Soviet Union are summarized in Table 1.6.

Table 1.6: Clinical applications of phage therapy in Eastern Europe and the Soviet Union. Adapted from Sulakvelidze et al. (2001)

Reference(s)	Infection(s)	Etiologic agent(s)	Comments
(Babalova et al., 1968; Miliutina and Vorotyntseva, 1993; Tolkacheva et al., 1981)	Bacterial dysentery and salmonellosis	*Shigella, Salmonella, E. coli* and *Proteus*	The combination of phages and antibiotics was reported to be effective in treating cases where antibiotics alone were ineffective (Miliutina and Vorotyntseva, 1993).
(Bogovazova et al., 1992; Cislo et al., 1987; Kochetkova et al., 1989; Sakandelidze, 1991; Weber-Dabrowska et al., 2000; Zhukov-Verezhnikov et al., 1978)	Infections of skin	*Pseudomonas , Staphylococcus., Klebsiella* spp*., Proteus, E. coli* and *Streptococcus*	31 patients having chronically infected skin ulcers were treated orally and locally with phages. The success rate was 74% (Cislo et al., 1987). 65 patients received phages and the rest received antibiotics. Phage treatment was successful in 82% of the cases, and antibiotic treatment was successful in 61% of the cases (Kochetkova et al., 1989).
(Ioseliani et al., 1980; Meladze et al., 1982)	Lung and pleural infections	*Staphylococcus, Streptococcus, E. coli* and *Proteus*	Phages were used to treat 223 patients and the results were compared to 117 cases where antibiotics were used. Full recovery was observed in 82% of the patients in the phage-treated group, as opposed to 64% of the patients in the antibiotic-treated group (Meladze et al., 1982).
(Perepanova et al., 1995)	Inflammatory urologic diseases	*Staphylococcus, E. coli,* and *Proteus*	Adapted phages were used to treat acute and chronic urogenital inflammation in 46 patients. The efficacy of phage treatment was 92% (marked clinical improvements) and 84% (bacteriological clearance) (Perepanova et al., 1995).
(Sakandelidze, 1991)	Infectious allergoses (rhinitis, pharyngitis, dermatitis, and conjunctivitis)	*Staphylococcus, Streptococcus, E. coli, Proteus, enterococci,* and *P. aeruginosa*	360 patients were treated with phages, 404 patients with antibiotics 576 patients with combination of phages and antibiotics improvement was observed in 86, 48 and 83% of the cases, respectively (Sakandelidze, 1991).
(Stroj et al., 1999)	Cerebrospinal meningitis	*K. pneumonia*	Orally administered phages were used successfully to treat meningitis in a newborn (after antibiotic therapy failed) (Stroj et al., 1999).

1.4.4.2 West Europe

Phage therapy research will gain momentum, while traditional antibiotic research has come to a stop in West Europe. Appropriately selected phages can easily be used to help prevent bacterial diseases in humans or animals, with potential for alternative applications and special interest for developing countries (Lorch, 1999).

The use of bacteriophage therapy requires, however, a detailed understanding of the phage-bacteria interaction and of the awareness of various novel kinetics phenomena not known in conventional drug treatments and not considered in the Eastern Europe studies (Bull et al., 2002; Levin and Bull, 2004). Kinetics theory of phage therapy predicts that the average number of phage per bacterium, that is, the multiplicity of infection (MOI), the number of phage dose applications and the timing of the phage application are important in phage therapy and are now being studied in the west countries.

In-vitro test

One critical parameter that affects phage therapy is the initial phage dose that is the multiplicity of infection (MOI). High MOI is used when the experiment requires that every cell in the culture is infected, that is, the case of phage therapy. By contrast, low MOI is used when multiple cycles of infection are required. *In vitro* studies allows to study what the most appropriate MOI in order to obtain an effective inactivation of the host. It has been shown *in vitro* conditions that the reduction of pathogenic bacteria increased with the increase of the MOI (Table 1.7). Tanji et al. (Tanji et al., 2005) showed that, *in vitro*, *Escherichia coli* concentration did not change after phage addition at a MOI of 1. When applied at a MOI of 10^4, the bacterial density decreased 5 logs. Andreatti Filho et. al (Andreatti Filho et al., 2007) showed that the number of viable *Salmonella enteritidis* decreased 4 logs at a MOI of 100. However, at a MOI of 10^6 the bacterial density decreased 7 logs.

All the literature reviewed, the number of phage doses applications and the timing of the phage application were not tested *in vitro*.

Another critical parameter that should be tested *in vitro* is the host resistance developed to the phages. In most studies, the resistance of bacteria to the phage is not tested (Table 1.7). In several *in vitro* studies (Andreatti Filho et al., 2007; Kumari et al., 2010; Tanji et al., 2005; Watanabe et al., 2007) it was observed a gradually increased in the bacterial number during the experiments of phage therapy. The authors speculate that these results may suggest the emergence of strains resistant to the phage. However, they do not actually test experimentally the development of bacterial resistance. Nevertheless, Loc Carrillo et al. (2005) concluded that *Campylobacter jejuni* develop resistance to two different phages after a phage therapy experiment.

Table 1.7: *In vitro* study recently developed in West Europe

Host	Phage	MOI	Result	Observation		Reference
Escherichia coli	SP15, SP21, SP22	1	No reduction	---	Resistance: Speculate (no tested)	(Tanji et al., 2005)
		10^4	Reduction of 5 logs after 8 hours of incubation	A gradual increase in bacterial was observed		
Klebsiella pneumoniae	Kpn5	0.1	Reduction of 6 logs after 3 hours of incubation	A gradual increase in bacterial was observed	Resistance: Speculate (no tested)	(Kumari et al., 2010)
Salmonella enteritidis	WT45Ø	100	Reduction of 4 logs after 6 hours of incubation	A gradual increase in bacterial was observed	Resistance: Speculate (no tested)	(Andreatti Filho et al., 2007)
		10^6	Reduction of 7 logs after 6 hours of incubation			
Campylobacter jejuni	CP34	300	Reduction of 3 logs after 8 hours of incubation	A gradual increase in bacterial was observed	Resistance: 11%	(Loc Carrillo et al., 2005)
Pseudomonas aeruginosa	KPP10	1	Reduction of 4 logs after 150 min of incubation	A gradual increase in bacterial was observed	Resistance: Speculate (no tested)	(Watanabe et al., 2007)
Pseudomonas aeruginosa	MPK1	10	Reduction of 5 logs after 30 min of incubation	A gradual increase in bacterial was observed	Resistance: not referred	(Heo et al., 2009)
	MPK6	10	Reduction of 4 logs after 1.5 hours of incubation			

Ex-vivo test

To understand the phage-bacteria interaction *in vitro* tests are not sufficient, being necessary to resort to in *ex vivo* tests. The *ex vivo* tests do not fully mimic the *in vivo* growth conditions, but these tests allow experimentation under identical conditions than *in vivo* and under more controlled conditions. This methodology combines the advantages of *in vivo* with the flexibility of the *in vitro.*

Up to my knowledge, there are a few *ex vivo* studies available in current literature (Atterbury et al., 2003; Goode et al., 2003). Goode et. al (Goode et al., 2003) showed that no *Salmonella* spp. or *Campylobacter jejun* were recovered, when they distributed on the surface of the chicken skin phage and their hosts at a MOI of 10^5. Atterbury et. al (Atterbury et al., 2003) demonstrated that Campylobacter jejuni decreased 1 log, after application of phage at a MOI of 10, on artificially contaminated chicken skin.

All the literature reviewed, the number of phage doses applications, the timing of the phage application and the host resistance developed to the phages were not tested *in ex vivo*.

In-vivo test

As *in vivo*, the appropriate MOI must be tested to effectively reduce the number of viable pathogenic bacteria and increase the survival rate of the animal model. It has been shown *in vivo* conditions that the survival rates increased with the increase of the MOI (Table 1.8). Huff et. al (Huff et al., 2005) demonstrated that mortality was significantly reduced from 85 to 35% at a MOI of 1, and the birds were completely protected when the challenge culture was mixed with 10^8 PFU/ml of bacteriophage, MOI of 10,000. Wang et. al (Wang et al., 2006) studied the dose effect of phage in rescuing mice from lethal imipenem-resistant *P. aeruginosa* bacteremia and showed that higher doses of the phage, MOI of 0.01-200, 100% of the animals survived. As the phage dose decreased, MOI of 0.0001 and 0.001, the animals became critically ill, showing survival rates of 0 and 20%, respectively.

The *in vivo* studies have been showed that the application of a single dose appears to be sufficient to control bacterial growth, contrarily to antibiotics (Table 1.8). Biswas et. al (Biswas et al., 2002) demonstrated that a single intraperitoneal injection of 10^8 PFU of the phage rescued 100% of *Enterococcus faecium* bacteremic mice. A similar study conducted by Smith and Huggins (Smith and Huggins, 1982) showed that all mice infected by *E. coli* survived with a single intramuscular dose of anti-K1 phage.

Another critical parameter that can be well evaluated *in vivo* studies is the timing of the phage treatment. When the phage is administered too early, the phage will be released from the body before it reaches the replication threshold. When the phage is administered too late, the phage will not be effective (Table 1.8). Study by Smith and Huggins (Smith and Huggins, 1982) showed that when phages were administered in the same time that the mouse was infected with the *E. coli*, all mice survived. However, if the phage was administered two days after, 19 mice survived, and this number decreased to 1, when the phage was administered 7 days later. Another study done by this group showed that administration of phage 6 hours before or 18 hours after infection with *E. coli*, the mice developed diarrhea (Smith et al., 1987). This symptom did not happen if the phage was administered between 10 minutes before and 12 hours after infection with *E. coli*.

The ability of phage to reach the infection site and access the host is another critical parameter that affects the phage therapy that can be studied *in vivo* using animal models (Table 1.8). Several studies show that for the same type of infection, the phage can be applied through different routes and some of them are more suited than others (Jikia et al., 2005; McVay et al., 2007). McVay et. al (McVay et al., 2007) showed that the location where the phages were injected change the survival rate of mice. The mice was subjected to burn wound injury and to fatal infection with *P. aeruginosa*. Then, a phage cocktail was administered intramuscular (i.m.), subcutaneous (s.c.) or intraperitoneal (i.p.). The i.p. route providing the most significant protection (87%) of the routes tested. The phages administered by the i.p. route were delivered at a higher dose, earlier and for a more sustained period of time than the phages administered by the i.m. or s.c. route. Moreover, studies have already been made in implementing the phage locally to eliminate wound

infections instead of injecting, also showing good results. Study by Jikia et. al. (Jikia et al., 2005) demonstrated that infections in human skin caused by *Staphylococcus aureus* were eliminated with the application of polymers embedded in a phage solution.

Table 1.8: *In vivo* study recently developed in West Europe

Host	Animal model	Phage/ administration	MOI	Results	Reference
Pseudomonas plecoglossicida	fish	Cocktail phage Orally	1	Survival rates: 80%	(Park and Nakai, 2003)
Pseudomonas aeruginosa	mice	KPP10 Orally	100	Survival rates: 66.7%	(Watanabe et al., 2007)
Pseudomonas aeruginosa	mice	Cocktail phage intraperitoneal injection	10^6	Survival rates: 87%	(McVay et al., 2007)
		intramuscular injection	10^6	Survival rates: 28%	
		subcutaneous injection	10^6	Survival rates: 22%	
Pseudomonas aeruginosa	fly	MPK6 Orally	1	Survival rates: 20%	(Heo et al., 2009)
Pseudomonas aeruginosa	mice	ΦA392 intraperitoneal injection	0,01	Survival rates: 100%	(Wang et al., 2006)
			0,0001	Survival rates: 0%	
Escherichia coli	mice	K12.K1 intramuscular injection	10	Survival rates: 94%	(Smith and Huggins, 1982)
Escherichia coli	chickens and calves	R Orally intramuscular injection	1	Survival rates: 100%	(Lavigne et al., 2003)
Escherichia coli	chickens	SPR02 Aerosol	1	Survival rates: 65%	(Huff et al., 2005)
			10^4	Survival rates: 100%	
Escherichia coli	chickens	F78E Orally	10	Survival rates: 25%	(Oliveira et al., 2010)
Escherichia coli	mice	Cocktail phage Orally	1	Survival rates: 50%	(Smith et al., 1987)
Enterococcus faecium	mice	ENB6 intraperitoneal injection	0.1	Survival rates: 100%	(Biswas et al., 2002)
Staphylococcus aureus	mice	MSa intraperitoneal injection	10	Survival rates: 97%	(Capparelli et al., 2007)
Klebsiella pneumonia	mice	KΦ1 intraperitoneal injection	100	Survival rates: 100%	(Malik and Chhibber, 2009)
Klebsiella pneumonia	mice	SS intranasal inhalation	100	Survival rates: 100%	(Chhibber et al., 2008)
Klebsiella pneumoniae	mice	Kpn5 intraperitoneal injection	10-200	Survival rates: 96.6%	(Kumari et al., 2010)
			0.1	Survival rates:53.33%	
			0.01	Survival rates:13.33%	
			0.001	Survival rates: 0%	

Clinical trails

Although the process of reintroduction of phage therapy in the West has been delayed, recently clinical cases in the West were conducted, which show the advances in clinical application of phage therapy (Table 1.9).

A recent a study, done in Switzerland, with human volunteers receiving phage T4 indicated that it is safe for oral administration (Bruttin and Brussow, 2005). No phage or phage T4-specific antibodies was detected in feces and in the serum of the human subjects. The number of *E. coli* in feces did not decrease and no adverse events related to phage application were reported.

In the United Kingdom, Marza and colleagues (Marza et al., 2006) reported the case of a 27 year old male with 50% surface area burns and skin grafts was applied. After several months, the patient became infected with *P. aeruginosa* and grafted areas broke down rapidly, despite appropriate antibiotic treatment. Therefore, treatment with phages was started. Three days after phage application, *P. aeruginosa* could no longer be isolated from swabs and subsequent extensive grafting was successful.

Chronic otitis is a very common disease and very difficult to treat. Here, *P. aeruginosa* are often largely organized into biofilms and relatively protected from both antibiotics and immune cells, being particularly hard to eradicate. The *Biocontrol* scientists conducted a successful trial of phage against *Pseudomonas* dog ear infections (Wright et al., 2009). The results of that trial were necessary to obtain regulatory approval for a phase I/II in human trial. In humans infected with *Psedudomonas* sp., they applied a single dose of a phage cocktail with six different phages. The controlled clinical trial of a therapeutic bacteriophage preparation showed efficacy and safety. The company is now pursuing a phase III trial in the near future.

Another phase I clinical study was performed to treat patients with venous leg ulcers (Rhoads et al., 2009). The cocktail phage, developed by *Intralytix*, contained eight individual phages (five were lytic for *P. aeruginosa*, two for *S. aureus* and one for *E. coli*).

Forty two patients with full thickness venous leg ulcers of over 30 days duration were included in the study. Patients received 50 ml of either diluted phage preparation or of sterile saline. Results of the study revealed no significant differences was determined between the test and control groups for frequency of adverse events, rate of healing, or frequency of healing. Efficacy of the preparation will need to be evaluated in a phase II. Some pre-clinical studies are already in study to inactive different bacteria, namely methicillin-resistant *S. aureus* strains (Table 1.9).

Table 1.9: Clinical trials of phage therapy in West Europe

Clinical infection/ bacterial agent	Product	Stage of development	References
Healthy human	Orally phage	Pre-clinical	(Bruttin and Brussow, 2005)
P. aeruginosa Burns infections	Discs soaked with phage solution	Phase I/ II	(Marza et al., 2006)
P. aeruginosa Ears infections	Cocktail of phage	Phase I/ II	(Wright et al., 2009)
Venous leg ulcer infections	Cocktail of phage	Phase I	(Rhoads et al., 2009)
Mycoplasma mycoides	Vaccines (orally phage)	Phase I/ II	(March et al., 2006)
E. coli, Staphylococcus sp., *Streptococcus* sp., *Pseudomonas* sp.	Phage for clinical trials	Pre-clinical	(BiophagePharma)(Canada)
S. aureus	Phage for clinical trials	Pre-clinical	(Gangagen) (India and USA)
Methicillin-resistant *S. aureus*	Phage for clinical trials	Pre-clinical	(Novolytics) (United Kingdom)
Methicillin-resistant *S. aureus, C . difficile*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*	Phage products	Pre-clinical and phase I	(PhicoTherapeutics) (United Kingdom)
Pseudomonas sp	Phage for clinical trials	Pre-clinical	(PhageBiotech) (Israel)
Methicillin-resistant *S. aureus* and *P. aeruginosa*	Phage for clinical trials	Pre-clinical	(SpecialPhageHoldings) (Australia)
S. aureus. Wound, systemic and respiratory infections	Phage for clinical trials	Pre-clinical	(Viridax) (USA)

In Portugal, there are two major companies also involved in the investigation of phage products, the *Technophage* SA in Lisbon and the *Innophage* in Oporto.

1.4.5 Phage therapy studies against Pseudomonas aeruginosa

Different phages have been tested to inactivate a variety of *P. aeruginosa* strains (Table 1.10) and in general, all these phage-bacteria interaction studies reveal that phages are capable of decreasing the number of viable bacteria, increasing the survival rate of the hosts (*in vivo* studies). Most of these studies did not evaluate the development of resistance by the bacteria (Table 1.10). The resistance development was only studied in two of the 28 studies considered in this revision and the results are very discrepant. Further studies are necessary to evaluate the importance of resistance development during phage therapy.

Table 1.10: Use of bacteriophages to control *Pseudomonas aeruginosa*

References	*Infections*	*Tested*	*Comments*	*Resistance*	*Phage(s)*
In vivo					
(Merabishvili *et al.*, 2009)	burn wound	Humans	Stable, sterile and no cytotoxic	-	Cocktail-BFC1
(Rhoads et al., 2009)	venous leg ulcers	Humans	Efficacy and safety	-	-
(Wright *et al.*, 2009)	chronic otitis	Humans	Efficacy and safety	-	Biophage-PA
(Marza *et al.*, 2006)	burn wound	Humans	No recovered *P. aeruginosa*	-	-
(Marza *et al.*, 2006)	chronic otitis	Dog	No recovered *P. aeruginosa*	-	-
(Hawkins *et al.*, 2010)	otitis	Dog	Redution: 56%	-	Cocktail
(Heo *et al.*, 2009)	systemic infection	Fly	Survival: 20%	-	MPK6
(Soothill, 1994)	burn wound	Guinea-pig	Control *P. aeruginosa*	-	-
(Velásquez, 2011)	thermal injuries	Mice	Survival:100%-28%	-	Pa02
(Heo *et al.*, 2009)	peritonitis sepsis	Mice	Survival:100%-40%	-	MPK6, MPK1
(McVay *et al.*, 2007)	burn wound	Mice	Survival:87%-22%	Resistance: 0%	Cocktail
(Vinodkumar *et al.*, 2008)	septicemia	Mice	Survival:100%	-	CSV-31.
(Wang *et al.*, 2006)	bacteremia	Mice	Survival:100%	-	ΦA392
(Watanabe *et al.*, 2007)	gut-derived Sepsis	Mice	Survival:67%	-	KPP10

(Soothill, 1992)	septicemia	Mice	Survival:100%	-	BS24
(Morello *et al.*, 2011)	cystic Fibrosis	Mice	Survival:100%-20%	-	PAK-P3
(Debarbieux *et al.*, 2010)	lung infections	Mice	Survival:100%	-	PAK-P
(Hagens *et al.*, 2004)	septicemia	Mice	Survival:100%	-	Pf3R, Pt1
In vitro					
(Ripp and Miller, 1998)	-	-	Reduction: 2 logs	-	UTI
(Watanabe et al., 2007)	-	-	Reduction: 4 logs	Speculated (no tested)	KPP10
(Hagens *et al.*, 2004)	-	-	Reduction: 4 logs	-	Pt1
(Hagens *et al.*, 2004)	-	-	Reduction: 3 logs	-	Pf3R
(Heo *et al.*, 2009)	-	-	Reduction: 5 logs	-	MPK1
(Heo *et al.*, 2009)	-	-	Reduction: 4 logs	-	MPK6
(Hanlon *et al.*, 2001)	-	Biofilm	Redution: 60%	-	F116
(Fu *et al.*, 2010)	catheter	Biofilm	Redution: 99%	Resistance: 90%	M4
(Ahiwale *et al.*, 2011)	catheter	Biofilm	Redution: 67%	-	BVPaP-3
(Glonti *et al.*, 2010)	-	**Biofilm**	-	-	PT-6

1.5 Final considerations

The phages have several characteristics that make them attractive for use as therapeutic agents, however more studies will be done. Bacteriophages may be administered alone or in combination with antibiotics, and can be given prophylactically or as a therapy of infection. They offer many advantages, as they are very specific, replicate at the site of infection and no serious adverse effects of their administration have been described and they have low resistance. As a result of the emergence of MDR strains of pathogenic bacteria, we need a solution quickly.

The aim of my work is to explore the potential of phages in controlling multidrug-resistant *P. aeruginosa* strains *in vitro* and *ex vivo* (human skin).

Material and methods

2. Material and methods

2.1 Bacterial strains, growth conditions and identification

MDR *Pseudomonas aeruginosa* strains were isolated at the microbiology laboratory of the University Hospital of Coimbra (Hospital Universitário de Coimbra - HUC) (4 isolates), Local Health Unit of Matosinhos (Unidade Local de Saúde de Matosinhos - Pedro Hispano - ULSM) (27 isolates), Infante D. Pedro Hospital (Hospital Infante D. Pedro Aveiro-HIDP) (17 isolates) and Avelab Laboratory (3 isolates). The MDR *Pseudomonas aeruginosa* 709 isolated by HUC was used as host to produce the phage stock suspension. The system BioMerieux Vitek 2® (BioMerieux) was employed to identify and characterize the profile of antibiotic susceptibility of the bacteria isolates.

The 51 MDR isolates used in this study were cultivated in Tryptic Soy Broth (TSB; Merck) with agitation of 160 rpm at 37ºC for 4 hours. For each assay, an aliquot of this culture (240 µL) was subcultured in 30 mL of TSB medium and grew overnight at 37 °C under stirring (120 rpm) to reach an optical density (OD_{620}) of ≈1.0, corresponding to ≈10^8 cells mL^{-1}.

2.2 Genotyping of bacterial isolates

For the molecular typing of the isolates a repetitive sequence PCR using a BOX A1R primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3') was followed (Rademaker et al., 2004). DNA was extracted using InstaGene Matrix kit (Bio Rad). BOX-PCR profiles were visualized after separation of PCR amplicons by electrophoresis in 1,5% agarose gel using 1x TAE (Tris-Acetate-EDTA) at 100 V for 3 hours, stained with red gel.

2.3 Phage isolation

Sewage water samples collected from HUC wastewater treatment plant were filtered by 0.45 µm-pore-size polycarbonate membranes (Millipore). The filtrate was added to a fresh bacterial culture in TSB. The mixture was incubated at 37ºC for 5 hours at 100 rpm and then centrifuged (10,000 x g, 10 minutes) (Thermo Haraeus Pico). The phage titer was determined by the double-layer method (Adams, 1959) using the centrifugated supernatant as phage suspension and Tryptic Soy Agar (TSA; Difco) as culture medium. The plates were incubated at 37ºC and examined for lysis plaques after 12 hours. Two more successive single-plaque isolations were performed to obtain pure phage stocks. The diameter of the lysis plaques was determined after incubation of the double-layer cultures. Phage stocks were stored at 4°C with 1% chloroform.

2.4 Phage nucleic acid extraction and characterization

Before nucleic acid extraction, phage particles were precipitated with polyethylene glycol (PEG) and separated by centrifugation (10,000 x g, 10 minutes). The pellets was resuspended in TE buffer [10 mM Tris HCl, 1mM ethylenediamine tetraacetic acid (EDTA), pH 8.0] (Sambrook and Russell, 2001). Extractions of nucleic acid from phage particles was performed using the protocol of Griffiths et al. (Griffiths et al., 2000). In order to amplify the phage nucleic acid kit was used the TempliPhi DNA Sequencing Template Amplification kit (Amersham Biosciences) was used as described by the manufacturer, with some adaptations. The template (2 µL) was added to 2 µL of distilled water and to 6 µL of sample buffer. A reaction premix was prepared (12 µL reaction buffer, 0.8 µL BSA (0.25 µg/µL) and 0.5 µL enzyme mix) and after denaturation, 7 µL of this premix was added to each sample and incubated at 30°C for 4.5 hours. The reaction was terminated by heating at 65°C for 10 min.

In order to characterize the phage nucleic acid, the nucleic acid extracts were subjected to digestion with DNase I (Ambion) and RNase I (Sigma Aldrich) as described by manufacturer. The reactions were terminated by heating at 80ºC for 5 min. Nucleic acid was visualized through agarose gel electrophoresis (1% agarose gel electrophoresis at 80 V).

2.5 Preparation of phages for transmission electron microscopy (TEM)

The phage suspension was centrifuged directly onto formvar-coated carbon-stabilized 400 mesh copper electron microscopy grids (Labometer) as described by Almeida et al. (Almeida et al., 2001). The viruses were ultracentrifuged at 100,000 x g for 1.30 hour at 25ºC in a Beckman L8-80K ultracentrifuge equipped with a swing-out rotor (SW28). After centrifugation, the grids were negatively stained with 1.5% (w/v) uranyl acetate for 60 seconds (Bratbak and Heldal, 1993), air-dried and examined with a Hitachi H9000 transmission electron microscope at a magnification of 100,000× and an accelerating voltage of 200 kV.

2.6 Phage host range analysis

The 51 isolates of MDR *P. aeruginosa* were used for the determination of phage host range. Fresh bacterial cultures were separately added to molten soft TSA, overlaid on solid TSA and spotted with 10 µL of the phage suspension. Cultures were incubated at 37ºC for 6 hours. Positive infection detected by the occurrence of lysis plaques. Each strain was tested in two independent assays.

2.7 Phage therapy in vitro

In order to obtain 0.5, 10 and 50 MOI, 10^5 CFU mL^{-1} of *P. aeruginosa* 709 and a set of serial dilutions of phage suspension (10^4 to 10^6 PFU mL^{-1}) were inoculated in TSB and incubated at 37ºC without agitation. For the MOI 10 assay, a second phage dose application (10^6 PFU mL^{-1}) was also tested. The second dose was applied 4 hours after the first one. In the controls, the phage was not added to the bacterial cultures. Aliquots were collected at time 0 and 2, 4, 6, 8, 12 and 18 h of incubation for host and phage quantification. For quantification of the host, aliquots were serially diluted, and pour-plated in duplicate, and incubated at 37ºC for 24 h. The phage title was determined in duplicate, by double-layered method, followed by incubation 37ºC for 18 h. Two independent experiments were conducted and the results were averaged.

2.8 Phage therapy in ex-vivo human skin

Samples of human skin, obtained from surgical procedures, were cut into sections of 10 cm^2, disinfected with povidone-iodine (PVPI) 10% and washed with saline solution. The skin sections were individually transferred to sterile petri dishes. An aliquot of 200 µL of an overnight culture of strain *P. aeruginosa* 709 was inoculated on the skin samples and the inoculum was evenly spread over the surface using a glass spreader. Gauze bandages soaked with phage solution (pH=7.3) were placed directly on the inoculated skin which was then incubated at 37°C in a humid atmosphere. Surface samples from the skin were collected from a defined area with cotton swabs at time 0 and 2, 4, 6, 8, 12 and 18 h of incubation and washed in PBS. The sample suspension was pour-plated, in duplicate on MacConkey agar (Merck) for quantification of the host bacteria and the results were expressed as CFU cm^{-2}. For the quantification of the phage, the PBS suspension was serially diluted, plated by the double-layer method, incubated at 37°C during 18 h and the results expressed as PFU cm^{-2}. The phage suspension was not added to the controls that were

otherwise treated as the tests. Experiments were conducted in duplicate and the results were averaged.

2.9 Phage survival in vitro

An aliquot of 1 mL of phage suspension (10^7 PFU mL^{-1}) was inoculated in PBS and incubated at 37ºC without agitation. Sub-samples were collected at time 0 and 9, 15, 21, 24, 27, 30 and 36 days incubations for phage quantification. The phage was quantified, in duplicate, by the double-layer method. Two independent assays were conducted and the the results were averaged.

2.10 Phage survival in ex-vivo human skin

Gauze bandages soaked in the phage solution (10^7 PFU mL^{-1}) were place over samples of human skin and kept at 37°C in a humid atmosphere. Skin samples were collected at time 0 and 4, 5, 6 and 7 days of incubation for phage quantification. The phage was quantified, in duplicate, using the double- layer method. Two independent assays were conducted and the results were averaged.

2.11 Detection of prophages in the host

Mitomycin C (Sigma Chemical) was added to a *P. aeruginosa* 709 culture (1 µg mL^{-1}) before and after 10 hours of incubation at 37°C with the phage PA709. The culture was incubated overnight at 37ºC and centrifugated (10,000 x g, 10 minutes) (Thermo Haraeus Pico). The spot test was performed using the supernatant as phage suspension. In the

control, mitomycin C was not added. Two independent assays were conducted and the results were averaged.

2.12 Screening of host resistant strains

After 8 and 10 hours of incubation at 37°C with the phage PA709, for MOI 10, an aliquot was plated in TSA in order to isolate bacteria colonies. Cultures were incubated at 37ºC overnight. Five isolated colonies were purified by the streak-plating technique (3-5 steps) and the purified isolates were cultured in TSA, in order to remove phage particles from the bacteria. To check if the strain remained sensitive to the phage, the isolated colonies were inoculated separately in TSB and tested by the spot procedure. Two independent assays were conducted for each isolate.

2.13 Statistical analyses

Statistical analysis was performed by using SPSS (SPSS 15.0 for Windows, SPSS Inc., USA). Normal distributions were assessed by the Kolmogorov-Smirnov test and homogeneity of variances was assessed by the Levene test. The significance of the difference in phage inactivation with different MOI and with different doses was evaluated by one-way ANOVA with the Bonferroni post hoc test. A value of $P < 0.05$ was considered as significant.

Results

3 Results

3.1 Bacterial strains

MDR *Pseudomonas aeruginosa* strains isolated at the microbiology laboratory of the University Hospital of Coimbra (Hospital Universitário de Coimbra - HUC), Local Health Unit of Matosinhos (Unidade Local de Saúde de Matosinhos - Pedro Hispano - ULSM), Infante D. Pedro Hospital (Hospital Infante D. Pedro Aveiro -HIDP) and Avelab Laboratory (3 isolates) are presented in Table 3.1.

Table 3.1: The 51 bacterial strains used in this study. ULSM, Unidade local de saude de Matosinhos; HUC, Hospitais da universidade de Coimbra; HIDP, Hospital Infante D. Pedro

Bacteria	Assigned by	Bacteria	Assigned by	Bacteria	Assigned by
PA 709	HUC	**PA 261.1**	ULSM	**PA 961**	HIDP
PA 826	HUC	**PA 088**	ULSM	**PA 304**	HIDP
PA 519	HUC	**PA 800**	ULSM	**PA 108**	HIDP
PA 548	HUC	**PA 845**	ULSM	**PA 766**	HIDP
PA 17567	AVELAB	**PA 89**	ULSM	**PA 1**	HIDP
PA 5531	AVELAB	**PA 876**	ULSM	**PA 2**	HIDP
PA 372	AVELAB	**PA 512**	ULSM	**PA 3**	HIDP
PA 025	ULSM	**PA 782**	ULSM	**PA 4**	HIDP
PA 433	ULSM	**PA 1003**	ULSM	**PA 5**	HIDP
PA 338	ULSM	**PA 722**	ULSM	**PA 6**	HIDP
PA 531	ULSM	**PA 3169**	ULSM	**PA 7**	HIDP
PA 154	ULSM	**PA 028**	ULSM	**PA 8**	HIDP
PA 103	ULSM	**PA 916**	ULSM	**PA 9**	HIDP
PA 701	ULSM	**PA 252**	ULSM	**PA 10**	HIDP
PA 489	ULSM	**PA 777**	ULSM	**PA 11**	HIDP
PA 61	ULSM	**PA 310**	ULSM	**PA 12**	HIDP
PA 352	ULSM	**PA 926**	ULSM	**PA 13**	HIDP

3.2 Resistance of bacterial strains to antibiotics

Among the 51 bacteria studied, 70% of isolates showed resistance to ciprofloxacin, piperacillin/tazobactam, piperacillin and ticarcillin/clavulanic acid, 50% to 70% were

resistant to ceftazidime, imipenem, meropenem and ticarcillin. The most effective antibiotics against these strains are amikacin, tobramycin and colistin with a of 80%, 80% and 100%, respectively (Figure 3.1).

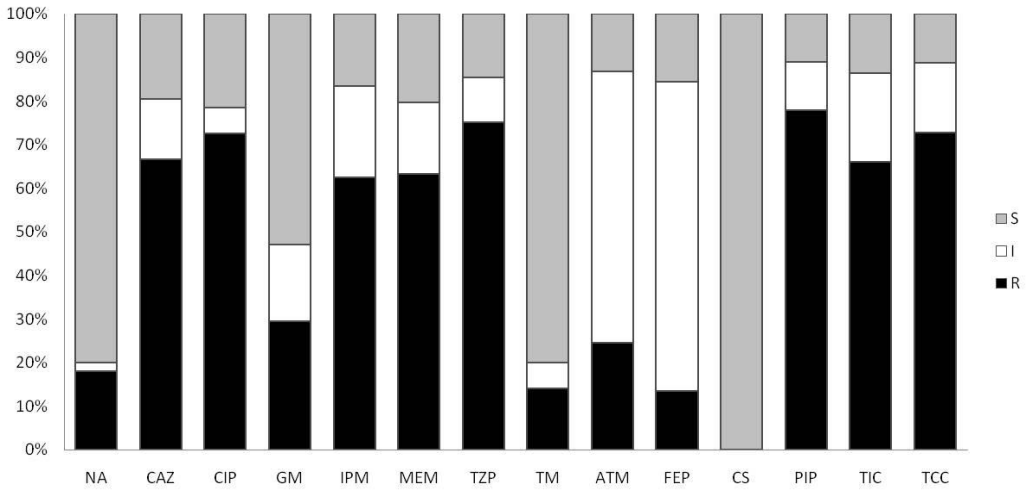

Figure 3.1: Relative frequency (%) of susceptibility, intermediate susceptibility and resistance to a range of common antibiotics among the 51 isolates. R: resistant; S: sensitive and I: intermediate; NA: amikacin; CAZ: ceftazidime; CIP: ciprofloxacin; GM: gentamicin; IPM: imipenem; MEM: meropenem; TZP: piperacillin/tazobactam; TM: tobramycin; ATM: aztreonam; FEP: cefepime; CS: colistin; PIP: piperacillin; TIC: ticarcillin; TCC: ticarcillin/clavulanic acid.

3.3 Phage isolation and characterization

The phage isolated from sewage water was referred as PA709. The stock of PA709 used in this study had a title of 1 x 10^8 PFU mL^{-1}. The lysis plaques were round with a 1 mm diameter after 6h of incubation in TSA with 0.6% of agar at 37°C (Figure 3.2 a). The TEM results showed that the phage PA709 had an icosahedral head (60 nm) and a long contractile tail typical of the family Myoviridae (Figure 3.2 b). The analysis of the nucleic acid of phage was assessed using enzyme digestion (DNase I, RNase I). The nucleic acid was digested by DNase I but not by RNase I. This results indicate that the phage has DNA as nucleic acid (Figure 3.2 c).

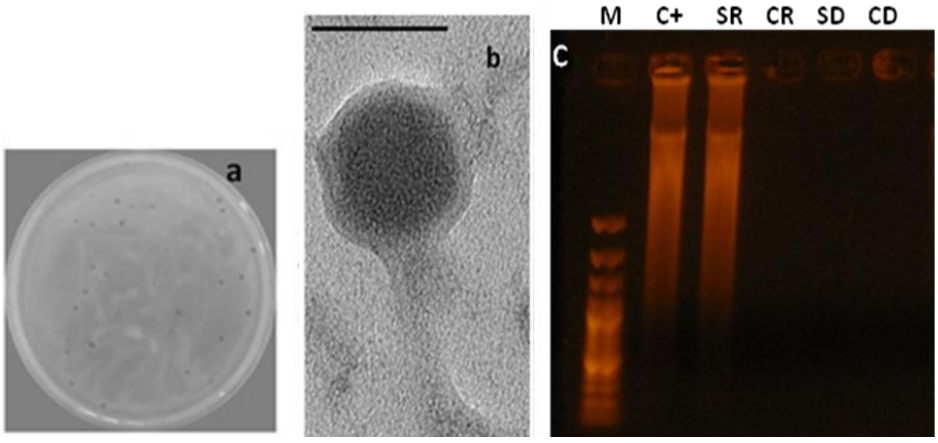

Figure 3.2: Characterization of phage PA709. a) phage plaques; b) transmission electron micrograph image of phage particles. The bar represents a length of 50 nm; c) analysis of nucleic acid of phage; Lane M, molecular length marker; C+, control reaction with nucleic acid samples; SR, nucleic acid samples digested with Rnase I; CR, Control of Rnase I; SD, nucleic acid samples digested with Dnase I; CD, Control of Dnase I.

The phage survival tests conducted *in vitro* show that phage PA709 is viable for more than 30 days, in the absence of the host. The concentration decreased 1 log in the first 24 days and then reached a plateau (Figure 3.3 a). The *ex vivo* experiments of phage survival showed that phage PA709 remained viable in human skin during the study period of 7 days. The phage titer decreased 1 log in the absence of the host (Figure 3.3 b).

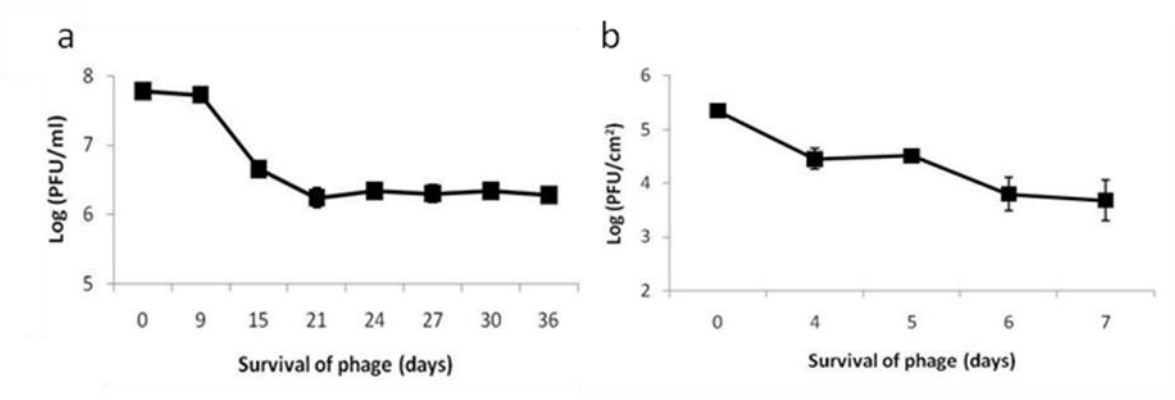

Figure 3.3: Survival of phage PA 709. a) *In vitro*. Phage was added to PBS and incubated at 37 °C; b) *Ex vivo.* Phage was added to human skin and incubated at 37 °C; Error bars indicate the standard deviation.

3.4 Host range determination

Phage PA709 exhibited a broad host spectrum. Thirty per cent of the 51 MDR *P. aeruginosa* clinical isolates were infected by the phage, 3 from HUC, 7 from ULSM, 4 from HIDP and 1 from Avelab Laboratory (Table 3.2).

Table 3.2: Host range of the isolated bacteriophage against *P. aeruginosa* isolates

Bacteria	Assigned by	Host range
PA 17567	AVELAB	+
PA 709	HUC	+
PA 548	HUC	+
PA 519	HUC	+
PA 028	ULSM	+
PA 61	ULSM	+
PA 777	ULSM	+
PA 088	ULSM	+
PA 926	ULSM	+
PA 103	ULSM	+
PA 154	ULSM	+
PA 766	HIDP	+
PA 1	HIDP	+
PA 3	HIDP	+
PA 6	HIDP	+

Of the 15 isolates infected by phage, nine were chosen from different Portuguese hospitals (Aveiro, Coimbra and Matosinhos) for molecular typing. The genotyping box results showed that six of the nine isolates display different band patterns, which correspond to different genotypes (Figure 3.4).

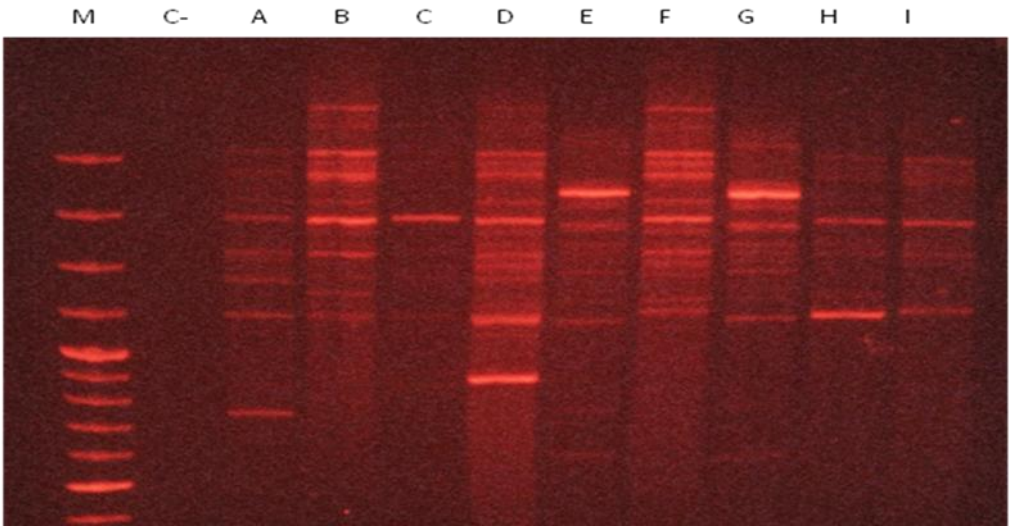

Figure 3.4: BOX PCR for 9 isolates of *P. aeruginosa*. Lane M, molecular length marker. Lane C-, control reaction with no template DNA included during PCR. Lanes A to I, isolates PA 709 (HUC), PA 028 (ULSM), PA 17567 (AVELAB), PA 61 (ULSM), PA 548 (HUC), PA 777 (ULSM), PA 088 (ULSM), PA 926 (ULSM) and PA 103 (ULSM), respectively.

3.5 Identification of bacteria strains

The six genetically different strains were sequenced. The results of the analysis of the gene sequences rRNA 16S are presented in Table 3.3. The results showed that, the 16s rDNA sequences of all strains show high homology with the *Pseudomonas aeruginosa*.

Table 3.3: Results of the analysis of the gene sequences rRNA 16S

Bacteria	Assigned by	Lane letter (Figure 3.4)	Classificação RDP [a] Classe/Ordem/Family	Identity BLAST-N	%
PA 709	HUC	A	Gammaproteobacteria Pseudomonadales Pseudomonadaceae	*Pseudomonas aeruginosa*	100%
PA 028	ULSM	B and F			100%
PA 17567	AVELAB	C			100%
PA 61	ULSM	D			99%
PA 548	HUC	E and G			100%
PA 926	ULSM	H and I			65%

[a] RDP classification - Ribossomal Database Project

3.6 Phage therapy in vitro

In the control cultures (without phages), *P. aeruginosa* 709 reached densities of 10^7 CFU mL^{-1} after 6 hours of incubation (Figure 3.5 a). In presence of the phage, for higher MOI (10 and 50), the number of viable bacteria decreased from 10^5 CFU mL^{-1} to 10^2 CFU mL^{-1}, after 4 hours of incubation (Figure 3.5 a). For these MOI the decrease of the target bacteria was more pronounced than for a MOI 0.5 ($P < 0.05$, ANOVA). With MOI 0.5, the number of viable bacteria decreased to 10^2 CFU mL^{-1} after 8 hours of incubation (Figure 3.5 a). The inoculation of a single dose of phage, for the three tested MOI, reduced the concentration of target bacteria by 5 logs, within the first 6 hours, but during the subsequent incubation the non infected bacteria grew and reached densities similar to the control.

The number of inoculated phage remained constant after 2 hours of incubation increased with further incubation to 10^8 PFU ml^{-1} (Figure 3.5 b).

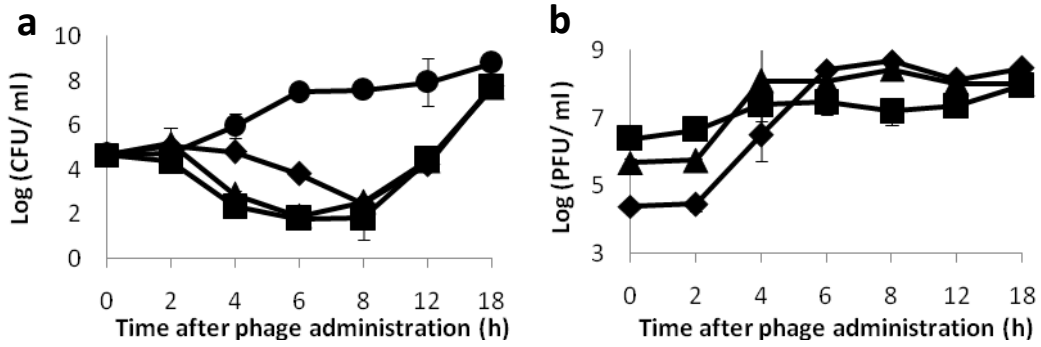

Figure 3.5: Infection of *P. aeruginosa* at different MOI*, in vitro*. **a)** bacterial concentration; **b**) phage concentration. The results are the average of two independent experiments. Error bars indicate the standard deviation. ●control PA709, ♦MOI 0.5, ▲MOI 10, ■ MOI 50

The administration of a second dose of phage, tested only for the MOI of 10, of incubation did not increase the efficiency of phage therapy within 4 hours of incubation. The pattern of bacterial growth was similar to that observed when only one phage dose was applied ($P>0.05$, ANOVA) (Figure 3.6).

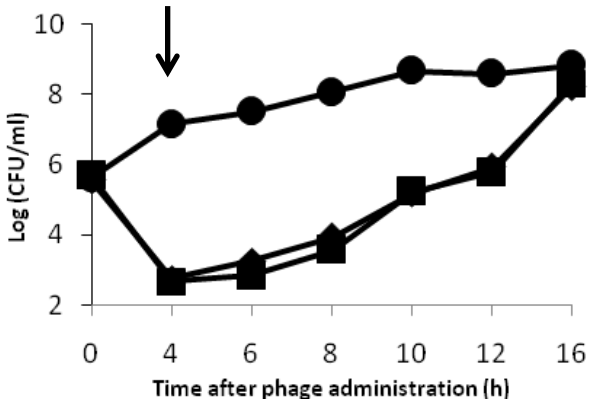

Figure 3.6: Control of *P. aeruginosa* by phage infection at a MOI of 10, *in vitro*. The results are the average of two independent experiments. Error bars indicate the standard deviation; ●control PA709, ▲ one dose of phage, ■ two doses of phage. At the time indicated by arrow, a second dose of PA709 phage was added to the culture.

3.7 Phage therapy in ex vivo human skin

At a MOI of 10, the number of viable bacteria decreased from 10^5 CFU cm^{-2} (0h) to 10^2 CFU cm^{-2} after 4 hours of incubation, corresponding to a reduction of 4 logs. In the control samples (without phage), the *P. aeruginosa* 709 reached concentrations of 10^6 CFU cm^{-2} after the same period of incubation (Figure 3.7).

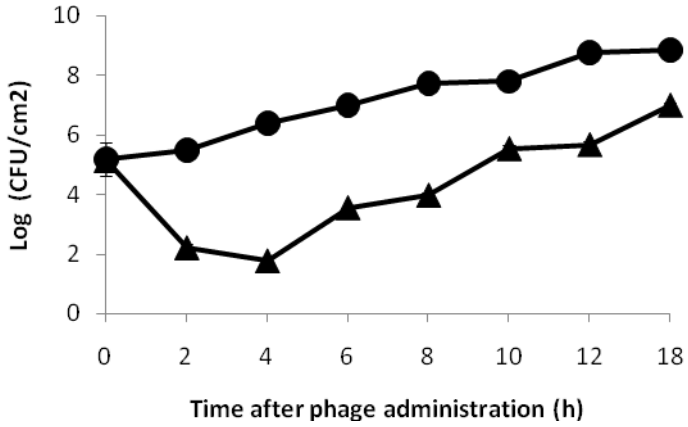

Figure 3.7: Control of *P. aeruginosa* by phage infection at a MOI of 10, in human skin (*ex vivo*). The results are the average of two independent experiments. Error bars indicate the standard deviation; ● control PA709, ▲ MOI 10.

3.8 Detection of prophages in the host

After the addition of mitomycin C to the *P. aeruginosa* 709 culture, no lysis plaques were observed before and after incubation with the phage suspension. As in the control, without mitomicin C, the spot test was negative, i.e, no lysis plaques were observed.

3.9 Screening of host resistant strains

The host bacteria did not develop resistance to phage infection after incubation in the presence of the phage suspension. All the isolated colonies collected after 8 and 10 hours of phage therapy *in vitro* at a MOI of 10, showed a clear zone in the TSA plates after 3-5 successive bacterial replications in TSA. For the isolated colonies of the sample subjected to phage therapy after the first and second replications in TSA no lysis plaques were observed. After the first and second replications in TSA a clear zone was observed only in the control samples.

Discussion

4 Discussion

Pseudomonas aeruginosa is one of the most common opportunistic human pathogenic responsible for wound infections (Luzzaro et al., 2004). Systemic antibiotic therapy has little utility in patients with deep wounds, because of poor penetration of the antibiotic into the wound, being the infection difficult to eliminate (Gilakjan and Kropinski, 1999; Kutter et al., 2010). On the other hand, as seen in this study and in other works (Wang et al., 2006; Wright et al., 2009), the most commonly used topical antibiotics are not effective against *P. aeruginosa*, alternative topical antimicrobial treatments are needed. Phage therapy represents a potential approach to inactivate pathogenic microorganisms and has already show to be efficient against bacterial infections in humans and animals (Almeida et al., 2009; Housby and Mann, 2009; Wiggins and Alexander, 1985).

The results of this study show that 1) the topical application of phage PA709 may effectively control human skin infections by *P. aeruginosa* 709 (bacteria reduction of 4 logs, after 2 hours of incubation); 2) the MOI of 10 and 50 are more effective than the MOI of 0.5; 3) the application of a single phage dose may be sufficient; 4) the application of phage PA709 is safe (no lysogenic conversion was detected); 5) the phage PA709 is suitable for phage therapy, has a broad host range (infection of 30% of the tested *P. aeruginosa* isolates), infecting different genotypes of *P. aeruginosa* and a high survival (*in vitro* and in *ex-vivo*).

The application of the bacteriophage PA709 on human skin artificially contaminated with *P. aeruginosa* 709 clearly reduces the number of viable bacteria by 4 logs after 2 hours of incubation. Although after 2 hours of incubation 2 logs of bacteria remain viable, the bacterial density may have decreased to a level low enough to be eliminated by the host immunitary system (Levin and Bull, 2004). As demonstrated by the mathematical model proposed by these authors the combination of phage and the host defenses are sufficient to keep the bacterial density below lethal threshold after phage therapy (Levin and Bull, 2004).

According to the literature, the non-inactivation of the remaining bacteria can be due to the difficulty of viruses to find the host bacteria (Wiggins and Alexander, 1985), to non-replicating condititon of the surviving bacteria that is physiologically refractory to phage infection (Bull et al., 2002; Levin and Bull, 2004) and to the development of resistance of the host to the phages (Levin and Bull, 2004). The results of this study show clearly that bacteria that re-grow after phage therapy were not resistant to PA709 phage. Similar results have been observed in other studies (Oliveira et al., 2009; Sillankorva et al., 2004). However, some authors attributed bacterial re-growth to the development of host resistance to the phages without actually testing the development of resistance (Andreatti Filho et al., 2007; Kutter et al., 2010). On the other hand, in this study, all the tested bacteria that re-grew after phage therapy regained sensitivity to the phages only after 3-5 streak-plating steps on solid medium, which means that these bacteria are yet infected with viruses which prevent their infection by the newly added phages after their isolation. Thus, the hypothesis of re-growth due to the low probability of encounter between viruses and hosts bacteria is not likely. In fact, the increase of MOI of 10 to 50 did not increase the efficiency of phage therapy, which means that almost all bacteria are infected at the MOI of 10 that was the MOI used *in vitro* and in *ex vivo* phage therapy experiments. The occurrence of lysogeny which can also render the bacterium immune to not only the original phages but also to related phages (Skurnik and Strauch, 2006) could also explain the bacterial re-growth, but no evidence of lysogeny was observed during the phage therapy experiments. Induction by the addition of mitomycin C to isolated bacteria that remained viable after phage therapy was not observed. The hypothesis of non-replicating bacteria to be physiologically refractory to phage infection can possibly explain the bacterial re-grow after phage therapy treatment.

The topical application of phage therapy by using gauze bandages soaked in phage suspension is suitable for topical use on wounds and was effective in the reduction of the concentration of infectious bacteria. Similar results were obtained when phage therapy was applied locally (Goode et al., 2003; Jikia et al., 2005). In addition, it was found that the normal skin microflora was not affected when this procedure was used (data not show) and the phage remain active for more than 7 days on the skin, which can help to avoid re-

infection by the same bacteria. Up to our knowledge studies on phage survival in *ex vivo* experiments with human skin, are not available in current literature. However, a study of Kumari et al. (Kumari et al., 2010) using phage to treat burn wound infection caused by *Pseudomonas aeruginosa* PAO in BALB/c Mice showed that the titer of five different Pseudomonas phage injected in mice kept on decreasing in skin with increase in time and no phage could be isolated in skin samples after 36 h of phage treatment.

Three MOI were tested in order to optimize the phage dose for the treatment of *P. aeruginosa* infections. High MOI are used when the experiments requires that most cells in the culture are infected, such as in the case of phage therapy. By contrast, low MOI are used when multiple cycles of infection are required. The MOI of 10 and 50 improved bacterial reduction relatively to the MOI of 0.5 such as observed in other studies (Goode et al., 2003; Huff et al., 2005). The reduction of *Salmonella enteritidis* and of *Campylobacter jejuni* by specific phages on excised chicken skin was investigated at different MOI (Goode et al., 2003). At a MOI of 1, phage reduced the two pathogens by < 1 log. At a MOI of 100-1000, the number of bacteria was reduced by up 2 logs. Huff et al., also showed that application of phages in birds, to reduce *Escherichia coli* is only effective at a MOI of 10,000 (Huff et al., 2005). When the culture was mixed with equal number of bacteriophage, mortality was reduced from 85 to 35%. However, in this study the difference of bacterial inactivation with MOI of 10 and of 50 was not significantly different.

Several studies indicate that the application of a single dose of phage suspension is enough to inactive pathogenic bacteria (Biswas et al., 2002; Sulakvelidze et al., 2001). Contrarily to antibiotics, repeated administration is unnecessary because as long as the target bacterium is present, the phage will be able to replicate. The local proliferation of the phages at the site of infection may require less frequent phage administration in order to achieve the optimal therapeutic effect. The results obtained in the present experiments confirm this assumption, encouraging a single dose of phage administration. The application of a second dose of phages did not increase the effectiveness of *P. aeruginosa* inactivation.

The results indicate that phage PA709 has a broad host range (30% of the 51 MDR *P. aeruginosa* clinical isolates were infected by the phage), infecting different genotypes of *P. aeruginosa* collected in the three studied cities of Portugal (Aveiro, Coimbra and Matosinhos). The bacterial host molecular typing by BOX-PCR with the primer BOXA1R showed that the strains of *P. aeruginosa* isolated from the different cities, which were infected by the phage PA709, correspond to different genotypes and, consequently, this phage may be widely used to treat *P. aeruginosa* infections.

The phage PA709 has an icosahedral head with a long contractile tail and a double stranded DNA molecule as nucleic acid, a morphology characteristic of members of the Myoviridae family. The morphology of the studied phage and the characteristics of the nucleic acid suggested that PA709 may be closely related to the *P. aeruginosa* phages KZ Lin68, Lin21, PTB80, NN, EL, and RU (Krylov et al., 2003) rather than with *P. aeroginosa* phages D3112 and B3 (Ceyssens et al., 2009; Gilakjan and Kropinski, 1999) and KMV, LKD16 and LKA1 (Lavigne et al., 2003; Lavigne et al., 2008) which were classified as Siphoviridae and Podoviridae family, respectively.

Phage PA709 presents some interesting features, namely high efficiency in the inactivation of MDR *P. aeruginosa,* a broad host range and high stability in stock suspensions and in *ex vivo* human skin. All these attributes make this phage very promising for the treatment of *P. aeruginosa* skin wound infections. However, more phages should be isolated in the future, for the formulation of cocktails which might improve the inactivation efficiency against *P. aeruginosa* human skin infections.

Annex

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Topical application of phage therapy to inactivate multidrug-resistant strain of *Pseudomonas aeruginosa* in skin infections: in vitro and in ex vivo experiments

A. Vieira, Y. J. Silva, Â. Cunha , N.C.M. Gomes, A. Almeida*

CESAM and Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 AVEIRO – PORTUGAL

*Correspondence: A. Almeida, Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 AVEIRO – PORTUGAL;
E-Mail: aalmeida@ua.pt,
Fax: +351 234372587,
Tel.: +351 234 370784

Keywords
Phage therapy, bacteriophage, *Pseudomonas aeruginosa*, multidrug resistant bacteria, human skin, wound infections.

Abstract

The rising prevalence of antibiotic resistant bacterial strains represents a serious therapeutic challenge for clinicians. Phage therapy has been regarded as a viable alternative to inactivate multidrug-resistant (MDR) strains. *Pseudomonas aeruginosa* is one of the most common MDR Gram-negative bacterium causing wound infections. The main goal of this study was to evaluate the efficiency of phage therapy against MDR *P. aeruginosa* in vitro and in ex vivo (human skin) experiments. A phage suspension (10^8 PFU mL^{-1}) was obtained using the clinical strain *P. aeruginosa* 709 as host. The ability of the phage to cause inactivation of different *P. aeruginosa* isolates was evaluated in vitro and in ex vivo, using a multiplicity of infection of 0.5 to 50. The number of MDR *P. aeruginosa* 709 in the human skin in the presence of the phage decreased 4 logs after 2 hours of incubation. The application of a second dose of phage did not increase the efficiency of the therapy. The results of this study indicate that the topical application of phage PA709 efficiently inactivates MDR *P. aeruginosa 709* clinical strain, being a good candidate as therapeutic agent for the treatment of human wound infections caused by MDR *P. aeruginosa* 709. The broad host range of the phage, including different genotypes of *P. aeruginosa*, suggests that this phage can be widely used to treat *P. aeruginosa* infections.

Introduction

Chemotherapy has shown to be a rapid and effective method to treat microbial infections but the regular use of antimicrobials has lead to the development of drug resistance in common pathogenic strains (Pai et al., 2001; Wróblewska, 2006). Although novel classes of antibiotics may be developed, the perspective that bacteria will eventually develop resistance to the new drugs (Walsh, 2003) emphasizes the risk that effective antibiotics may not be available to treat seriously ill patients in the near future. Because of the increasing costs of the research on new antimicrobials, the pharmaceutical industry has diminished its investment in this field, despite the increasing frequency and severity of antimicrobial resistance cases. This has dramatically weakened the so-called pipeline for new antibiotic compounds, particularly against Gram-negative bacteria.

As a result, the development of a novel, convenient and inexpensive methods, such as phage therapy, for fighting microbial diseases is required. Phage therapy research is as alive and full of promises nowadays as it was during its apogee of the 1960s and early 1970s (Comeau et al., 2008). Coincidentally, there was a parallel reawakening of interest in exploiting the enormous diversity of viruses that infect bacterial pathogens for phage therapy approaches to the control or prevention diseases of bacterial origin (Housby and Mann, 2009; Sulakvelidze et al., 2001).

The use of bacteriophage therapy requires, however, a detailed understanding of the phage-bacteria interaction and awareness of various novel kinetics phenomena not known in conventional drug treatments.

The understanding of phage-bacteria interaction helps to know how the phage can control bacterial growth. This control can be achived by maintaining the density of phage convenient values, so that the phages can effectively reduce the rate of replication of the host (Levin and Bull, 2004), avoiding their re-growth. However, studies in vitro have shown that bacteria can re-grow after some hours of phage therapy (Andreatti Filho et al., 2007; Heo et al., 2009; Kumari et al., 2010; Loc Carrillo et al., 2005). Some authors interpreted that this gradual increase of bacterial density is due to the emergence of strains resistant to the phages. Nevertheless, some of these studies did not actually experimentally test the development of bacterial resistance, only consider that the bacterial re-growth can be due to the development of resistance to phage infection (Andreatti Filho et al., 2007; Kumari et al., 2010). Other authors interpreted the re-growth as an indication that bacteriophages do not infect the host

bacteria when viral density is below the host cell threshold (Wiggins and Alexander, 1985). After several hours of incubation, the host may become a non-replicating bacterial population (slow bacterial growth or bacterial populations structured as biofilms) preventing the phage induced cell lysis (Bull et al., 2002; Levin and Bull, 2004). It is important to note that this increase is not observed in vivo since the combination of phage and the host defenses are sufficient to keep the bacterial density below lethal threshold after phage therapy (Levin and Bull, 2004). Phage therapy only needs to decrease the numbers of infecting bacteria to a level from which the host defenses can take care of the remaining bacteria (Levin and Bull, 2004).

Kinetics theory of phage therapy predicts that the average number of phage per bacterium, that is, the multiplicity of infection (MOI), the number of phage doses applied and the route how the phages are applied, could be critical to the efficiency of bacterial inactivation.

It has been shown in vitro conditions that the reduction of pathogenic bacteria increased with the increase of the MOI (Goode et al., 2003). The same was observed in vivo experiments with mice infected with a lethal imipenem-resistant *P. aeruginosa* bacteremia (Huff et al., 2005).

Contrarily to antibiotics, the application of a single dose appears to be sufficient to control bacterial growth. In vivo studies demonstrated that, through different routes of phage application, a single dose rescued 100% of the bacteremic tested animals (Smith and Huggins, 1982).

Several studies have shown that for the same type of infection, the phage can be applied through different routes, some more suitable than others (McVay et al., 2007; Watanabe et al., 2007). McVay et al. (McVay et al., 2007) showed that the location where the phages were injected change the survival rate of mice. The application of phage locally to eliminate wound infections in human skin (Jikia et al., 2005) and in chicken skin (Goode et al., 2003) instead of injecting, has shown good results.

To understand the phage-bacteria interaction, in vitro tests are not sufficient. In vivo tests are as necessary as cumbersome and expensive. Tests ex vivo do not fully mimic the in vivo growth conditions, but allow experimentation under conditions identical to in vivo but under more controlled conditions. This methodology combines the advantages of in vivo with the flexibility of the in vitro. Ex vivo conditions allow experimentation under more controlled conditions than possible in in vivo experiments, at the expense of altering the natural environment.

Pseudomonas aeruginosa is the main pathogen that colonize the skin injury, cause wound infections (Church et al., 2006; McVay et al., 2007) and other diseases such as pneumonia, bacteremia, meningitis, urinary tract infection in immunocompromised individuals and hospitalized patients (Rossolini et al., 2007; Wróblewska, 2006). Currently, this is one of the most common multidrug-resistant (MDR) strains in hospitalized patients (Bergogne-Berezin and Towner, 1996; Rossolini et al., 2007). Although there are several reports available in literature where phages have been used to treat variety of bacterial infections in animal model systems (Huff et al., 2005; Kumari et al., 2010; McVay et al., 2007; Wang et al., 2006), the information on the treatment of skin wound infection is scanty. Phages therapy was successfully tested

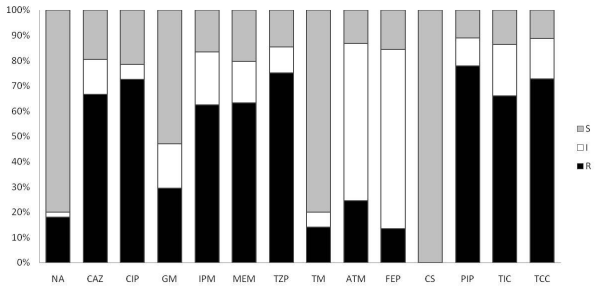

Fig. 1. Relative frequency (%) of susceptibility, intermediate susceptibility and resistance to a range of common antibiotics among the 51 isolates. R: resistant; S: sensitive and I: intermediate; NA: amikacin; CAZ: ceftazidime; CIP: ciprofloxacin; GM: gentamicin; IPM: imipenem; MEM: meropenem; TZP: piperacillin/tazobactam; TM: tobramycin; ATM: aztreonam; FEP: cefepime; CS: colistin; PIP: piperacillin; TIC: ticarcillin; TCC: ticarcillin/clavulanic acid.

in the prevention of the rejection of skin implants in pigs (Soothill, 1992) and were also used in the treatment of skin wound infections by *P. aeruginosa* (Kumari et al., 2010; McVay et al., 2007). Recently, a cocktail of three different *P. aeruginosa* specific phages induced protection against fetal infection with *P. aeruginosa* in mice (McVay et al., 2007). From these, a clinical trial in which a cocktail of phages targeted against *P. aeruginosa* and *Staphylococcus aureus* was initiated in Belgium (Merabishvili et al., 2009).

The main objective of this study was to evaluate the efficiency of phage therapy to control MDR P. aeruginosa infections in human skin wounds. The kinetics of phage-bacteria interactions were examined in vitro and in ex vivo in order to determine the course of the infection and provide basis to select the most suitable protocol for subsequent in vivo experiments.

Experimental

Bacterial strains, growth conditions and identification

MDR *Pseudomonas aeruginosa* strains were isolated at the microbiology laboratory of the University Hospital of Coimbra (Hospital Universitário de Coimbra - HUC) (4 isolates), Local Health Unit of Matosinhos (Unidade Local de Saúde de Matosinhos - Pedro Hispano - ULSM) (27 isolates), Infante D. Pedro Hospital (Hospital Infante D. Pedro – Aveiro -HIDP) (17 isolates) and Avelab Laboratory (3 isolates). The MDR *Pseudomonas aeruginosa* 709 isolated by HUC was used as host to produce the phage stock suspension. The system BioMerieux Vitek 2® (BioMerieux) was employed to identify and characterize the profile of antibiotic susceptibility of the bacteria isolates.

The 51 MDR isolates used in this study were cultivated in Tryptic Soy Broth (TSB; Merck) with agitation of 160 rpm at 37ºC for 4 hours. For each assay, an aliquot of this culture (240 µL) was subcultured in 30 mL of TSB medium and grew overnight at 37 °C under stirring (120 rpm) to reach an optical density (OD620) of ≈1.0, corresponding to $\approx 10^8$ cells mL^{-1}.

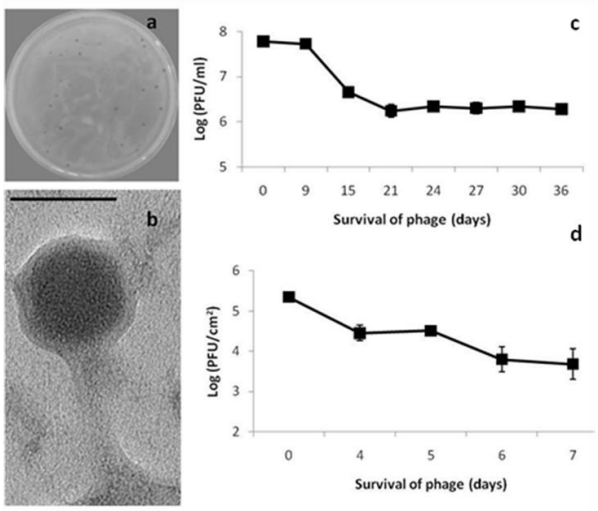

Fig. 2 Macroscopic and microscopic characterization of phage PA709. **a** phage plaques; **b** transmission electron micrograph image of phage particles. The bar represents a length of 50 nm; **c** survival of phage PA 709 in vitro**.** Phage was added to PBS and incubated at 37 °C; **d** survival of phage PA 709 ex vivo**.** Phage was added to human skin and incubated at 37 °C; Error bars indicate the standard deviation.

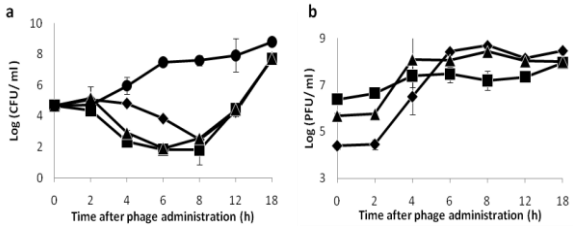

Fig. 3 Infection of *P. aeruginosa* at different MOI, in vitro. **a** bacterial concentration; **b** phage concentration. The results are the average of two independent experiments. Error bars indicate the standard deviation; ● control *P. aeruginosa* 709, ♦ MOI 0.5, ▲MOI 10, ■ MOI 50.

Phage isolation

Sewage water samples collected from HUC wastewater treatment plant were filtered by 0.45 µm-pore-size polycarbonate membranes (Millipore). The filtrate was added to a fresh bacterial culture in TSB. The mixture was incubated at 37ºC for 5 hours at 100 rpm and then centrifuged (10,000 x g, 10 minutes) (Thermo Haraeus Pico). The phage titer was determined by the double-layer method (Adams, 1959) using the centrifugated supernatant as phage suspension and Tryptic Soy Agar (TSA; Difco) as culture medium. The plates were incubated at 37ºC and examined for lysis plaques after 12 hours. Two more successive single-plaque isolations were performed to obtain pure phage stocks. The diameter of the lysis plaques was determined after incubation of the double-layer cultures. Phage stocks were stored at 4°C with 1% chloroform.

Phage nucleic acid extraction

Before nucleic acid extraction, phage particles were precipitated with polyethylene glycol (PEG) and separated by centrifugation (10,000 x g, 10 minutes). The pellets was resuspended in TE buffer [10 mM Tris HCl, 1mM ethylenediamine tetraacetic acid (EDTA), pH 8.0] (Sambrook and Russell, 2001). Extractions of nucleic acid from phage particles was performed using the protocol of Griffiths et al. (Griffiths et al., 2000). In order to amplify the phage nucleic acid kit was used the TempliPhi DNA Sequencing Template Amplification kit (Amersham Biosciences) was used as described by the manufacturer, with some adaptations. The template (2 µL) was added to 2 µL of distilled water and to 6 µL of sample buffer. A reaction premix was prepared (12 µL reaction buffer, 0.8 µL BSA (0.25 µg/µL) and 0.5 µL enzyme mix) and after denaturation, 7 µL of this premix was added to each sample and incubated at 30°C for 4.5 h. The reaction was terminated by heating at 65°C for 10 min.

Phage nucleic acid characterization

The nucleic acid extracts were subjected to digestion with DNase I (Ambion) and RNase I *(Sigma* Aldrich) as described by manufacturer. The reactions were terminated by heating at 80ºC for 5 min. Nucleic acid yield was observed through agarose gel electrophoresis (1% agarose gel electrophoresis at 80 V).

Preparation of phages for transmission electron microscopy (TEM)

The phage suspension was centrifuged directly onto formvar-coated carbon-stabilized 400 mesh copper electron microscopy grids (Labometer) as described by Almeida et al. (Almeida et al., 2001). The viruses were ultracentrifuged at 100,000 x g for 1.30 h at 25ºC in a Beckman L8-80K ultracentrifuge equipped with a swing-out rotor (SW28). After centrifugation, the grids were negatively stained with 1.5% (w/v) uranyl acetate for 60 seconds (Bratbak and Heldal, 1993), air-dried and examined with a Hitachi H9000 transmission electron microscope at a magnification of 100,000× and an accelerating voltage of 200 kV.

Phage host range analysis

The 51 isolates of MDR *P. aeruginosa* were used for the determination of phage host range. Fresh bacterial cultures were separately added to molten soft TSA, overlaid on solid TSA and spotted with 10 µL of the phage suspension. Cultures were incubated at 37ºC for 6 hours. Positive infection detected by the occurrence of lysis plaques. Each strain was tested in two independent assays.

Phage therapy in vitro

In order to obtain 0.5, 10 and 50 MOI, 10^5 CFU mL^{-1} of *P. aeruginosa* 709 and a set of serial dilutions of phage suspension (10^4 to 10^6 PFU mL^{-1}) were inoculated in TSB and incubated at 37ºC without agitation. For the MOI 10 assay, a second phage dose application (10^6 PFU mL^{-1}) was also tested. The second dose was applied 4 hours after the first one. In the controls, the phage was not added to the bacterial cultures. Aliquots were collected at time 0 and 2, 4, 6, 8, 12 and 18 h of incubation for host and phage

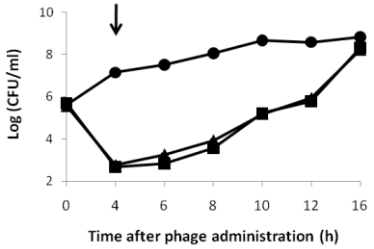

Fig. 4 Control of *P. aeruginosa* by phage infection at a MOI of 10, in vitro**.** The results are the average of two independent experiments. Error bars indicate the standard deviation; ● control *P. aeruginosa* 709, ▲ one dose of phage, ■ two doses of phage. At the time indicated by arrow, a second dose of PA709 phage was added to the culture.

quantification. For quantification of the host, aliquots were serially diluted, and pour-plated in duplicate, and incubated at 37ºC for 24 h. The phage title was determined in duplicate, by double- layered method, followed by incubation 37ºC for 18 h. Two independent experiments were conducted and the results were averaged.

Phage therapy in ex-vivo human skin

Samples of human skin, obtained from surgical procedures, were cut into sections of 10 cm^2, disinfected with povidone-iodine (PVPI) 10% and washed with saline solution. The skin sections were individually transferred to sterile petri dishes. An aliquot of 200 µL of an overnight culture of strain *P. aeruginosa* 709 was inoculated on the skin samples and the inoculum was evenly spread over the surface using a glass spreader. Gauze bandages soaked with phage solution (pH=7.3) were placed directly on the inoculated skin which was then incubated at 37°C in a humid atmosphere. Surface samples from the skin were collected from a defined area with cotton swabs at time 0 and 2, 4, 6, 8, 12 and 18 h of incubation and washed in PBS. The sample suspension was pour-plated, in duplicate on MacConkey agar (Merck) for quantification of the host bacteria and the results were expressed as CFU cm^{-2}. For the quantification of the phage, the PBS suspension was serially diluted, plated by the double- layer method, incubated at 37°C during 18 h and the results expressed as PFU cm^{-2}. The phage suspension was not added to the controls that were otherwise treated as the tests. Experiments were conducted in duplicate and the results were averaged.

Phage survival in vitro

An aliquot of 1 mL of phage suspension (10^7 PFU mL^{-1}) was inoculated in PBS and incubated at 37ºC without agitation. Sub-samples were collected at time 0 and 9, 15, 21, 24, 27, 30 and 36 days incubations for phage quantification. The phage was quantified, in duplicate, by the double- layer method. Two independent assays were conducted and the the results were averaged.

Phage survival in ex-vivo human skin

Gauze bandages soaked in the phage solution (10^7 PFU mL^{-1}) were place over samples of human skin and kept at 37°C in a humid atmosphere. Skin samples were collected at time 0 and 4, 5, 6 and 7 days of incubation for phage quantification. The phage was quantified, in duplicate, using the double- layer method. Two independent assays were conducted and the results were averaged.

Detection of prophages in the host

Mitomycin C (Sigma Chemical) was added to a *P. aeruginosa* 709 culture (1 µg mL^{-1}) before and after 10 hours of incubation at 37°C with the phage PA709. The culture was incubated overnight at 37ºC and centrifugated (10,000 x g, 10 minutes) (Thermo Haraeus Pico). The spot test was performed using the supernatant as phage suspension. In the control, mitomycin C was not added. Two independent assays were conducted and the results were averaged.

Screening of host resistant strains

After 8 and 10 hours of incubation at 37°C with the phage PA709, for MOI 10, an aliquot was plated in TSA in order to isolate bacteria colonies. Cultures were incubated at 37ºC overnight. Five isolated colonies were purified by the streak-plating technique (3-5 steps) and the purified isolates were cultured in TSA, in order to remove phage particles from the bacteria. To check if the strain remained sensitive to the phage, the isolated colonies were inoculated separately in TSB and tested by the spot procedure. Two independent assays were conducted for each isolate.

Statistical analyses

Statistical analysis was performed by using SPSS (SPSS 15.0 for Windows, SPSS Inc., USA). Normal distributions were assessed by the Kolmogorov-Smirnov test and homogeneity of variances was assessed by the Levene test. The significance of the difference in phage inactivation with different MOI and with different doses was evaluated by one-way ANOVA with the Bonferroni post hoc test. A value of $P < 0.05$ was considered as significant.

Results

Resistance profiling of the bacterial strains

Among the 51 bacteria studied, 70% of isolates showed resistance to ciprofloxacin, piperacillin/tazobactam, piperacillin and ticarcillin/clavulanic acid, 50% to 70% were resistant to ceftazidime, imipenem, meropenem and ticarcillin. The most effective antibiotics against these strains are amikacin, tobramycin and colistin with a of 80%, 80% and 100% respectively (Fig. 1).

Phage characterization

The phage isolated from sewage water was referred as PA709. The stock of PA709 used in this study had a title of 1 x 10^8 PFU mL^{-1}. The lysis plaques were round with a 1 mm diameter after 6h of incubation in TSA with 0.6% of agar at 37°C (Fig. 2a). The analysis of the nucleic acid of

phage was assessed using enzyme digestion (DNase I, RNase I). The nucleic acid was digested by DNase I but not by RNase I. This results indicate that the phage has DNA as nucleic acid. The TEM results showed that the phage PA709 had an icosahedral head (60 nm) and a long contractile tail typical of the family *Myoviridae* (Fig. 2b).
The phage survival tests conducted in vitro show that phage PA709 is viable for more than 30 days, in the absence of the host. The concentration decreased 1 log in the first 24 days and then reached a plateau (Fig. 2c). The ex vivo experiments of phage survival showed that phage PA709 remained viable in human skin during the study period of 7 days. The phage titer decreased 1 log in the absence of the host (Fig.2d).

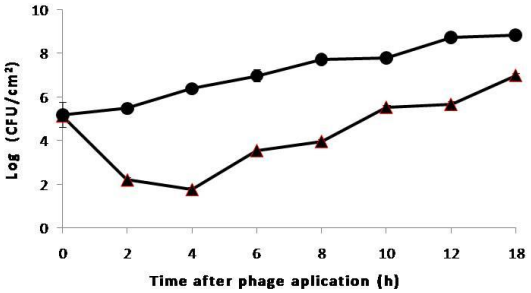

Fig. 5 Control of *P. aeruginosa* by phage infection at a MOI of 10, in human skin (ex vivo)**.** The results are the average of two independent experiments. Error bars indicate the standard deviation; ● control *P. aeruginosa* 709, ▲ MOI 10.

Host range determination

Phage PA709 exhibited a broad host spectrum. Thirty per cent of the 51 MDR *P. aeruginosa* clinical isolates were infected by the phage, 3 from HUC, 7 from ULSM, 4 from HIDP and 1 from Avelab Laboratory.

Phage therapy in vitro

In the control cultures (without phages), *P. aeruginosa* 709 reached densities of 10^7 CFU mL^{-1} after 6 hours of incubation (Fig. 3a). In presence of the phage, for higher MOI (10 and 50), the number of viable bacteria decreased from 10^5 CFU mL^{-1} to 10^2 CFU mL^{-1},after 4 hours of incubation (Fig. 3a). For these MOI the decrease of the target bacteria was more pronounced than for a MOI 0.5 ($P < 0.05$, ANOVA). With MOI 0.5, the number of viable bacteria decreased to 10^2 CFU mL^{-1} after 8 hours of incubation (Fig. 3a).
The inoculation of a single dose of phage, for the three tested MOI, reduced the concentration of target bacteria by 5 logs, within the first 6 hours, but during the subsequent incubation the non infected bacteria grew and reached densities similar to the control. The number of inoculated phage remained constant after 2 hours of incubation increased with further incubation to 10^8 PFU ml^{-1} (Fig. 3b).

The administration of a second dose of phage, tested only for the MOI of 10, of incubation did not increase the efficiency of phage therapy within 4 hours of incubation. The pattern of bacterial growth was similar to that observed when only one phage dose was applied ($P>0.05$, ANOVA) (Fig. 4).

Phage therapy in ex vivo human skin

At a MOI of 10, the number of viable bacteria decreased from 10^5 CFU cm^{-2} (0h) to 10^2 CFU cm^{-2} after 4 hours of incubation, corresponding to a reduction of 4 logs. In the control samples (without phage), the *P. aeruginosa* 709 reached concentrations of 10^6 CFU cm^{-2} after the same period of incubation (Fig. 5).

Detection of prophages in the host

After the addition of mitomycin C to the *P. aeruginosa* 709 culture, no lysis plaques were observed before and after incubation with the phage suspension. As in the control, without mitomicin C, the spot test was negative, i.e, no lysis plaques were observed.

Screening for resistance to phage infection

The host bacteria did not develop resistance to phage infection after incubation in the presence of the phage suspension. All the isolated colonies collected after 8 and 10 hours of phage therapy in vitro at a MOI of 10, showed a clear zone in the TSA plates after 3-5 successive bacterial replications in TSA. For the isolated colonies of the sample subjected to phage therapy after the first and second replications in TSA no lysis plaques were observed. After the first and second replications in TSA a clear zone was observed only in the control samples.

Discussion

Because of the poor penetration of antibiotics in skin wounds, systemic antibiotherapy has often a limited success in the treatment of this type of infections (Jikia et al., 2005; Kutter et al., 2010). The results of this study show that 1) the topical application of phage PA709 may effectively control human skin infections by *P. aeruginosa* 709 (bacteria reduction of 4 logs, after 2 hours of incubation); 2) the MOI of 10 and 50 are more effective than the MOI of 0.5; 3) the application of a single phage dose may be sufficient; 4) the application of phage PA709 is safe (no lysogenic conversion was detected); 5) the phage PA709 is suitable for phage therapy, has a broad host range (infection of 30% of the tested *P. aeruginosa* isolates), infecting different genotypes of *P. aeruginosa* and a high survival (in vitro and in ex-vivo).
The application of the bacteriophage PA709 on human skin artificially contaminated with *P. aeruginosa* 709 clearly reduces the number of viable bacteria by 4 logs after 2 hours of incubation. Although after 2 hours of incubation 2 logs of bacteria remain viable, the bacterial density may have decreased to a level low enough to be eliminated by the host immunitary system (Levin and Bull, 2004). As demonstrated by the mathematical model proposed by these authors the combination of phage and the host defenses are sufficient to keep the bacterial

density below lethal threshold after phage therapy (Levin and Bull, 2004).

According to the literature, the non-inactivation of the remaining bacteria can be due to the difficulty of viruses to find the host bacteria (Wiggins and Alexander, 1985), to non-replicating condition of the surviving bacteria that is physiologically refractory to phage infection (Bull et al., 2002; Levin and Bull, 2004) and to the development of resistance of the host to the phages (Levin and Bull, 2004). The results of this study show clearly that bacteria that re-grow after phage therapy were not resistant to PA709 phage. Similar results have been observed in other studies (Oliveira et al., 2009; Sillankorva et al., 2004). However, some authors attributed bacterial re-growth to the development of host resistance to the phages without actually testing the development of resistance (Andreatti Filho et al., 2007; Kutter et al., 2010). On the other hand, in this study, all the tested bacteria that re-grew after phage therapy regained sensitivity to the phages only after 3-5 streak-plating steps on solid medium, which means that these bacteria are yet infected with viruses which prevent their infection by the newly added phages after their isolation. Thus, the hypothesis of re-growth due to the low probability of encounter between viruses and hosts bacteria is not likely. In fact, the increase of MOI of 10 to 50 did not increase the efficiency of phage therapy, which means that almost all bacteria are infected at the MOI of 10 that was the MOI used in vitro and in ex vivo phage therapy experiments. The occurrence of lysogeny which can also render the bacterium immune to not only the original phages but also to related phages (Skurnik and Strauch, 2006) could also explain the bacterial re-growth, but no evidence of lysogeny was observed during the phage therapy experiments. Induction by the addition of mitomycin C to isolated bacteria that remained viable after phage therapy was not observed. The hypothesis of non-replicating bacteria to be physiologically refractory to phage infection can possibly explain the bacterial re-grow after phage therapy treatment.

The topical application of phage therapy by using gauze bandages soaked in phage suspension is suitable for topical use on wounds and was effective in the reduction of the concentration of infectious bacteria. Similar results were obtained when phage therapy was applied locally (Goode et al., 2003; Jikia et al., 2005). In addition, it was found that the normal skin microflora was not affected when this procedure was used (data not show) and the phage remain active for more than 7 days on the skin, which can help to avoid re-infection by the same bacteria. Up to our knowledge studies on phage survival in ex vivo experiments with human skin, are not available in current literature. However, but a study of Kumari et al. (Kumari et al., 2010) using phage to treat burn wound infection caused by *Pseudomonas aeruginosa* PAO in BALB/c Mice showed that the titer of five different Pseudomonas phage injected in mice kept on decreasing in skin with increase in time and no phage could be isolated in skin samples after 36 h of phage treatment.

Three MOI were tested in order to optimize the phage dose for the treatment of *P. aeruginosa* infections. High MOI are used when the experiments requires that most cells in the culture are infected, such as in the case of phage therapy. By contrast, low MOI are used when multiple cycles of infection are required. The MOI of 10 and 50 improved bacterial reduction relatively to the MOI of 0.5 such as observed in other studies (Goode et al., 2003; Huff et al., 2005). At a MOI of 1, specific phages reduced the concentration of *Salmonella enteritidis* and *Campylobacter jejuni* on excised chicken skin by < 1 log but with MOI of 100-1000, the concentration of bacteria was reduced by as much as 2 logs (Goode et al., 2003). The application of phages in birds, to reduce *Escherichia coli* was only effective at a MOI of 10,000 and when the culture was mixed with equal number of bacteriophage, mortality was reduced from 85 to 35% (Huff et al., 2005). However, in this study the difference of bacterial inactivation with MOI of 10 and of 50 was not significantly different (Huff et al., 2005).

The observation that that the application of a single dose of phage suspension may be sufficient to inactivate pathogenic bacteria is not new (Biswas et al., 2002; Sulakvelidze et al., 2001). Contrarily to antibiotics, repeated administration is unnecessary because as long as the target bacterium is present, the phage will be able to replicate. The local proliferation of the phages at the site of infection may require less frequent phage administration in order to achieve the optimal therapeutic effect. The results obtained in the present experiments confirm this assumption, encouraging a single dose of phage administration. The application of a second dose of phages did not increase the effectiveness of *P. aeruginosa* inactivation.

The results indicate that phage PA709 has a broad host range (30% of the 51 MDR *P. aeruginosa* clinical isolates were infected by the phage), infecting different genotypes of *P. aeruginosa* collected in the three studied cities of Portugal (Aveiro, Coimbra and Matosinhos). The bacterial host molecular typing by BOX-PCR with the primer BOXA1R (data not show) showed that the strains of *P. aeruginosa* isolated from the different cities (North and Center of Portugal), which were infected by the phage PA709, correspond to different genotypes and, consequently, this phage may be widely used to treat *P. aeruginosa* infections.

The phage PA709 has an icosahedral head with a long contractile tail and a double stranded DNA molecule as nucleic acid, a morphology characteristic of members of the Myoviridae family. The morphology of the studied phage and the characteristics of the nucleic acid suggested that PA709 may be closely related to the *P. aeruginosa* phages KZ Lin68, Lin21, PTB80, NN, EL, and RU (Krylov et al., 2003) rather than with *P. aeroginosa* phages D3112 and B3 (Ceyssens et al., 2009; Gilakjan and Kropinski, 1999) and KMV, LKD16 and LKA1 (Lavigne et al., 2003; Lavigne et al., 2008) which were classified as Siphoviridae and Podoviridae family, respectively.

Phage PA709 presents some interesting features, namely high efficiency in the inactivation of MDR *P. aeruginosa*, a broad host range and high stability in stock suspensions and in ex vivo human skin. All these attributes make this phage very promising for the treatment of *P. aeruginosa* skin wound infections. However, more phages should be isolated in the future, for the formulation of cocktails which might improve the inactivation efficiency against *P. aeruginosa* human skin infections.

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