Universidade de Aveiro Departamento de Biologia
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Sara Raquel do Souto Pereira

Viruses as therapeutic and infectious agents

Vírus como agentes terapêuticos e infeciosos

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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 Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Vírus, bacteriófagos, terapia fágica, cocktails fágicos, *Enterobacter cloacae*, hepatite A, hepatite C, infeção, seroprevalência, epidemiologia

resumo

O presente trabalho está dividido em duas partes: a primeira parte centra-se na utilização de vírus, mais concretamente bacteriófagos como meio terapêutico, já a segunda parte centra-se num estudo epidemiológico feito em parceria com o laboratório de análises clínicas Avelab, em que a prevalência dos vírus da hepatite A e C entre os anos 2002 e 2012 é evidenciada.

Enterobacter cloacae faz parte da flora microbiana normal do trato gastrointestinal de 40-80% da população. Este microrganismo oportunista é capaz de causar infeção em pacientes debilitados e hospitalizados. *E. cloacae* é resistente a um vasto número de antibióticos e por conseguinte as infeções causadas por esta bactéria são difíceis de controlar. Neste sentido, a aplicação da terapia fágica poderá ser uma possível alternativa no controlo da infeção. A sobrevivência e a dinâmica fago-hospedeiro foram testadas em três fagos previamente isolados (E-2, E-3 e E-4). A sobrevivência foi testada em PBS pelo método de quantificação em dupla camada de agar. A dinâmica de interação fago-hospedeiro foi caracterizada em meio de cultura Caldo de Soja Tripticaseína e da quantificação dos fagos pela técnica da dupla camada de agar. A dinâmica de interação fago-hospedeiro foi testada em PBS e urina. Relativamente à sobrevivência dos fagos observou-se o decréscimo da concentração de E-2 em duas ordens de grandeza em 105 dias. A concentração de E-3 diminuiu em seis ordens de grandeza em 160 dias, enquanto a concentração de E-4 diminuiu apenas em uma ordem de grandeza em 255 dias. A bactéria foi inibida pelos três fagos, resultando no decréscimo de ≈ 3 log após 4-10 h de incubação. O uso de cocktails foi mais eficaz observando-se uma redução de ≈ 4 log após 4 h. Na urina a inativação do hospedeiro não foi tão eficaz, sendo aproximadamente 2 log. Os fagos E-2, E-3 e E-4 demonstraram ser eficientes na inativação de *E. cloacae* sendo potenciais agentes no controlo de infeções nosocomiais.

A hepatite A é uma doença hepática viral comum e tem trazido graves problemas de saúde e económicos à medida que o seu padrão epidemiológico muda ao longo do tempo. Inquéritos sorológicos de países desenvolvidos indicam um declínio da seroprevalência do vírus da hepatite A (VHA) devido à melhoria das condições económicas e de saneamento. O vírus da hepatite C (VHC) é uma causa importante de doença do fígado em todo o mundo e causa de morbilidade e mortalidade substanciais. Na ausência de uma vacina, a prevenção é extremamente importante, especialmente para os grupos de risco. A taxa de infeção da hepatite C e a taxa de imunidade à hepatite A foram pesquisadas ao longo de um período de onze anos, por sexo e faixa etária no Distrito de Aveiro. Neste estudo retrospetivo, amostras de sangue de pacientes do Distrito de Aveiro, em regime ambulatório, provenientes do Laboratório de Análises Clínicas Avelab, foram analisadas para a presença de anticorpos contra antigénios do VHA e do VHC através de um imunoensaio de quimiluminescência. A imunidade para o VHA foi de 60%. A imunidade foi dependente da idade ($p < 0.05$) mas não se observaram diferenças significativas entre sexos nem durante o período de estudo ($p > 0.05$). Cerca de 4% dos pacientes apresentaram anticorpos anti-VHC. A infeção pelo VHC foi dependente da idade e variou entre os sexos ($p < 0,05$), para além disso diminuiu durante o período de estudo ($p < 0,05$). Os resultados apresentados neste estudo indicam que o número de pacientes jovens imunes ao VHA é baixo podendo indicar um maior impacto da doença no futuro. Relativamente à infeção pelo VHC conclui-se que os homens de meia-idade são mais afetados do que as mulheres o que pode indicar que esse grupo é mais propenso a comportamentos de risco. Para além disso, a diminuição dos casos positivos ao longo do período de estudo pode indicar uma diminuição na exposição a fatores de risco.

keywords

Viruses, phage therapy, phage cocktails bacteriophages, *Enterobacter cloacae*, multidrug resistant bacteria, hepatitis A, hepatitis C, infection, seroprevalence, epidemiology, surveillance study.

abstract

The present study is divided into two parts: the first part focuses on the use of viruses, specifically bacteriophages as an antimicrobial therapy, and the second part focuses on an epidemiological study conducted in partnership with the Clinical Laboratory Avelab, in which the prevalence of hepatitis A and C viruses between 2002 and 2012 is demonstrated.

Enterobacter cloacae is part of the normal flora of the gastrointestinal tract of 40 - 80% of people. This opportunistic microorganism is capable of causing infection in debilitated and hospitalized patients, such as urinary tract infections (UTI) associated with the use of urethral catheters. *E. cloacae* is resistant to a broad number of antibiotics therefore infections caused by this bacterium are difficult to control. Phage therapy may be a useful tool to control infections caused by antibiotic resistant strains. Three previously isolated phages E-2, E-3 and E-4 produced on *E. cloacae* were used to examine survival and host-phage dynamics in a buffer solution and in urine in order to evaluate their ability to treat UTI. The survival was determined in phosphate buffered saline (PBS) through quantification by soft agar overlay technique. The host-phage dynamics was characterized in Tryptic Soy Broth (TSB) medium and in human urine samples. The concentration of E-2 decreased by two orders of magnitude in the first 105 days. E-3 was not detectable after 160 days. E-4 concentration only decreased by one order of magnitude after 255 days. The results show that the growth of the *E. cloacae* was inhibited by the three phages, resulting in a decrease of ≈ 3 log after 4 - 10 h of incubation. The use of cocktails with two or three phages was significantly more effective, namely the phage cocktail E-2/E-4, with reductions of ≈ 4 log after only 2 hours of treatment. In urine, although the phage cocktail E-2/E-4 was less efficient in *E. cloacae* inactivation than in PBS, the inactivation was effective, bacterial reduction of 2.3 log after 4 h of incubation. Phages E-2, E-3 and E-4 showed an efficient inactivation of *E. cloacae*, namely when used as phage cocktails, being potential candidates as agents for the control of nosocomial urinary tract infections caused by *Enterobacter cloacae*.

Hepatitis A is a common viral liver disease and brings serious health and economic problems as its epidemiologic pattern changes over time. National serosurveys from developed countries have indicated a decline in HAV (hepatitis A virus) seroprevalence over time due to the improvement of economic and sanitation levels. The hepatitis A virus (HAV) immunity rate was surveyed throughout an eleven-year period by sex and age group in Aveiro District. Hepatitis C virus (HCV) is a major cause of liver disease worldwide and causes substantial morbidity and mortality. The common absence of symptoms associated leads to uncertainty to the geographic distribution of this disease. In the absence of a vaccine and effective treatment, prevention is extremely important, especially for at risk groups. The immunity rate of hepatitis A and the infection rate of Hepatitis C were surveyed over a period of eleven years by sex and age group in the district of Aveiro. In this retrospective study, blood samples from patients in ambulatory regime from the Laboratory of Clinical Analysis Avelab, were analyzed for the presence of antibodies against antigens of HAV and HCV using a chemiluminescence immunoassay. The HAV immunity was 60%. The immunity was age dependent ($p < 0.05$) but no significant differences were noted between sexes or during the study period ($p > 0.05$). About 4% of patients had anti-HCV antibodies. HCV infection was age and gender-dependent ($p < 0.05$) and decreased during the study period ($p < 0.05$). The results presented in this study indicate that the immunity for HAV in young patients is low and may indicate a greater impact of the disease in the future. Regarding HCV infection is concluded that middle-aged men are more affected by HCV than women which may indicate that this group is more prone to risky behavior. In addition, the reduction of infection rate throughout the study period may indicate a decrease in exposure to risk factors.

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Acronyms and Abbreviations

ANOVA	Analysis of variance
CFU	Colony forming units
CFU mL^{-1}	Colony forming units per milliliter
ds	Double-stranded
HAV	Hepatitis A virus
HCV	Hepatitis C virus
ICU	Intensive care units
MOI	Multiplicity of infection
PFU	Plaque forming units
PFU mL^{-1}	Plaque forming units per milliliter
ss	Single-stranded
TSA	Tryptone Soy Agar
TSB	Tryptic Soy Broth
WHO	World Health Organization

Thesis outline

This thesis documents two different studies, both related to viruses. In these studies, viruses are considered both as therapeutic and infectious agents.

This document is divided into two parts, with different general purposes. Both parts begin with general literature reviews serving as a basis for the following experimental work (chapters 1 and 3).

In the first part (chapter 1 and 2) the efficiency of phage therapy in growth control of a pathogen with increasing importance in the hospital environment is tested, in order to assess their applicability in the treatment of urinary tract infections.

In the second part (chapter 3 to 5) a serosurvey was conducted in partnership with the clinical laboratory Avelab in order to assess the prevalence of hepatitis A and hepatitis C in the District of Aveiro between 2002 and 2012.

Part I

Chapter 1 - Introduction

1. Bacterial resistance to antibiotics

The resistance of a bacterium to an antimicrobial drug can result from intrinsic or acquired mechanisms. The intrinsic mechanisms occur naturally in genes located on the bacterial chromosome and are therefore inherent to bacteria. These intrinsic mechanisms are related to the absence of the target or with the presence of low affinity targets, low cell permeability and also to multidrug efflux systems of multiresistant bacteria (Ammor, Gueimonde et al. 2008). Thus, in the natural resistance, the bacterium is always resistant to the antimicrobial drug. On the other hand, in acquired resistance, the bacterium is initially sensitive to a particular antibiotic but due to various mechanisms it becomes resistant (Bockstael and Van Aerschot 2009). Acquired antimicrobial resistance can occur due to a mutation in the gene of the chromosome consisting of the antibiotic target or by horizontal transfer of genes, specifically mobile genetic elements that are most likely to be transmitted (Alekshun and Levy 2007). The antimicrobial resistance mechanisms and examples of resistant bacteria are described in Table 1 (Mulvey and Simor 2009).

Table 1 - Antimicrobial resistance mechanisms and respective examples (adapted from Mulvey & Simor, 2009)

Mechanism	Resistant microorganisms	Antibiotics affected by bacterial resistance
Antimicrobial inactivation		
β-lactamase	• *Staphylococcus aureus* • *Haemophilus influenza* • *Enterobacteriaceae*	• Penicillins • Cephalosporins
Enzymatic inactivation of aminoglycosides	• *Enterobacteriaceae*	• Gentamicin • Tobramicin
Altered target site		
Altered penicillin-binding proteins	• *S. pneumonia* • Methicillin-resistant *S. aureus*	• Penicillin • Methicillin • Cloxacillin
Altered DNA gyrase or topoisomerase	• *S. pneumoniae* • *Enterobacteriaceae* • *Pseudomonas aeruginosa*	• Ciprofloxacin • Levofloxacin • Moxifloxacin
Decreased access to the target site		
Change in outer membrane proteins or porins	• *Enterobacteriaceae* • *P. aeruginosa*	• Gentamicin • Tobramycin • Tetracycline
Efflux pump	• *S. aureus* • *Streptococci*	• Clindamycin • Erythromycin

Genes that confer bacterial resistance to antimicrobial drugs can be disseminated. With some exceptions, the intrinsic resistance and the resistance resulting from a mutation are resistance mechanisms that are unlikely to be transmitted (Alekshun and Levy 2007). The most frequent mechanism of acquisition of resistance genes corresponds to horizontal transfer which may occur within the same strain or between different strains or even different bacterial genus. There are three different mechanisms of horizontal gene transfer (Figure 1).

During transformation bacteria capture and incorporate segments of DNA from bacterial lysates, the resistance gene may be incorporated into the chromosome or plasmid in the recipient cell. When the transfer of these resistance genes is mediated by bacteriophages is called transduction. In conjugation acquisition of resistance genes occurs as a result of direct contact of two bacterial cells. Plasmids, transposons or integrons move into the recipient cell. Plasmids are the only ones able to replicate autonomously.

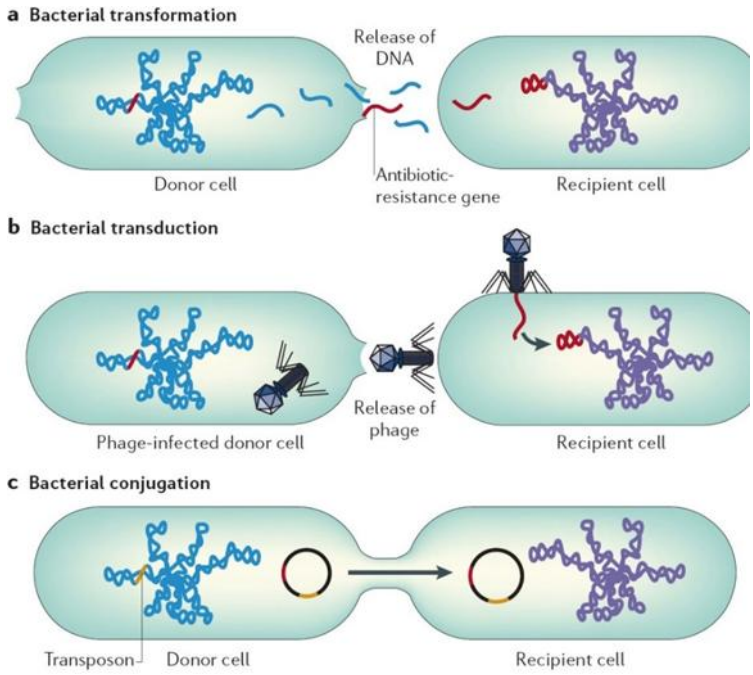

Figure 1 - Horizontal gene transfer between bacteria (adapted from Furuya et al., 2006)

2. Importance of *Enterobacter cloacae* infection

Enterobacter cloacae belongs to *Enterobacter* genus and *Enterobacteriaceae* family. It is distinguished from the genus *Klebsiela* by its motility and is usually ornithine decarboxylase positive and urease negative (Kosako, Tamura et al. 1996). The most frequent human pathogens from *Enterobacter* genus are *E. aerogenes* and *E. cloacae. E. cloacae* is several times a cause of nosocomial infections. There is not much information

about the factors leading to its pathogenicity and virulence. As a Gram-negative pathogen, *E. cloacae* possess endotoxins and thus may have pathogenic characteristics similar to other Gram-negative pathogens. *E. cloacae* is responsible for respiratory tract, urinary tract and intra-abdominal infections, endocarditis, septic arthritis, osteomyelitis and skin and soft tissue infections (Jeong, Lee et al. 2003).

In preantibiotic era, it was not considered in surveys of nosocomial bacteremia (McGowan 1988). However, the importance of this infection was highlighted in the most recent National Healthcare Safety Network at the Centers for Disease Control and Prevention (NHSN) data. In this survey, 2,039 hospitals reported nosocomial infections and 5% of them were caused by *Enterobacter spp.* Nearly 20% of total pathogens reported were multidrug resistant and 2% of total pathogens were extended-spectrum cephalosporin–resistant *Enterobacter spp* (Sievert, Ricks et al. 2013).

In another study the increasing importance of *Enterobacter spp*. was most apparent when isolates from intensive care units (ICU) were considered separately from the hospital at large. *Enterobacter spp*. was the third most common pathogen isolated from respiratory tract, the fourth most common from cirurgical wounds and the fifth most common from the urinary tract. Patients at increased risk of acquiring *E. cloacae* infection are those with a prolonged hospital stay, especially at ICU (Sanders and Sanders 1997).
There are several studies proving *E. cloacae* resistance to β-lactams, fluoroquinilones, aminoglycosides and tigecycline (Périchon, Courvalin et al. 2007, Pitout and Laupland 2008).

3. *Enterobacter cloacae* and urinary tract infections

The bladder is typically sterile, but the epithelial cells coating the urethra, downstream, are colonized by rods and aerobic facultative gram-negative *cocci* (Madigan et al 2009). The presence of microorganisms in the terminal urethra, and its consequent multiplication and invasion of tissues can cause the onset of a UTI (urinary tract infection). A set of structural, functional and physiologic factors in the urinary tract can play a key role in the development of the infection. Bacterial growth may be promoted by the presence of certain nutrients, such as glucose, amino acids and uric acid in urine and by an increase in its pH (Zasloff 2007). The presence of 10^5 CFU mL^{-1} of the same organism in a sample of urine is defined by significant bacteriuria (Raju and Tiwari 2001).

Pathogens belonging to the genus *Enterobacter* are some of the most commonly found in urinary infections Linhares et al., conducted a study in ambulatory regime in Aveiro wherein *Enterobacter spp* showed high resistance (greater than 80%) to 1st and 2nd

generation cephalosporins, amoxicillin and amoxicillin + clavulanic acid (Linhares, Raposo et al. 2013).

Urinary tract infection associated with urethral catheters are the most common infections occurring during hospitalization accounting for up to 40% of all nosocomial infections (Kunin 1997). The *Enterobactericae* are the most common pathogen in catheterized patients. There are reports indicating a relation between catheterized patients and bloodstream infection caused by *E. cloacae* (Tambyah and Maki 2000). *E. cloacae* infections have the highest mortality rate compared to other *Enterobacter* infections (Kanemitsu, Endo et al. 2007).

4. Bacteriophages

4.1 Discovery of Bacteriophages

In 1896 Ernest Hanking in England reported the observation of high antibacterial activity against *Vibrio cholerae* in the waters of the Ganges (Deresinski 2009). Faced with this phenomenon Ernest Hanking suggested an unknown substance that would be primarily responsible for the high antibacterial activity, thus restricting the spread of epidemics of cholera (Summers 2005). Two years later Gamaleya, Russia, observed a similar phenomenon with *Bacillus subtilis* (Dublanchet and Fruciano 2008). 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 and developed the method of quantification of viruses and some theories, including the replication cycle of the phage (Bratbak, Egge et al. 1993). The name bacteriophage was formed from “bacteria” and “phagein” (“to eat”, in Greek) (Sulakvelidze, Alavidze et al. 2001).

4.2 Properties and classification of bacteriophages

Bacteriophages are constituted by one order and 17 families (Table 2). Bacteriophage taxonomy is based on their shape, size and proteins as well as on their nucleic acid. Bacteriophages are tailed, polyhedral, filamentous, or pleomorphic (Figure 2). Most bacteriophages contain dsDNA, but there are phages with ssDNA, ssRNA or dsRNA (Ackermann 2003, Ackermann 2007).

Table 2 - Basic properties of bacteriophages and their classification (adapted from Ackermann, 2003).

Shape	*Nucleic acid*	*Order and families*	*Characteristics*
Tailed	dsDNA linear	*Myoviridae*	Tail contractile
		Siphoviridae	Tail long, noncontractile
		Podoviridae	Tail short
Polyhedral	ssDNA, Circular	*Microviridae*	Capsomers
	dsDNA, Circular, Superhelical	*Corticoviridae*	Complex capsid, lipids
	dsDNA, Linear	*Tectiviridae*	Inner lipoprotein vescicle, pseudotail
	ssRNA, Linear	*Leviviridae*	Polivirus-like
Filamentous	dsRNA, Linear, Segmented	*Cystoviridae*	Envelope, lipids
	ssDNA, Circular	*Inoviridae*	Long filaments, short rods
	dsDNA, Linear	*Lipothrixviridae*	Envelope, lipids
	dsDNA, Linear	*Rudiviridae*	TMV-like
Pleomorphic	dsDNA, circular superhelical	*Plasmaviridae*	Envelope, lipids, no capsid
	dsDNA, Circular superhelical	*Fuselloviridae*	Lemon-shaped
	dsDNA, linear superhelical	*Saltepovirus*	Lemon-shaped
	dsDNA, circular superhelical	*Guttaviridae*	Droplet-shaped

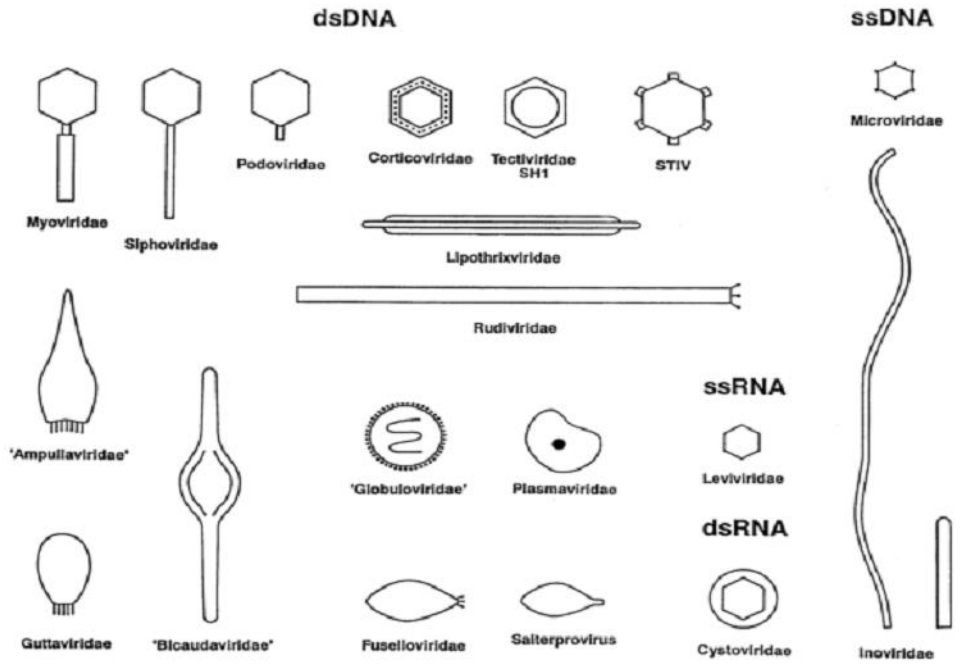

Figure 2 - Representation of phage families (Ackermann, 2007).

4.2.1 Bacteriophage infection

Phages are metabolically inert in their extra cellular form. They are only able to self-reproduce as long as the host is present and their replication depends exclusively on

the host intracellular machinery to translate their own genetic code (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 (Figure 3) (Almeida, Cunha et al. 2009). Only lytic phages are suitable candidates for phage therapy since they may destroy bacteria (Weinbauer and Rassoulzadegan 2004).

In the lytic cycle, firstly phages bind to specific receptors of bacteria. This stage 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 they multiply in the host cell after the genome is injected into the host bacterium. 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 (Weinbauer 2004, Goodridge 2010). The expression of the phage late proteins involved in the formation of new phage particles is responsible for the cell lysis (Duckworth and Gulig 2002). Fifty to 200 particles are released during lysis (Huff, Huff et al. 2005).

In the lysogenic cycle, the virus enters its genetic material into the host cell, that is, the viral DNA becomes part of the infected cell DNA. The genetic material of the bacteriophage, called prophage, can be transmitted to daughter cells. Occasionally, due to external factors (e.g. UV light), spontaneous lysis occurs and progeny phage is released (Hanlon 2007). Lysogeny might be a viral strategy that allows survival in periods of low host density during nutrient starvation (Weinbauer 2004).

Pseudo-lysogeny is another phenomenon that describes a phage-host cell interaction in which the nucleic acid of the phage, upon infection of an appropriate host cell, neither establishes a stable relationship nor triggers a lytic response. The phage nucleic acid simply resides within the cell in a non-active state. In situations of low nutrition there is insufficient energy available for the phage to initiate the typical responses of infection. However, when the level of nutrient availability increases, the phage acquires the necessary energy to allow gene expression, leading to either the establishment of a state of true lysogeny or replication and expression of the viral genome, leading to virion formation and lysis of the host cell (Ripp and Miller 1998).

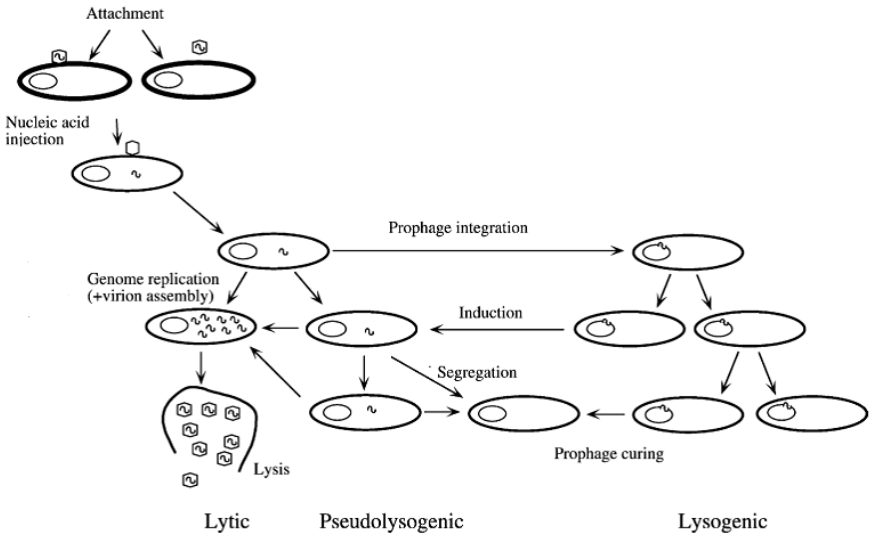

Figure 3 - Representation of phage families (Ackermann, 2007).

4.3 Phage Therapy

Phage therapy consists in the use of bacteriophages to inactivate bacteria (Almeida, Cunha et al. 2009). Since bacteriophages discovery, phage therapy was vigorously investigated, but after antibiotic advent the interest in phage-based therapeutics declined in the western world (Sulakvelidze, Alavidze et al. 2001). Nevertheless, in former Soviet Union countries, this therapy was continuously used and many progresses have been made (Summers 2001).

4.3.1 Prerequisites for phage therapy

Phage production is not simple and when this therapy was first applied clinically side effects were difficult to control. There are some requirements in the production of phages (Skurnik and Strauch 2006).

- Phages must be free from microorganisms;
- Phages must be well characterized. The genome must be sequenced in order to avoid resistance and toxin transmission to bacteria;
- Phages must be lytic and non-temperate;
- Phages must not transduce virulence factor genes of the host;
- The receiver of the phage must be known. In a population of 10^6 to 10^8 there is a great possibility of spontaneous mutants resistant to phages displaying an altered receptor;
- The broad host range must be well known;
- Stability of the phages over storage and application must be tested;
- Amenability to scale up.

4.3.2 Advantages and disadvantages of phage therapy

The emergence of pathogenic bacteria resistant to most, if not all, currently available antimicrobial agents has become a critical problem in modern medicine. Since apparently we are reentering the “preantibiotics” era the development of alternative antibacterial therapies is imperative. Nowadays, western countries renewed the interest in phage therapy (Sulakvelidze, Alavidze et al. 2001).

Advantages

Phage therapy represents a potentially viable alternative to antibiotics and to other antibacterial compounds to inactivate pathogenic bacteria (Almeida, Cunha et al. 2009). There are many advantages of bacteriophages over antibiotics (Table 3).

Table 3 - Major advantages of phage therapy against antibiotics (Adapted from Abedon & Thomas-Abedon, 2010, Hagens and Loessner, 2010, Harcombe and Bull, 2005, Matsuzaki et al., 2005, Skurnik and Strauch, 2006).

Advantages of phage therapy against antibiotics
• Phages are specific to target. The broad host-range usually includes the level of strain and rarely the level of genus. The restricted host specificity means that phages generally will not affect beneficial bacteria, like commensals in the gastrointestinal tract; • Phages have limited resistance development compared to antibiotics; • No serious side-effects have been described since phages do not affect eukaryotic cells neither natural microflora. The target specificity decrease patient’s side effects since the natural flora is not disturbed; • When resistance occurs, the selection of new phages is a relatively fast process; • Attenuates the virulence of bacteria that become resistant to phage; • Phages multiply as long as bacteria are present; • Low-cost production.

Disadvantages

Despite the many advantages, there are still some problems associated to this therapy.

It is possible that the release of large amounts of bacterial endotoxins bound to the membrane during cell lysis causes side effects, though, purification of the phage suspensions is a simple process (Tanji, Shimada et al. 2005). The use of phage therapy is

more effective when applied locally, although there are several studies in which systemic application has promising results (Gregoracci 2006).

The lysogenic conversion can be another problem associated to this therapy. The lysogeny can induce a phenotype modification of the host cell such as toxin production and antibiotic resistance but also resistance to infection by similar phages (Alisky, Iczkowski et al. 1998, Skurnik and Strauch 2006). In phage infection there is an attachment of the phage onto specific receptors of bacteria. If the mutation of these receptors occurs, the phage fails to recognize bacteria (Levin and Bull 2004). Despite this obstacle to the application of phage therapy, the induction of resistance of bacteria is not considered a major concern, since a wide range of studies show that the mutation rate of phage accompanies the mutation rate of bacteria, sufficiently to maintain the effectiveness of phage therapy (Parisien, Allain et al. 2008). Furthermore, it is possible that the receptor used by the phage is a virulence determinant thus the loss of receptor would reduce the virulence of bacteria, then it would be easier for the host immune system to overcome the infection (Skurnik and Strauch 2006). It is also possible to use cocktails of phages in order to avoid resistance (Chan and Abedon 2012, Mateus, Costa et al. 2014).

4.3.3. Clinical applications of phage therapy

Immediately after the discovery of bacteriophages, although surrounded in mysticism regarding the nature and potential therapeutic properties, phage therapy was used in benefit of man (Gregoracci 2006).

During the summer of 1919, d'Herelle applied phage therapy in five children with bacillary dysentery admitted at the hospital *Necker-Enfants-Malades* in France, achieving therapeutic success in all cases (Dublanchet and Bourne 2007).

Since western countries abandoned phage therapy, the majority of studies demonstrating the efficacy of phage therapy in clinical settings came from research groups in Eastern Europe and the former Soviet Union and were published in non-English journals (Sulakvelidze, Alavidze et al. 2001).

Most of the actual knowledge regarding clinical applications of phages comes Eliava Institute of Bacteriophage, Microbiology and Virology in Georgia. Thousands of monophages and cocktail of phages (*pyophage* and *intestiphage*) for pathogenic bacteria strains, such as *Staphylococcus*, *Streptococcus*, *Proteus, Pseudomonas aeruginosa* and *Clostridium* were prepared in this Institute (Lorch 1999, Kutateladze and Adamia 2008). They continually renewed the cocktail *pyophage* and *intestiphage* with new phages against recently virulent strains for wound infections and enteric bacteria, respectively. For

deeper wounds, phages embedded in polymer called *PhageBioderm* is often used in addition to pyophage. *PhageBioderm* is a biodegradable, non-toxic polymer developed by Georgian chemists and microbiologists in 1995 and approved for commercial release in 2000 (Kutter, De Vos et al. 2010).

In the Institute for Immunology and Experimental Medicine in Poland there is a bank of phages constituted by 300 specific bacteriophage strains against S*taphylococci, Enterococci, Escherichia sp., Klebsiella sp., Salmonella sp., Shigella sp., Enterobacter sp., Proteus sp., Serratia sp., Acinetobacter sp.* and *Pseudomonas sp.* (Kutter, De Vos et al. 2010).

The literature provided by these countries show that the bacteriophages have potential as antimicrobial agents. Soviet and Polish studies were mostly clinical, contrary to most Western Europe countries studies. They found efficacy against mucosal, systemic and cutaneous infections, including in immunosuppressed patients. These studies showed rare and reversible side effects (Slopek, Durlakowa et al. 1983, Sulakvelidze, Alavidze et al. 2001).

In western countries phage therapy is recently gaining interest. Studies related to phage kinetics, especially the multiplicity of infection (MOI) that is, the prediction on the number of phage dose applications and the timing of application, are currently being done (Levin and Bull 2004).

4.3.4. Other applications of phage therapy

Phage therapy is also being extensively studied in other industries. In table 4 are described companies that commercialize phages or phage products for various sectors.

- **Aquaculture**

Aquaculture is characterized by production of all types of cultured aquatic animals and plants in brackish water, freshwater and marine water (Pillay and Kutty 2005). Despite the rapid development of this industry, its vulnerability to infections leads to considerable losses (Flegel 2006, Saksida 2006). The development of infections caused by microorganisms, including multiresistant bacteria, is readily transmitted by water, infecting a variety of fish species (Almeida, Cunha et al. 2009). Since the rate of resistance to antibiotics in this sector is increasingly high, there has been much interest in finding phages to treat the main diseases affecting aquaculture. A group of Japanese researchers studied the potential use of phages to prevent infections in fish caused by *Lactococcus*

garviae and concluded that the use of phages can be useful to control infections in fish cultures (Skurnik and Strauch 2006, Almeida, Cunha et al. 2009).

- **Veterinary**

Phage therapy is also used to control animal infections. The most successful cases reported relate to the use of phage against *Salmonella* infections in poultry and in cases of enteritis neonatal caused by *Escherichia coli* in calves, piglets and lambs, with promising results (Atterbury 2009, Henriques, Sereno et al. 2013).

- **Agriculture**

The main infections in plants are of fungal origin, however bacterial infections are responsible for the major losses in agriculture (Balogh, Jones et al. 2010). These infections are often difficult to control due to lack of effective bactericide (Obradovic, Jones et al. 2005). In order to overcome this problem phages have been recently evaluated to control some phytobacteria (Balogh, Canteros et al. 2008, Balogh, Jones et al. 2010). An available example of a phage suspension is agrifago that is effective and safe for prevention and control of harmful bacteria in tomato and pepper cultures (Housby and Mann 2009).

- **Food industry**

The use of phage in the food industry is currently applied as a biological control method for undesirable pathogens, increasing food security, in particular fresh and ready-to-eat foods (Hagens and Loessner 2010). The food industry focuses its interest in four pathogens (*Listeria monocytogenes*, *Salmonella, Campylobacter* and *Escherichia coli*) (Rees and Dodd 2006). In 2006, the FDA and the American Agriculture Department approved a phage suspension anti-*Listeria* that can be used as an aid in processing of all food products susceptible to *Listeria monocytogenes* (Hagens and Loessner 2010).

Table 4 - Leading companies related to the commercialization of phage products (adapted from Housby and Mann, 2009).

Company (location)	Product area	Stage of development
BigDNA (Edinburgh, UK)	Bacteriophage DNA vaccination via phage encoded DNA delivered intravenously or orally	R&D
Blaze Venture Technologies (Hertfordshire, UK)	Phage immobilization technology, MRSA, licensing	Licensing
JSC Biochimpharm (Tbilisi, Republic of Georgia)	Various phage lysates are mixed and used for intestinal problems, for example, Dysentery, salmonellosis,	Commercialization

	dyspepsia, colitis and enterocolitis and for bacterial infections.	
Biopharm L Limited (Tbilisi, Republic of Georgia)	Products include Pyobacteriophage and Intesti-bacteriophage that are mixtures of phage lysates for bacterial intestinal and infection control-sold to pharmacies as Over The Counter drugs.	Commercialization
BioControl (Southampton, UK)	*Pseudomonas* infections of the ear	Phase II trial completed
Biophage Pharma Inc. (Montreal, Canada)	Environmental therapies and diagnostics, phage products geared towards antibacterial resistance problems and as a weapon against bioterrorism	Research and development
EBI Food Safety (Wageningen, Netherlands)	Food Safety. A cocktail of phage against *Listeria*	LISTEX P100^{TM}, Product available
Gangagen (Bangalore, India and Palo Alto, California, USA)	*Staphylococcus aureus*	Pre-clinical
Innophage (Porto, Portugal)	Environment, Cosmetic and Medical bacterial infections	Unknown
Intralytix (Baltimore, USA)	Food safety, *Listeria*	FDA and EMEA approval on ready to eat meats and cheeses
Neurophage Pharmaceuticals (Cambridge, USA)	Brain changes, for example Alzheimer	Startup company
Novolytics (Coventry, UK)	Prevention and treatment of MRSA infection	Pre-clinical
Omnilytics (Salt Lake City, Utah, US)	AgriPhage is a natural, safe, effective treatment that prevents and controls harmful bacteria on tomato and pepper plants	Product available
Phage-Biotech (Rehovot, Israel)	Anti-Pseudomonas infectives	R&D
Phico Therapeutics (Cambridge, UK)	Anti MRSA products	Pre-clinical
Phage International (San Ramon, California,USA; Tbilisi, Republic of Georgia)	Phage treatment center	Distributor
Viridax (Boca Raton, Florida, USA)	Staphylococcal aureus—respiratory, systemic, topical, wound care	Pre-clinical

4.3.5. Phage cocktails

The simultaneous use of two or more phages has been proven more effective than the use of monophage therapy (Chan and Abedon 2012).

The monophage therapy is used when a sufficiently wide host range phage is found. This approach is simpler since the preparation of the phage suspension is easier and the risk of immunological interaction is lower. The combinations of individual phages

isolates broaden the typical narrow spectrum of activity of the single phage. This results in increased magnitude of utility for individual formulations to treat specific bacterial diseases and prevention of phage-resistant bacteria (Thiel 2004)**.**

Resistance may result from the alteration or loss of the bacterial cell surface receptors, inhibition of phage DNA penetration and production of restriction endonucleases which degrade the phage DNA (Labrie, Samson et al. 2010). This limitation may be overcame by the combined use of two or more phages (Chan, Abedon et al. 2013).

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Chapter 2 - Potential of phage cocktails in the inactivation of *Enterobacter cloacae*

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1. Abstract

Enterobacter cloacae is part of the normal flora of the gastrointestinal tract of 40 - 80% of people. This opportunistic microorganism is capable of causing infection in debilitated and hospitalized patients, such as urinary tract infections (UTI) associated with the use of urethral catheters. *E. cloacae* is resistant to a broad number of antibiotics therefore infections caused by this bacterium are difficult to control. Phage therapy may be a useful tool to control infections caused by antibiotic resistant strains. Three previously isolated phages E-2, E-3 and E-4 produced on *E. cloacae* were used to examine survival and host-phage dynamics in a buffer solution and in urine in order to evaluate their ability to treat UTI. The survival was determined in phosphate buffered saline (PBS) through quantification by soft agar overlay technique. The host-phage dynamics was characterized in Tryptic Soy Broth (TSB) medium and in human urine samples, through quantification of phages by soft agar overlay technique and host quantification in Tryptic Soy Agar (TSA) medium. The concentration of E-2 decreased by two orders of magnitude in the first 105 days. The concentration of E-3 decreased by one order of magnitude in the first 20 days and reached a plateau until 77 days. Afterwards, the phage titer decreased by three orders of magnitude until 156 days. E-4 concentration only decreased by one order of magnitude after 255 days. The results show that the growth of the *E. cloacae* was inhibited by the three phages, resulting in a decrease of ≈ 3 log after 4 - 10 h of incubation. The use of cocktails with two or three phages was significantly more effective, namely the phage cocktail E-2/E-4, with reductions of ≈ 4 log after only 4 hours of treatment. In urine, although the phage cocktail E-2/E-4 was less efficient in *E. cloacae* inactivation than in PBS, the inactivation was effective, bacterial reduction of 2.3 log after 4 h of incubation. Phages E-2, E-3 and E-4 showed an efficient inactivation of *E. cloacae*, namely when used as phage cocktails, being potential

candidates as agents for the control of nosocomial urinary tract infections caused by *Enterobacter cloacae*.

Keywords: Viruses, phage therapy, phage cocktails bacteriophage, *Enterobacter cloacae*, multidrug resistant bacteria, urinary tract infections

2. Introduction

Enterobacter cloacae is a Gram-negative pathogen responsible for respiratory tract, urinary tract and intra-abdominal infections, endocarditis, septic arthritis, osteomyelitis and skin and soft tissue infections and these bacteria are responsible for healthcare-associated infections or nosocomial infections (Jeong, Lee et al. 2003). Nosocomial infections are acquired during hospital admission or medical procedures and have been increasing due to the overuse of antibiotics. According to the latest European Centre for Disease Prevention and Control report, from a total of 231 459 patients from 947 hospitals, the prevalence of patients with at least one nosocomial infection was 6.0%. Of a total of 264 infections by *E. cloacae* reported during 2011, 94 were multi-drug resistant (ECDC 2013).

Urinary tract infection (UTI) associated with urethral catheters are the most common infections occurring during hospitalization, accounting for up to 40% of all nosocomial infections (Kunin 1997). There are reports indicating a relation between catheterized patients and infections caused by *E. cloacae* (Tambyah and Maki 2000). Moreover, *E. cloacae* infections have the highest mortality rate compared to other *Enterobacter* infections (Kanemitsu, Endo et al. 2007). Phage therapy (use of lytic phages to inactivate bacteria) can be used as an alternative to control this nosocomial infection.

Bacteriophages or phages are viruses that are capable to infect exclusively bacteria. Until the advent of antibiotics, phage therapy was widely used, especially in the Eastern Europe countries. It fell into disuse and now, due to the overuse of antibiotics and consequent appearances of resistant bacteria, a growing interest in this therapy approach can be noted (Sulakvelidze, Alavidze et al. 2001, Ackermann 2003, Hanlon 2007).

The selection of the appropriate bacteriophage is a key factor in the success of the phage therapy treatment. Among the main criteria required to select viruses for phage therapy are i) host range, ii) survival in the environment; iii) no potential for lysogenic conversion and/or generalised transduction and, of course, iv) the efficiency of bacterial inactivation (Skurnik and Strauch 2006).

Therapeutic phages should have a large broad host range which means that phages should be able to lyse majority of the strains of a given bacterial species (Almeida,

Cunha et al. 2009). Some studies that have focused on the host range of individual phages have shown a large variation in phage specificity, both within and across bacterial species. In fact, some phages that appear to be broader, in the sense that they can infect different genera of bacteria, fail to infect a subset of strains or species within the same genus. This is most likely the result of both specific phage adaptations and the subsequent evolution of bacterial resistance in some lineages, including via transfer of plasmids, which makes difficult to decipher specific rules concerning phage host range (Koskella and Meaden 2013). For instance, Jensen et al (1998) have shown that nine of ten phages of *E. coli*, *P. aeruginosa* and *Sphaerotilus natans* presented a broad-host-range, infecting their hosts and bacteria from another orders (such as, Pseudomonadales, Burkholderiales or Enterobacteriales) (Jensen, Schrader et al. 1998). However, the 'jumbo' phage RaK2, isolated using *Klebsiella* sp as host by Šimoliūnas et al (2013), was tested on 40 bacterial strains from different genera and 39 of these bacterial strains were resistant to the phage RaK2 (Šimoliūnas, Kaliniene et al. 2013).

The success of phage therapy to control pathogenic bacteria depends on viral survival and viability in environment to maintain their lytic attributes. Although there are some data available about the study of the mechanisms and rates of mortality or loss of infectivity of phages, little is known about their time of survival in the environment. De Paepe and Taddei (2006) by comparing life history traits of 16 phages infecting the bacterium *Escherichia coli*, showed that their mortality rate is constant with time and negatively correlated to their multiplication rate in the bacterial host. The authors showed that the capsid thickness and the density of the packaged genome account for 82% of the variation in the mortality rate (De Paepe and Taddei 2006). Tsonos et al (2013) used a cocktail of four different phages to cure avian pathogenic *Escherichia coli* (APEC) infected chickens. Although the phages in the cocktail were able to efficiently lyse the APEC strain in vitro, treated chickens did not show a significant decrease in mortality, lesion scores or weight loss compared to untreated groups, even though the APEC-specific phages could be re-isolated from the lung and heart of chickens that were euthanized. Moreover, the re-isolated bacteria from infected chickens had remained sensitive to the phage cocktail (Tsonos, Oosterik et al. 2013).

To select phages with therapeutic potential it is necessary to assure that there is any potential for lysogenic conversion of the phages in order to avoid the expression of genes that encoding toxins and to maintain their lytic characteristics so that inactivate efficientely the pathogenic bacteria. The potential of lysogenic conversion can be evaluated through the detection of genes encoding integrase enzymes. Another concern

having into account in the selection of the phages is their generalised transduction which can tansmit resistance genes to the bacteria such as antibiotic resistance. (Skurnik and Strauch 2006).

Another major concern regarding the use of phages to control infections is the emergency of phage-resistant mutants (Hyman and Abedon 2010). Resistance may result from the alteration or loss of the bacterial cell surface receptors; inhibition of phage DNA penetration; production of restriction endonucleases which degrade the phage DNA, clustered regularly interspaced short palindromic repeats (CRISPR) system, a widespread microbial response to by-pass the selective pressure exerted by phage infection, among others (Deveau, Garneau et al. 2010). This limitation can be overcame by the combined use of two or more phages or phage cocktails (Chan, Abedon et al. 2013). The mutation induced by phages may lead also to loss of pathogenic properties (Filippov, Sergueev et al. 2011). Another advantage of the use of phage cocktails is the ability of treating multiple pathogens, this feature broaden the spectrum of action of phage therapy (Cairns, Timms et al. 2009, Kunisaki and Tanji 2010, Chan, Abedon et al. 2013).

The aim of the present study was to evaluate the effectiveness and safety of phage cocktails in order to control UTI caused by *E. cloacae.* The isolated phages were tested separately and combined in PBS and the best phage cocktail was also tested in human urine samples.

3. Material and Methods

3.1. Bacterial strains and Growth conditions

The bacterial strain *E. cloacae* used in study as phage host was previously isolated in our laboratory (Pereira, 2014). The bacterial strains used in host range studies are listed in Table 5. *Salmonella typhimurium* (ATCC 13311 and ATCC14028), *Escherichia coli* (ATCC 25922 and 13706), *Aeromonas hydrophila* (ATCC 7966), *Staphylococcus aureus* (ATCC 6538 and DSM 25693), *Vibrio parahaemolyticus* (DSM 27657), *Shigella flexnexi* (DSM 4782), *Listeria innocua* (NCTC 11288) and *Listeria monocytogenes* (NCTC1194) were purchased from ATCC, DSM and NCTC collection, respectively. Four *S. aureus* enterotoxic strains (2065 MA, 2153 MA, 2095 MA and 2043 MA) were isolated from food (Baptista et al, 2015). Five strains *Salmonella enteriditis* were isolated from food given by Controlvet. The other bacterial strains used in this study were isolated in other study from water collected in Ria Aveiro (Louvado et al, 2012). The *E. cloacae* isolate were maintained in TSA (Merck, Darmstadt, Germany) at 4 ºC. Before each assay, one isolated colony was transferred to 10 mL of TSB (Merck, Darmstadt,

Germany) and was grown overnight at 37 °C. An aliquot of this culture (100 µL) was aseptically transferred to 10 mL of fresh TSB medium and grown overnight at 37 °C to reach an optical density (O.D. 600) of ≈ 0.8.

3.2. Phage isolation and host range

A sample of wastewater from a secondary-treated sewage plant near the city of Aveiro (Portugal) was used to isolate the *E. cloacae* bacteriophages according Costa et al. (2011). The sewage water was filtered sequentially by 3 µm and then 0.45 µm-pore-size polycarbonate membranes. The filtrate was added to a fresh bacterial culture in double concentrated TSB. The mixture was incubated at 37 ºC for 5 hours and then centrifuged (10,000 x g, 10 minutes). The phage titer was determined by the double-layer method (Adams 1959) using the centrifuged supernatant as phage suspension and TSA as culture medium. The plates were incubated at 37 ºC and examined for lysis plaques after 12 hours. Two more successive single-plaque isolations were performed to obtain pure phage suspensions. Phage suspensions were stored at 4 °C with 1% of chloroform (Scharlau, Spain).

Phage host range was determined by the spot test. Ten microliters of concentrated phage lysate (>10^9 PFU mL^{-1}) was dropped onto a TSA plate overlaid with *E. cloacae* (10^8 CFU mL^{-1}). The plate was allowed to dry and incubated for 8 - 12 h. According to the degrees of clarity, the spots were differentiated into three categories: large clear (++), small clear lysis (+), not lysis (-). Bacterial susceptibility to the three bacteriophages was assayed for 47 pathogenic bacterial strains (Table 5). The hosts tested included species from 15 genera.

3.3. Phage survival determination

Phage survival was tested in phosphate buffered saline (PBS). Phage suspensions were added (estimated final concentration 10^7 PFU mL^{-1}) and incubated at 25 °C without shaking. The phage titer was determined as described above, at time zero and at intervals of 12 h until day 1, 24 h until day 5, 48 h until day 9, 72 h until day 12, 120 h until day 45, and 240 h until the end of the experiment (day 255), by the double-layer method. The plates were incubated at 37 °C and examined for plaques after 4 - 8 h. For each phage, three independent experiments were done.

3.4. Phage DNA extraction

Bacteriophage lysates (10^9 plaque forming units, PFU mL^{-1}) were centrifuged 3 times at 6000 g for 10 min. The phage lysates were ultracentrifuged at 100000 g for 2 h at 10 ºC. Five hundred microliters of SM buffer were added to the pellet and was let to rest for 2 h. The suspension was then treated with DNase I and RNase A at 37 °C for 20 min to remove any free nucleic acids contamination. Nucleic acid extraction from phage particles was performed as described by Griffiths *et al.* [38]. Extraction was performed by the addition of 0.5 mL of hexadecyltrimethylammonium bromide (Sigma Aldrich, St. Louis, MO, USA) extraction buffer and 0.5 mL of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0; Sigma Aldrich) to the sample. The sample was lysed for 30 s in a FastPrep FP120 (BIO 101/Savant) at 5.5 ms^{-1} and centrifuged (16,000 g) for 5 min at 4 °C. The supernatant was pipetted into a clean vial, mixed 1:1 with chloroform-isoamyl alcohol (Sigma Aldrich) and centrifuged (16,000 g) for 5 min at 4 °C. The supernatant was removed to a clean tube and the nucleic acids were precipitated with two volumes of 30% (wt/v) polyethylene glycol 6000 – 1.6 M NaCl for 2 h at 25°C. This mixture was centrifuged (18,000 g) at 4 °C for 10 min, the pellet washed in ice cold 70% (v/v) ethanol, centrifuged (18.000 g) at 4 °C for 10 min and then the pellet was air dried prior to re-suspension in 30 µL Tris-EDTA buffer. Nucleic acid yield was quantified in the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The resulting product was electrophoresed through 0.8 % agarose gel at 80 V for 40 min.

3.5. Whole genome sequencing and bioinformatics analyses

Ten µg of DNA of each purified phage were subjected to a Roche/454 pyrosequencing approach on a GS FLX device with Titanium reagents. The six samples were barcoded and pooled on 1/4 of the sequencing plate. Reads were assembled using CLC Genomics Workbench version 7 (CLCbio, Aarhus, Denmark) and Roche GSAssembler version 2.7 (Roche, Switzerland).

3.6. Kill curves in PBS

The three phages (E-2, E-3 and E-4) were separately tested and phage cocktails were tested (two or three phages mixed together at the same concentration). In all assays *E. cloacae* was used as host at a MOI of 100. In order to obtain a MOI of 100, 2,5 µL of *E. cloacae* culture at a concentration of 10^8 CFU ml^{-1} and the phage suspension at a concentration of 10^9 PFU mL^{-1} were added to sterilized Erlenmeyers with 30 mL of TSB

medium and incubated at 37 ºC overnight. Before each phage therapy assays, the phage titer was determined using the double agar layer method, after an incubation period of 4–6 h at 37 °C. For each assay two control samples were preformed, the phage control (PC) and the bacterial control (BC) and the incubation conditions were exactly the same. After 0, 2, 4, 6, 8, 10, 12 and 24 hours aliquots of test samples and of bacterial and phage controls were collected to quantify bacteria and phages, respectively.

3.7. Kill curves in urine

To perform phage therapy in urine, the cocktail with higher efficiency was tested in urine instead of PBS at a MOI of 100 and the phages were also tested separately. The urine samples were ceded by Laboratory of Clinical Analysis Avelab (Aveiro, Portugal). The study was approved by the Ethical Committee of the Clinical Analysis Laboratory Avelab, specifically by Doctors Alberto Ferreira Neves, António Rodrigues and Maria Teresa Raposo. Early urine samples were collected, using the Avelab Laboratory protocol, by midstream clean-catch technique after patient daily hygiene. The middle jact was collected directly into the sterile recipient. The urine samples presented a pH of 6, a density of 1.021 and did not contain proteins, epithelial cells or bacteria. The urine was previously centrifuged (10,000 x g, 10 minutes) and filtered through a 0.45 µm-pore-size polycarbonate membrane. After 0, 2, 4, 6, 8, 10, 12 hours aliquots of test samples and of bacterial and phage controls were collected to quantify bacteria and phages.

3.8. Statistical analysis

Statistical analysis was performed using SPSS (SPSS 20.0 for Windows, SPSS Inc., USA). Normal distributions were checked by Shapiro-Wilk test. Homogeneity of variance was checked by Levene test. The existence of significant differences among the different phage therapy conditions was assessed by one-way analysis of variance (ANOVA) model. For each situation, the significance of the differences was done by comparing the results obtained in the test samples with the results obtained for the correspondent control samples for the different times of each of the three independent assays. A value of $p < 0.05$ was considered to be statistically significant.

4. Results

4.1. Phage host range

The isolated phages on *E. cloacae* infected bacteria from *Salmonella* spp., *Escherichia* coli, *Proteus*, *Citrobacter and Shigella flexneri*, all members of the *Enterobacteriaceae* family, but no bacteria belonging to the other families tested. Detailed results are described in Table 5.

Table 5 - Bacterial sensitivity to bacteriophages E-2, E-3 and E-4 isolated on E. cloacae.

Species	Infectivity of phage		
	E-2	E-3	E-4
Salmonella Typhimurium ATCC 14028	-	+	+
S. Typhimurium ATCC13311	-	-	-
Salmonella Enteriditis CVA	+	+	+
S. Enteriditis CVB	-	-	-
S. Enteriditis CVC	-	-	-
S. Enteriditis CVD	-	-	-
S. Enteriditis CVE	-	+	-
E. coli ATCC 25922	-	-	-
E. coli ATCC 13706	-	-	-
E. coli BC30	+	-	+
E. coli AE11	-	-	-
E. coli AD6	-	-	-
E. coli AF15	-	-	-
E. coli AN19	++	++	++
E. coli AC5	-	+	-
E. coli AJ23	-	+	-
E. coli BN65	-	-	-
E. coli BM62	+	-	-
Shigella flexneri DSM 4782	++	++	++
Citrobacter freundii 6F	+	-	-
C. freundii 10l	+	-	-
Providencia sp.	-	-	-
P. vermicola	-	-	-
Proteus vulgaris	-	-	-
Proteus mirabilis	+	+	-
K. pneumoniae	-	-	-
Enterococcus feacalis	-	-	-
E nterococcus faecium	-	-	-
S.aureus DSM 25693	-	-	-
S. aureus ATCC 6538	-	-	-
S. aureus 2065 MA	-	-	-
S. aureus 2153 MA	-	-	-
S. aureus 2043 MA	-	-	-
S. aureus 2043 MA	-	-	-
Listeria innocua NCTC 11288	-	-	-

L. monocytogenes NCTC 1194	-	-	-
Vibrio parahaemolyticus DSM 27657	-	-	-
V. anguillarum DSM 21597	-	-	-
V. fischeri ATCC 49387	-	-	-
Photobacterium damselae damselae DSM 7482	-	-	-
Aeromonas hydrophilla ATCC 7966	-	-	-
A. salmonicida CECT 894	-	-	-
Pseudomonas aeruginosa	-	-	-
P. fluorescens	-	-	-
P. putida	-	-	-
P. segetis	-	-	-
P. gingeri	-	-	-
Total number of strains infected	8	8	5

4.2. Phage survival determination

The results of the survival of *E. cloacae* phages on PBS are represented in Figure 4. The concentration of E-2 decreased by two orders of magnitude in the first 105 days. E-3 concentration decreased by two orders of magnitude in the first 20 days and reached a plateau until 77 days. Afterwards, the phage titer decreased by three orders of magnitude until 156 days. E-4 concentration only decreased by one order of magnitude after 255 days (Figure 4).

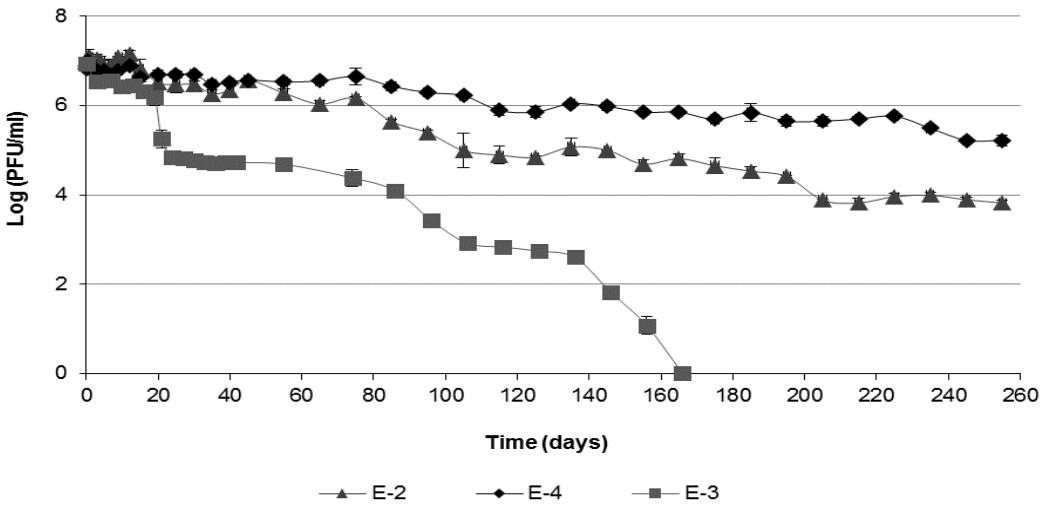


Figure 4 - Survival of E-2, E-3 and E-4 phages in PBS. Values represent the mean of three experiments; error bars represent the standard deviation.

4.3. Genome sequencing

A total of 23955 reads were produced, with 460 bp average read length, resulting in a total of 110'342'246 bases. Specifically, 22'256 reads were obtained for E-4, 11'5491 for E-2 and 31'628 for E-3. Phage genomes were de novo assembled using CLC Genomics Workbench 7 and Roche GSAssembler 2.7. Phages E-2 (1'367-fold coverage, 36'275 bp genome), E-3 (404-fold coverage 31'522 bp genome) and E-4 (235-fold coverage, 39'142 bp genome) were assembled with the GSAssember software.

4.4. *E. cloacae* inactivation in PBS

4.4.1. E. cloacae inactivation by single-phage suspensions

E. cloacae was challenged with the three phages, separately and in a cocktail, in order to assess their potential as therapeutic agents. The maximum of bacterial inactivation with the E-2 phage was 3.4 log after 4 h of incubation and after 12 h was still significantly high (1.6 log) relatively to the bacterial control (ANOVA, $p < 0.05$) (Figure 5A). No decrease of the phage survival (ANOVA, $p > 0.05$) was observed during the 24 h of the experiments for the phage controls, however, when the phage was incubated in the presence of the host, a significant increase in phage concentration was observed after 24 h relatively to phage control (1.1 log) (ANOVA, $p < 0.05$) (Figure 5B).

When the E-3 phage was tested, a maximum bacterium inactivation was observed after 4 h (3.8 log) and after 12 h the rate of inactivation was still significantly high (2.3 log) relatively to the bacterial control (ANOVA, $p < 0.05$) (Figure 5A). A significant increase of 1.2 log in phage concentration was observed after 24 h relatively to phage control (ANOVA, $p < 0.05$). Phage control remained constant during the treatment from the beginning of the treatment (ANOVA, $p > 0.05$) increasing significantly, 1.2 log, in the presence of the host after 24 h (ANOVA, $p < 0.05$) (Figure 5B).

The maximum of bacterium inactivation by the E-4 phage was 3.8 log after 6 h (ANOVA, $p < 0.05$) relatively to the bacterial control. After 12 h the bacterium inactivation was still high (2.5 log) relatively to the bacterial control (ANOVA, $p < 0.05$) (Figure 5A). The phage control remained constant since the beginning of the treatment (ANOVA, $p > 0.05$), but the phage concentration increased considerably in the presence of the host, by 1.8 log, (ANOVA, $p < 0.05$) (Figure 5B).

Phages E-2 and E-3 inactivation was similar during the treatment (ANOVA, $p > 0.05$). Compared to phage E-2 and phage E-3 the inactivation of the bacteria by the

phage E-4 was less efficient until 4 h of treatment but after 6 h it was significantly more efficient than the other phages inactivation (ANOVA, $p < 0.05$) (Figure 5A).

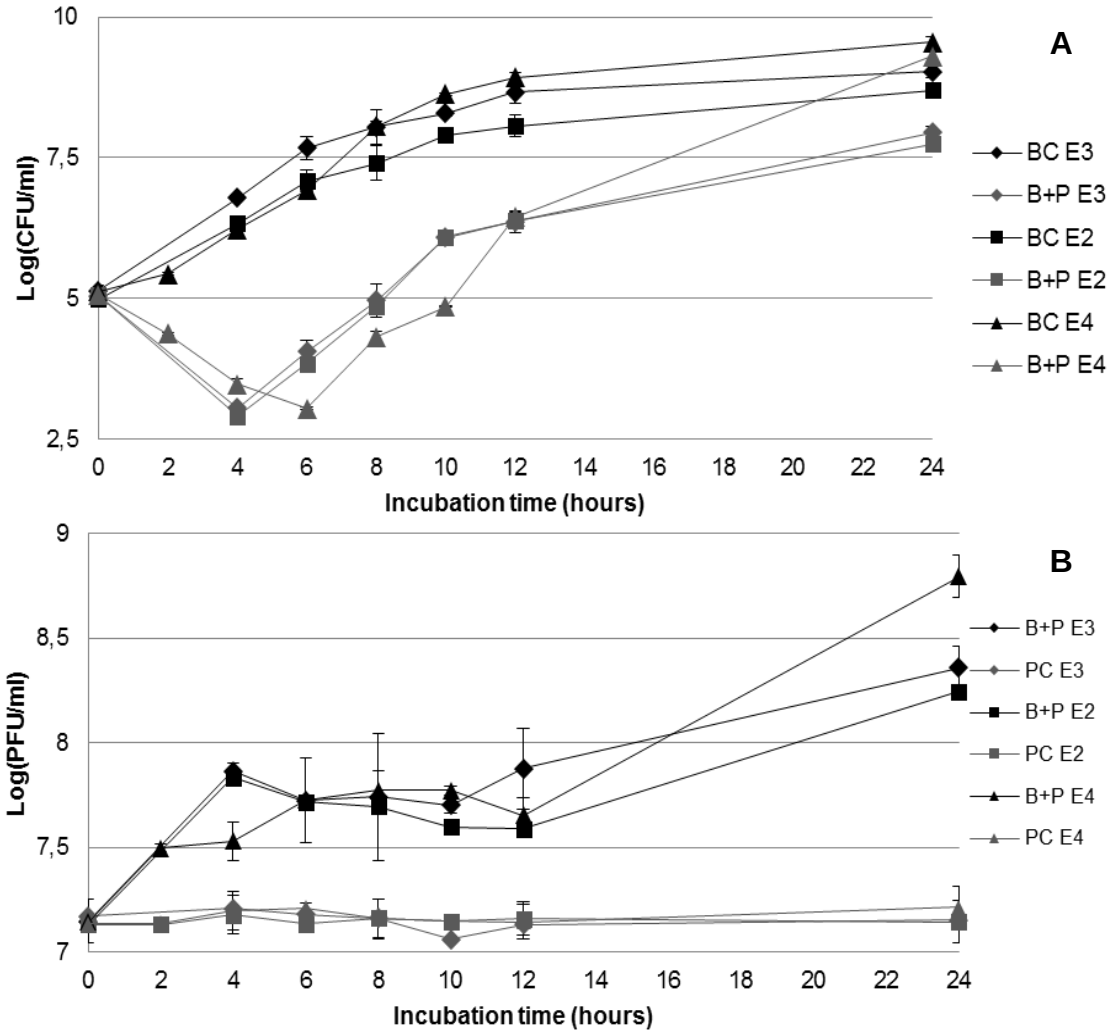


Figure 5 - Inactivation of E. cloacae by the three phages (E-2, E-3 and E-4) at a MOI of 100 during the 24 h. (A) Bacterial concentration: BC – Bacteria control; B+P – Bacteria plus phage. (B) Phage concentration: PC – phage control; B+P – Bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation.

4.4.2. E. Cloacae inactivation by phage cocktails

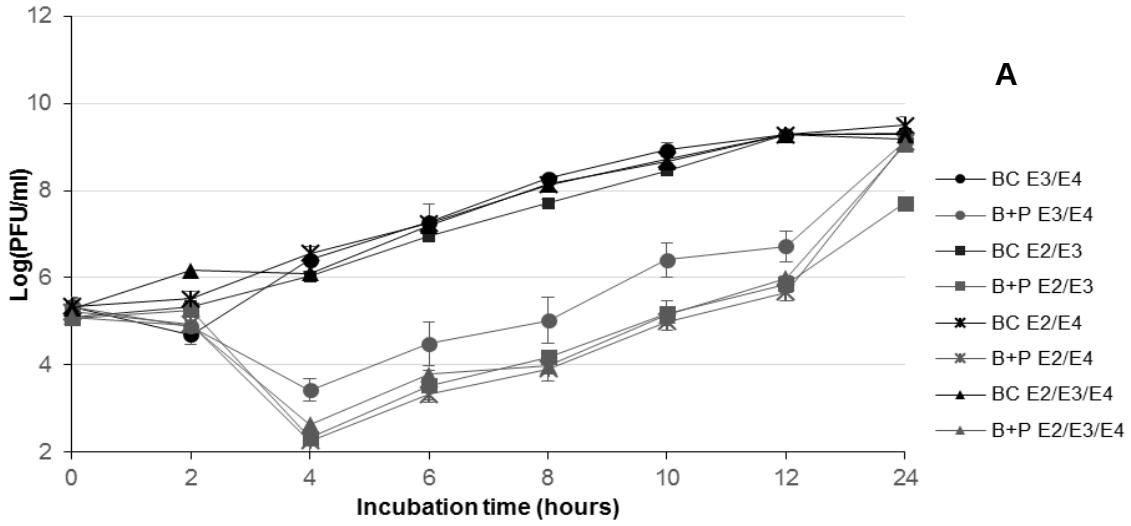
When the E-2/E-4 phage cocktail was used, the maximum of bacterial inactivation was 4.3 log after 4 h of incubation. After 12 h the inactivation was 3.6 log. These results are significantly different from the values obtained for the phages E-2 and E-4 alone (ANOVA, $p < 0.05$) (Figure 6A). No decrease in the phages survival was observed during study period (ANOVA, $p > 0.05$) but in the presence of its host phage cocktail concentration increased significantly (ANOVA, $p < 0.05$) (Figure 6B) by 1 log.

With the phage cocktail E-2/E-3 was tested, the maximum of bacterial inactivation was 3.7 and 3.5 log, respectively after 4 h and 12 h of incubation. The inactivation was significantly higher from the value obtained when phage E-2 was used alone (ANOVA, $p < 0.05$). However, when compared to E-3 inactivation the results were not significantly different (ANOVA, $p > 0.05$) (Figure 6A). No decrease in the phages survival was observed during study period (ANOVA, $p > 0.05$) and in the presence of its host the phage concentration increased significantly (ANOVA, $p < 0.05$) by 1 log (Figure 6B).

Using the phage cocktail E-3/E-4 the maximum rate of the bacterial inactivation occurred within 4 h of incubation and was about 3 log. After 12 h of incubation the inactivation rate was 1.3 log. These results were significantly different from results obtained in the assays with both phages separately (ANOVA, $p < 0.05$) (Figure 6A). The phages survival during the study period was constant (ANOVA, $p > 0.05$) and there was an increasing in phage concentration in the presence of the host during the study time (ANOVA, $p < 0.05$) of 1.3 log (Figure 6B).

With the phage cocktail E-2/E-3/E-4 the maximum of bacterial inactivation was 3.5 log achieved after 4 h of incubation and of 1 log after 12 h. These results are significantly different from the results obtained using E-3 and E-4 alone (ANOVA, $p < 0.05$), but are similar with the results obtained using E-2 phage (ANOVA, $p > 0.05$) (Figure 6A). The phages survival was constant during the study period (ANOVA, $p > 0.05$) and an increase of 1 log in phage concentration in the presence of bacterial host was observed (ANOVA, $p < 0.05$) (Figure 6B).

The inactivation of the bacteria by the phage cocktails E-2/E3, E-2/E-4 and E-2/E-3/E-4 was similar (ANOVA, $p > 0.05$) but the phage cocktail E-3/E-4 was less effective than the other 3 to inactivate the bacteria (ANOVA, $p < 0.05$) (Figure 6A).



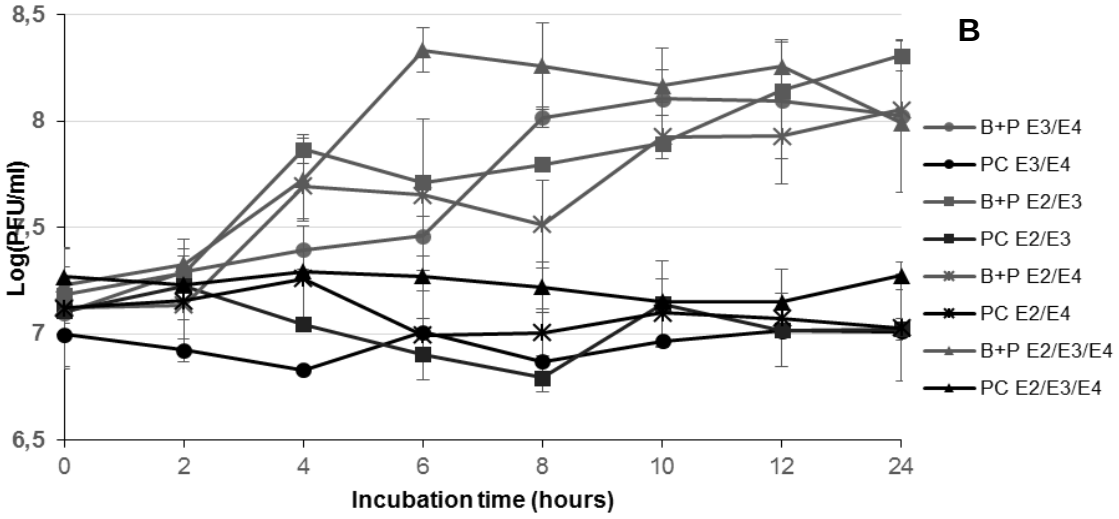


Figure 6 - Inactivation of E. cloacae by phage cockails (E-3/E-4, E2/E3, E2/E4 and E2/E3/E4) at a MOI of 100 during the 24 h. A Bacterial concentration: BC – Bacteria control; B+P – Bacteria plus phage .B Phage concentration: PC – phage control; B+P – Bacteria plus phage. Values represent the mean of three esperiments; error bars represent the standard deviation.

4.5. *E. Cloacae* inactivation in Urine

We wanted to assess the properties of the phages in question in the clinically relevant setting, therefore the killing assays were repeated in urine. The best results were obtained with phage cocktail E-2/E-4. The maximum bacterial inactivation was 2 log 4 h after the beginning of treatment. These results are significantly different from the results obtained in phage therapy in PBS (ANOVA, $p < 0.05$) (Figure 7A). The phage survival was constant during the 24 h of treatment (ANOVA, $p > 0.05$) and increase significantly, by 1 log, in the presence of the host (Figure 7B).

The maximum bacterial inactivation for E-2 in urine samples was 2 log after 4 h of treatment, these results are significantly different from those obtained in PBS (ANOVA, $p < 0.05$) (Figure 7A). The phage survival was constant during study period (ANOVA, $p > 0.05$), and increased significantly (1 log) in the presence of the host after 12 h of treatment (ANOVA, $p < 0.05$) (Figure 7B). The bacteria inactivation by the phage E-4 was significantly lower compared to the results obtained using the phage cocktail E-2/E-4 and the E-2 phage in urine (1 log after 4 hours) (ANOVA, $p < 0.05$) (Figure 7A). The phage survival was constant during the experiments (ANOVA, $p < 0.05$) and phage concentration increased significantly in the presence of the host bacteria (ANOVA, $p < 0.05$) (Figure 7B).

The inactivation of the bacteria in urine samples by the phage cocktail E-2/E4 and phage E-2 was similar (ANOVA, $p > 0.05$) but the phage E-4 was less effective than the cocktail and the phage E-2 (ANOVA, $p < 0.05$) (Figure 7A).

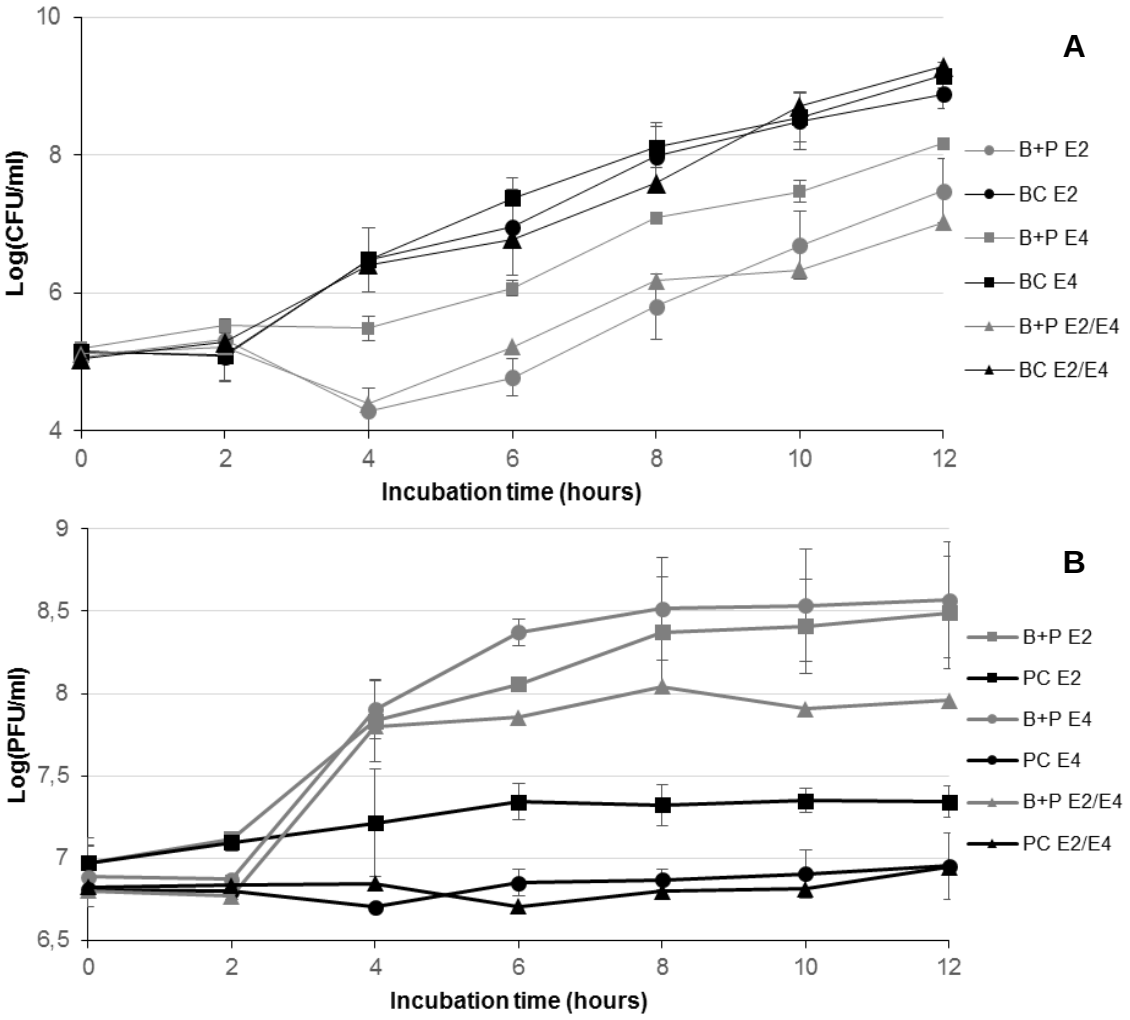


Figure 7 - Inactivation of E. cloacae by phage E-2 and E-4 and the cocktail E2/E4 in urine at a MOI of 100 during the 12 h. (A) bacterial concentration: BC – Bacteria control; B+P – Bacteria plus phage. (B) Phage concentration: PC – phage control; B+P – Bacteria plus phage. Values represent the mean of the three experiments; error bars represent the standard deviation

5. Discussion

The emergence of antibiotic resistant bacteria directed more research interest to alternative therapies. Several studies have demonstrated that phage therapy has great potential in the inactivation of infections caused by bacteria. The *E. cloacae* infection has major importance to be a cause of urinary tract infections difficult to control (Zhanel, Hisanaga et al. 2005). There is not yet any report on the application of phage therapy in *E. cloacae.*

The biggest challenge of phage therapy is the selection of suitable phages, that besides are not temperate and have broad activity and long survival in the environment, do not carry toxin genes and do not perform generalized transduction. Therefore, it is mandatory to perform whole genome sequencing and assesses specific properties of the phage. The genome sequence of the three phages isolated in this study did not detect the presence of genes encoding toxins, antibiotic resistance and integrase enzymes. Consequently, these phages can be considered safe to be used in phage therapy.

Other concern of this therapy is the emergence of phage resistant bacteria (Levin and Bull 2004, Vieira, Silva et al. 2012). However, resistance to phage can be overcome by the phage itself because it evolves together with the host and this aspect is one of the major advantages of a phage over the “static” antibiotic. Moreover, the use of phage cocktails has managed to overcome this problem (Zhang, Kraft et al. 2010, Chan and Abedon 2012, Chan, Abedon et al. 2013). In fact, the use of phage cocktails has been indicated as a good alternative for the treatment of bacterial infections. Since the beginnings of phage therapy use in the Eastern countries this approach has been preferred. However, one must keep into account that the more phages used in treatment, the higher the probability of non-specific infections to occur. Less complex cocktails, for example, two to ten distinct phages, are the best option (Chan, Abedon et al. 2013).

With the exception of the cocktail E-3/E-4, the phage cocktails of 2 and 3 phages presented a significant increase in the inactivation of bacteria compared to the use of isolated phages. These results can be explained by a delay in the development of phage bacterial resistance. Between 4 and 10 h of incubation, the increase in the bacterial concentration was of ≈ 3 log when phage cocktails were used but of ≈ 4 log when the single phage suspensions were tested. As the efficacy of bacterial inactivation by the three phages has been similar when they were used alone, the phages of cocktails E-2/E-3, E-2/E-4 and E-2/E-3/E-4 probably used different bacterial receptors to adsorb to *E. cloacae* and, consequently, the time necessary to develop resistance to the two or three phages is higher. However, the phages E-3 and E-4 probably used the same receptor to

infect *E. cloacae*. For the selection of phage cocktails it is important to consider the receptors used by the phage to infect the host to improve bacterial inactivation effectiveness and to limit the development of resistance (Filippov, Sergueev et al. 2011).

Although in all treatments the used phages were unable to control the host, after treatment some bacteria survive to the phage infection, probably due to phage resistance development by the host. However, the rate of inactivation achieved after 4 h of treatment, only two log of bacteria survive, can be enough to allow the immune system of the infected person to control the infection. Moreover, previous studies suggest that phage resistant bacteria tend to be less fit or lose their virulence properties (Wagner and Waldor 2002, Filippov, Sergueev et al. 2011).

The phage cocktail E-2/E-4 was effective to inactivate *E. cloacae* in human urine. However, although the phages of the cocktail E-2/E-4 survived in the urine, maintained their concentration in the absence of the host and significantly increased their titer in the presence of the bacterium during the study period, its effectiveness to inactivate the *E. cloacae* in urine was considerably lower compared to that observed in the experiment in PBS, 2.0 log against to 4.3 log after 4 h, respectively. Considering the results of bacterial inactivation in urine using the single suspensions of both phages it is clear that the decrease in the bacterial inactivation, relatively to the inactivation in PBS, was mainly due to phage E-4. The inactivation of the bacteria in urine samples by the phage E-4 was only of 1 log. The inactivation by the phage E-2 was of 2 log, similar to that observed with the phage cocktail in urine samples and 2 log lower that the inactivation with the same cocktail in PBS. As the phages maintain their stability during the incubation period in urine, and replicate in the presence of the host as well as in the PSB, the lower bacterial inactivation in urine seems not to be due to a lower phage replication of the phages. It has been shown that although phages survive in different values of pH, temperature, salt concentration, organic matter concentration, the efficiency of phage therapy is significantly affected by salt concentration and organic matter concentration (Silva et al., 2014). The increase in salt concentration and organic matter concentration increased the efficiency of phage therapy against *Vibrio parahaemolyticus* (Silva et al., 2014). As organic matter concentration in the human urine is higher than in the PBS and the salt concentration is lower in human urine (≈ 3 g L^{-1}) than in PBS (9 g L^{-1}), the lower efficiency of phage therapy in urine at a first glance, seems to be due to the lower values of salt. However, other studies have shown that the interplay between receptor-binding protein and cellular ligand is strongly dependent on Ca-ions and other salts (Binetti, Quiberoni et al. 2002, Harada, Yamashita et al. 2013). High salt concentrations can lead to unspecific binding

which is not followed by infection (Silva, Costa et al. 2013). However, in this study, the phage concentration increased significantly in the presence of the bacterium during the study period in urine. Further studies to determine the exact receptors and their binding protein are needed.

The results of this study show that the three phages can inactivate pathogenic bacteria from different genera of the family *Enterobacteriacea*, most of them also implicated frequently in UTI, which suggest that these phages can be used to inactivate these uropathogens. These three phages do not infect bacteria belonging to other families. The phage E-2 presented the broader host range for the *Enterobacteriaceae* strains, infecting 5 genera and the phage E-4 the smaller host range, infecting 3 genera. However, the E-4 phage was as effective as the other two to inactivate the *E. cloacae* and when this phage was used in combination with the broader host range E-2 phage, the rate of inactivation reached a higher value and a lower value of resistant bacteria proliferation. The results suggest that the combination of an effective phage with other, with a broader host range can be a practical approach to treat UTI caused by *Enterobacteriaceae*.

The results of this study showed that the three phages can survive in the buffer solution during several months (6 - 8) and in urine, at least 12 h, replicating efficiently in the presence of the pathogenic bacteria. Unlike antibiotics, phages are self-replicating as well as self–limiting, and, consequently, they replicate exponentially as bacteria replicate and decline when bacterial numbers decrease (Almeida et al., 2009). In this study it was possible to produce phages suspensions with high titers, up to 10^{10} PFU mL^{-1}, suitable to use in phage therapy, that declined when bacteria were removed, but maintaining their high concentrations.

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Part II

Chapter 3 – Introduction

The Hepatitis caused by viruses presents a great importance due to its worldwide distribution, reaching pandemic proportions. The Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus (HDV), Hepatitis E virus (HEV) and Hepatitis G virus (HGV) are biologically distinct although they all have in common the destruction of hepatic tissue (Araújo, Mendonça et al. 2007).

1. Hepatitis A

Hepatitis A first written account, according to a review by Cockayne (1912), was the description of an epidemic on the island of Minorca in the 18th century. Subsequently, many other reports of epidemics were made, and the name of catarrhal jaundice was given by Virchow due to the amount of bile thrombi observed from the autopsies (Cockayne 1912). The suspicion that the cause of the disease could be viral only emerged in 1931 by Findlay and Cols (Findlay 1932).

Hepatitis A virus (HAV) belongs to the *Hepatovirus* genus within the *Picornaviridae* family. It is a nonenveloped single-stranded RNA virus and has an icosahedral capsid of 30 nm (Feinstone, Kapikian et al. 1973). HAV replicates in hepatocytes and sparks an immune response causing liver inflammation. There is genetic variability between HAV isolates from different parts of the world and it is possible to classify HAV strains into 6 genotypes. Genotypes I to III are found in humans and genotypes IV to VI are found in simians. The phenotype I is the most common, being found in 80% of the infected people (Robertson, Jansen et al. 1992).

HAV causes acute liver disease worldwide, formerly known as infectious hepatitis, being annually estimated 1.4 million cases of new infections (Bell, Shapiro et al. 1998). Despite the low mortality rate (between 0.1% and 2.1%), it causes a very significant morbidity. Symptoms include in average a week of gastrointestinal and flulike symptoms, anorexia and dark urine; several weeks of jaundice, and then it follows a period of several weeks of convalescence (Cuthbert 2001). HAV may also lead to extrahepatic complications (e.g. pancreatitis, vasculitis and glomerulonephritis). The main cause of death is fulminant hepatitis associated to chronic liver disease (Ciocca 2000). Hepatitis A causes epidemic waves that are usually repeated at intervals varying in accordance with the virus circulation (Rosenthal 2003).

Hepatitis A is transmitted by fecal-oral route, either by direct contact with an HAV-infected person or by ingestion of HAV-contaminated food or water. As the period of

viremia is short and the concentration of virus in the blood is low, transmission by blood or contaminated material is rare, but it can occur if the injected material (serum or blood) have been derived from an individual in incubation period. Sporadic cases have been reported after blood transfusions. The infection rate is strongly correlated with sanitary conditions but also with the educational level indicators, food preparation by contaminated handlers and consume of contaminated shellfish grown in polluted water (Hutin, Pool et al. 1999, Staes, Schlenker et al. 2000, Shepard, Simard et al. 2006). There are also high-risk groups, including drug addicts, day-care employees and homosexuals due to the high promiscuity and the oro-anal practices (Desenclos and MacLafferty 1993). The risk of exposure is higher in military personnel then in civil community due to communal living standards, and also due to the fact that militaries are often recruited to high endemic areas (Hutin, Pool et al. 1999). For people susceptible to HAV, it is estimated that the risk of contracting the disease travelling to developing countries is 3 to 6 cases per 1000 travellers per month but in case of “backpacking” travellers and humanitarian action volunteers, the risk is 20 cases per 1000 travellers (Shepard, Simard et al. 2006).

There are three phases included on the course of the disease: incubation, symptomatic infection and convalescence. HAV fecal excretion occurs from the incubation to early symptomatic phase. The incubation period range from 15 to 50 days and the peak of infectivity occurs as serum alanine aminotransferase concentration increases (Bower, Nainan et al. 2000). The main serological marker, IgM anti-HAV, can be detected between 5 days and 6 months after exposure. IgG anti-HAV confers lifelong immunity, this antibody can also be detectable in the symptomatic phase (CDC 1999, Wasley, Samandari et al. 2005). (Figure 8).

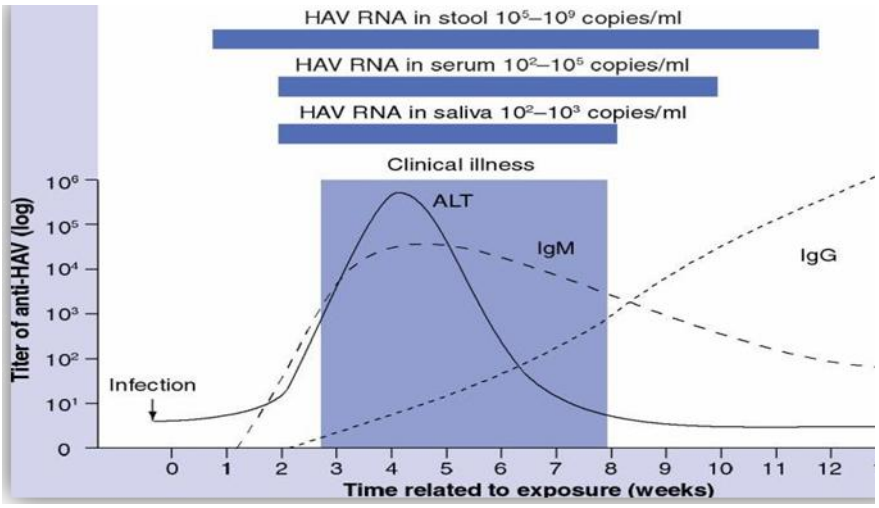

Figure 8 - Events that occur during HAV infection (adapted from Paula et al 2012).

Hepatitis A generally has a good prognosis. Mortality is shown to be very low in young, increasing if the disease is acquired from the fourth decade of life (Kemmer and Miskovsky 2000).

2. Hepatitis C

Hepatitis C has been studied for decades, even before the discovery of the agent responsible for the disease, the hepatitis C virus (HCV) (Houghton 2009). In 1970, serological screening tests to investigate the HAV and HBV, showed that 25% of cases of hepatitis associated with blood transfusions were associated with HBV. The remaining 75% were considered as hepatitis non-A and non-B (NANB) (Araújo, Mendonça et al. 2007). Only after observation of some cases of NANB hepatitis that progressed with elevated alanine aminotransferase (ALT) and cirrhosis, came a greater interest in studying a potential responsible of this "new disease" (Alter 1999).

HCV is a small linear RNA virus (50-60 nm) with a single filament classified in the *Flaviviridae* family and *Hepacivirus* genus, being transmitted through the bloodstream (Walsh and Alexander 2001, Noorali, Pace et al. 2010). It has half-life of 2.7 hours and preliminary data suggest that HCV is able to penetrate the hepatocyte through CD81 receptor site where it replicates preferentially (McHutchison 2004). It has a very high rate of replication (10^{12} virions per day). HCV does not replicate via a DNA intermediate nor interact within the genome of the host, *i.e.* is not cytopathogenic (Malkevich, Womack et al. 2001).

In developed countries, before the introduction of routine testing of donated blood, the HCV main course of transmission were blood and blood products transfusions. Nowadays the illicit use of injectable drugs is the main source of HCV transmission. The prevalence of HCV in drug users is higher than that of human immunodeficiency virus (HIV) (Champion, Taylor et al. 2004). In developing countries the use of re-used and non-sterilized syringes and needles in medical procedures is the main cause of transmission. Sexual transmission is less common than with Hepatitis B virus (HBV) and the risk of perinatal transmission is low (Noorali, Pace et al. 2010).

The main serological marker is anti-HCV, the presence of anti-HCV antibodies cannot be confirmed until 12-27 weeks after exposure, creating a window period of seronegativity and elevated risks of infectivity. In cases where the anti-HCV antibody is positive the quantification of viral RNA, the HCV core antigen EIA and the genotype screening are indicated tests (Mast, Alter et al. 1999). Apart from the detection/quantification of the genome, determination of viral genotype is highly relevant in

clinical practice, particularly in the selection of therapy, defining the duration of treatment and dosage adjustment of antivirals (Anjo, Café et al. 2014).

In Portugal, the combination of Pegylated interferon alpha (PEG IFN- α) and ribavirin (RBV) therapy is approved for the treatment of chronic HCV infection in individuals with HCV infection of non-1 genotypes (Anjo et al., 2014; Saludes et al., 2014). This combination therapy provides healing in approximately 60% of patients (Velosa, Caldeira et al. 2012), however has several serious side effects such as anemia, granulocytopenia, and depression, and is associated with long-term treatment and high cost. The effectiveness of the combined PEG IFN-α and RBV therapy is less than 50% for genotype 1 HCV, the most prevalent genotype (Lok, Gardiner et al. 2012).

The acute hepatitis C is commonly asymptomatic. When symptomatic, it includes anorexia, vague abdominal discomfort, nausea and vomiting, fever and fatigue, progressing to jaundice in about 25% of patients (Marcellin 1999). Eventually, 60% - 90% of infected people evolve to chronic disease. Chronic hepatitis is defined as a continuing disease without improvement for at least six months (Dubuisson, Hsu et al. 1994).

HCV infection is associated with a wide spectrum of liver damage, which can range from minimal histological changes to liver cirrhosis, hepatocellular carcinoma or eventually death (Veldt, Heathcote et al. 2007). Fulminant hepatitis is rare, except in co-infection with hepatitis A. The development of cirrhosis is age-dependent and the risk is higher when the infection occurs after 40 years old. Approximately 20% of those who develop cirrhosis will suffer hepatocellular carcinoma (Maasoumy and Wedemeyer 2012) (Figure 9).

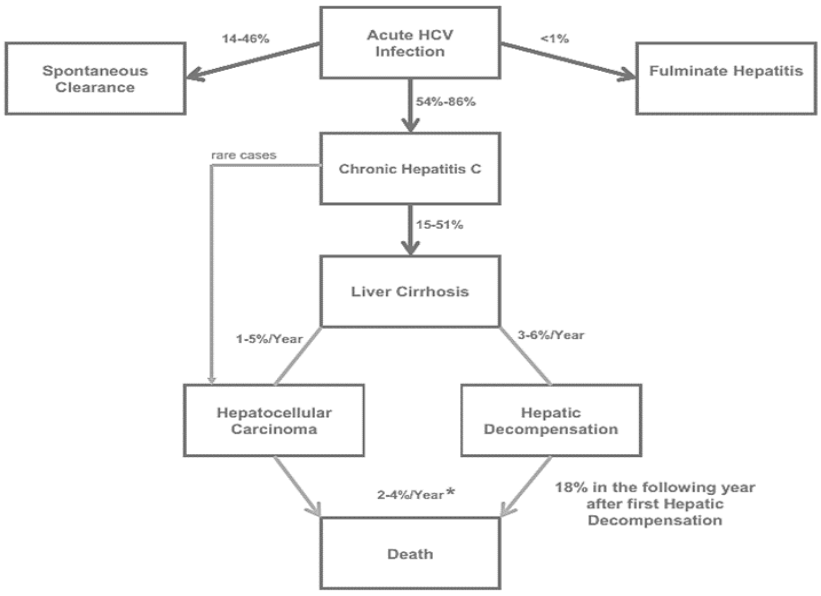

Figure 9 - Natural history of Hepatitis C virus infection (Maasoumy and Wedemeyer, 2012)

There are seven drugs licensed for the treatment of HCV – standard interferon (IFN) or pegylated interferon alpha (PEG-IFN), ribavirin (RBV), the protease inhibitors (PIs) boceprevir, simeprevir and telaprevir, the nucleotide analog polymerase inhibitor sofosbuvir and the most recently licensed treatment ABT-450/r–Ombitasvir and Dasabuvir with Ribavirin. The seventh is the most effective treatment with a sustained virologic response in 90% of cases. The limitations of HCV treatment include high cost, the need for sophisticated laboratory tests and trained clinicians, as well as the limited efficacy and high toxicity of most of the medicines (WHO 2014). To administer the appropriate treatment is important to determine the genotype of the virus (Velosa et al., 2012).

The public health importance of these two forms of hepatitis are increasingly being recognized. This study aims to evaluate the variation in prevalence of hepatitis A and hepatitis C in the district of Aveiro, in the period of 2002 to 2012, in order to broaden the knowledge of epidemiology of these diseases in Portugal and prevent spreading and future outbreaks.

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Chapter 4 - Hepatitis A Immunity in the District of Aveiro (Portugal): An Eleven-Year Surveillance Study (2002–2012)

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1. Abstract

Hepatitis A is a common viral liver disease and brings serious health and economic problems as its epidemiologic pattern changes over time. National serosurveys from developed countries have indicated a decline in HAV (hepatitis A virus) seroprevalence over time due to the improvement of economic and sanitation levels. The hepatitis A virus (HAV) immunity rate was surveyed throughout an eleven-year period by sex and age group in Aveiro District. In this retrospective study, blood samples from patients of Aveiro District, in ambulatory regime, collected at the Clinical Analysis Laboratory Avelab between 2002 and 2012 were screened for the presence of antibodies against HAV antigen using a chemiluminescence immunoassay. The global immunity (positive total anti-HAV) was 60% and only 0.3% of the patients presented recent infection by HAV (positive IgM anti-HAV). The HAV immunity was age-dependent ($p < 0.05$), but no significant differences ($p > 0.05$) between sexes were observed. The immunity was similar throughout the study period ($p > 0.05$). The results of this study indicate that young people (especially under 25 years old) from District of Aveiro are susceptible to HAV infection, constituting a high risk group. The elderly should be also a concern in the future of Hepatitis A infection.

Keywords: Hepatitis A; immunity; surveillance study; epidemiology; Portugal

2. Introduction

Hepatitis A is an acute disease transmitted by fecal-oral route, either by direct contact with an HAV-infected person or by ingestion of HAV-contaminated food or water.

During the course of the disease there are three phases: incubation, symptomatic infection and convalescence and the HAV excretion occurs from the incubation to early symptomatic phase. The incubation period ranges from 15 to 50 days (Feinstone, Kapikian et al. 1973). The main serological marker, IgM anti-HAV can be detected between five days and 6 months after exposure. Anti-HAV IgG confers lifelong immunity and this antibody can also be detectable in the symptomatic phase (Lemon, Jansen et al. 1992, Robertson, Jansen et al. 1992).

HAV causes liver disease worldwide, being annually estimated 1.4 million cases of new infections (Bower, Nainan et al. 2000). Despite the low mortality rate (between 0.1% and 2.1%), it causes a very significant morbidity (Wasley, Samandari et al. 2005). HAV may also lead to extrahepatic complications (e.g., pancreatitis, vasculitis and glomerulonephritis). The main cause of death is fulminant hepatitis associated to chronic liver disease (CDC, 1999). Hepatitis A causes epidemic waves that are usually repeated at intervals varying in accordance with the virus circulation (Bell, Shapiro et al. 1998).

In developed countries, the HAV incidence has declined essentially due to the great improvements of sanitary conditions (Rosenthal 2003). However, a decrease of immune individuals leads to an increased risk of potential outbreaks. In such circumstances, these outbreaks may be unpredictable and difficult to control. This concern is also focused on the fact that, in children, hepatitis A is mostly asymptomatic. In adults, 70%–89% of cases are symptomatic and with increasing age the symptoms are worsened. Thus, although the incidence in population has decreased, there is an increase in costs per case of HAV infection as a large segment of the population is now susceptible to it (Luyten and Beutels 2009). There is an evidence that many of the outbreaks are misdiagnosed (FitzSimons, Hendrickx et al. 2010). According to the World Health Organization (WHO), annual reports show about 100,000 cases of outbreaks in Europe, 500 of them being fatal. Knowledge of anti-HAV seroprevalence rates in the world and in each region of a country is of utmost importance to establish public health priorities and to adopt adequate vaccination policies (WHO, 2009).

In 2011, the WHO has compiled the immunity studies made across the world and a summary was made. In western countries, the overall immunity was 50%, and less than 20% to people aged less than 20 years. In this region, in which Portugal is included, there is a low child immunity rate and the adult susceptibility rate is high. In Central and Eastern Europe, there is a low-medium child immunity rate and the adult susceptibility rate is medium. In North America, there is a low child immunity rate and medium susceptibility in adults (WHO, 2009). Between 1996 and 2006, 1164 patients were hospitalized with HAV

in Portugal, 30% were younger than 15 years and 3% had severe liver failure (data of the Computing and Financial Management of Health) (Marinho, Valente et al. 2000). According to the largest epidemiological survey carried out in Portugal in 1984, comprising 1770 individuals distributed among several districts, the immunity rate for the Portuguese population was 84.9% and 93.4% to people aged less than 20 years. This country was considered highly endemic (Lecour, Ribeiro et al. 1984).

The vaccine is highly immunogenic, conferring protection against HAV in approximately 95% of the vaccinated patients (Rosenthal 2003). Protection is considered to be lifelong after a complete hepatitis A vaccination schedule (two doses) (Landry, Tremblay et al. 2000, Raczniak, Thomas et al. 2013). A combined hepatitis A/B vaccine with high immunogenicity is also available in some countries (Kallinowski, Knöll et al. 2000). The vaccine is not yet widely used but in more developed countries it is recommended for risk groups. Moreover, some studies have found that universal vaccination is cost-effective (Jacobs, Margolis et al. 2000, MacIntyre, Burgess et al. 2003, Rosenthal 2003, Bauch, Anonychuk et al. 2007). In Catalonia, the universal vaccination program of preadolescents began in 1998 and has avoided 90% of cases in young people aged 12–19 years (Navas, Salleras et al. 2005). In Portugal, the vaccine has been available since 1998 but is not yet widely used, and it is only recommended for specific at-risk groups. According to the national health regulatory authority (DGS) recommendations, children, adolescents or adults who travel to high or intermediate endemic countries, adolescents and adults with chronic liver disease or who belong to a community where an outbreak is detected should be primarily vaccinated (Cavaco, 2010). The vaccine is not yet included in the National Plan of Vaccination (DGS, 2012).

The public health importance of this form of hepatitis is becoming increasingly recognized as the epidemiological picture of the disease has been dramatically changing in recent years. In many countries, outdated data are still being used in policy decisions (FitzSimons, Hendrickx et al. 2010). Seroprevalence studies of hepatitis A as well as the evaluation of the cost-benefit of the vaccine may contribute to the decision of extending vaccination to the entire population. This study aims to evaluate the prevalence of hepatitis A in Aveiro District, from 2002 to 2012, in order to broaden knowledge on the epidemiology of this disease in Portugal and to assess the risk of outbreaks of HAV.

3. Material and Methods

3.1. Samples

In this retrospective study all serum samples analysed for total anti-HAV and IgM anti-HAV from patients of the District of Aveiro, in ambulatory regime, collected at the Clinical Analysis Laboratory Avelab (Aveiro, Portugal) during the period 2002-2012, were analysed. The Clinical Analysis Laboratory Avelab comprises delegations located in 14 municipalities in the district of Aveiro and Coimbra.

A total of 7894 samples (4357 from male and 3537 from female) were collected during the study period. All patients were grouped by age ranges: 0-5, 6-15, 16-25, 26-35, 36-45, 46-55, 56-65 and >66.

The age and sex of each patient were registered. The vaccination history of the patients was not considered. The study was approved by the Ethical Committee of the Clinical Analysis Laboratory Avelab, specifically by the Pharmacist Dr. António Ferreira Neves and Doctors Alberto Ferreira Neves, António Rodrigues and Maria Teresa Raposo.

3.2. Sampling

Samples were collected, using the Avelab Laboratory protocol. The venous blood of the patients was collected and reserved into a tube with separator spheres allowing clot formation. The samples were centrifuged at 1381 x g for 10 minutes. The blood samples were analyzed within one hour after collection. When this procedure was not possible the samples were stored at 2-8ºC and processed until 24 hours after collection. The samples were dismissed 7 days after sampling.

3.3. Antibodies Detection

The samples were analyzed in an automated Siemens ADVIA Centaur® XP immunoassay analyzer. It was used the acridinium ester (AE) as the chemiluminescent label. Samples were diluted in buffer and purified HAV antigen was added forming in the presence of specific antibodies (IgG and/or IgM) immune complexes. AE labeled mouse monoclonal anti-HAV antibodies, biotinylated mouse monoclonal anti-HAV Fab fragment and streptavidin coated paramagnetic capture particles were incubated with the immune complexes. The biotinylated conjugate and acridinium labeled conjugate bind to antigen sites not occupied by sample HAV antibodies. A magnet is used to separate the microparticles and the unbound material is washed. The bound acridinium ester conjugate

is then measured by a chemiluminescent reaction. The amount of light produced is inversely proportional to the antibodies concentration (Siemens, 2006).

Total anti-HAV and/ or IgM anti-HAV were analyzed in all samples. The presence of IgM anti-HAV indicates recently infected patients and the presence of total anti-HAV (IgG anti-HAV and IgM anti-HAV) indicates that patients had previous or ongoing infection. The samples were classified as non-infected, recently infected, immune and non-immune, according to the detected antibodies. The patients were classified as immune when the result of total anti-HAV was positive and infected when the result of IgM anti-HAV was positive. Patients with negative anti-HAV antibodies (negative total anti-HAV and negative IgM anti-HAV) were considered non-immune and patients with negative IgM anti-HAV were considered non-infected.

3.4 Statistical analysis

The data were treated using the Statistical Package for the Social Sciences (SPSS) 20.0 for Windows. To simplify the statistical analysis the patients were grouped by age ranges: 0-5, 6-15, 16-25, 26-35, 36-45, 46-55, 56-65 and >66. The absolute (n) and relative (%) frequencies were presented for qualitative variables. The normality of data was checked before analysis. As all the variables failed this statistical method assumption, the non-parametric Chi-square (X^2) test was used to check if the distribution of variables was similar in the different groups for immunity. The significance level established was 0.05.

4. Results

4.1. Hepatitis A

4.1.1. Characterization of the sample

The annual average of analyzed samples was 718. It was observed a decrease of about 7% on the number of analysis performed between 2009 and 2012 (Fig.10-A). From the total of 7894 serum samples analyzed between 2002 and 2012, 4357 (55.2%) were performed in male patients and 3537 (44.8%) in females. The age of the patients ranged between 0 and 99 years. The patients aged between 26-35 years represented the age group that carried out more analysis. In general, an increase of the percentage of analysis performed until the age of 26-35 years was observed, meanwhile a decrease was found for patients aged more than 35 years (Fig.10-B).

From the 7894 serum samples analyzed, more than half of the patients (60.4%) had HAV immunity and only 0.3% of patients were recently infected with HAV (Fig.11). About 30% of the patients did not showed immunity against HAV.

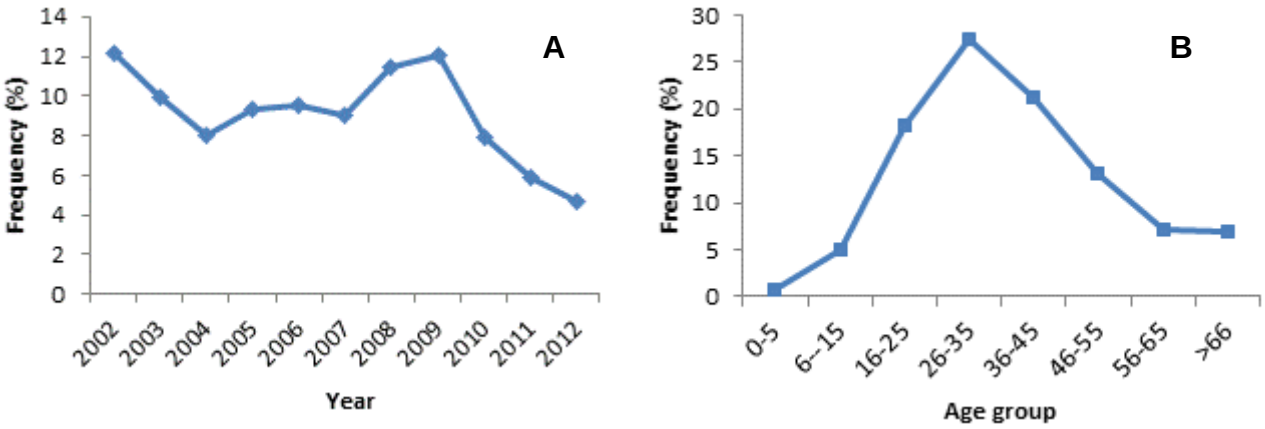

Figure 10 - Frequency (%) of the samples by year (A) and age group (B) during the study period.

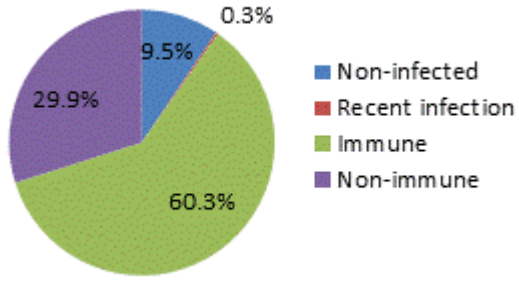

Figure 11 - Sample classification during the study period.

4.1.2. Characterization of HAV immune patients

There was an abrupt decrease on immunity from 2009 to 2012 but an inversion in the ratio of analysis required was also observed. Having into account both analyses (IgM anti-HAV and total anti-HAV), the percentage of immunity was 73% (n=966) in 2002, 63% (n=953) in 2009 and 28.6% (n=374) in 2012. However, for total anti-HAV detection, the percentage of immunity in 2002 was 73.6% (n=954), in 2009 65.2% (n=922) and in 2012, 67.7% (n=158). Throughout the study period, slight changes in the incidence of the overall immunity were observed, but considering only the results of total anti-HAV the percentage of immunity did not vary significantly ($p>0.05$) (Figure 12A).

The overall immunity was not significantly different between female and male patients ($p>0.05$). However, significant differences in seroprevalence among the different age groups were observed ($p<0.05$). The age of the immune patients ranged from 0 to 99

years old with a mean age of 42 years old. With the exception of patients aged less than 5 years, there was an overall increase of immunity with age. A gradually increase (54.8%) of immunity in patients with age comprised between 6 -15 years and between 56-65 years was observed, in spite of a decrease of immunity of about 5% in patients aged between 56-65 and with more than 66 years. For patients aged over 25 years, the percentage of immunity was higher than 50% (Figure 12B).

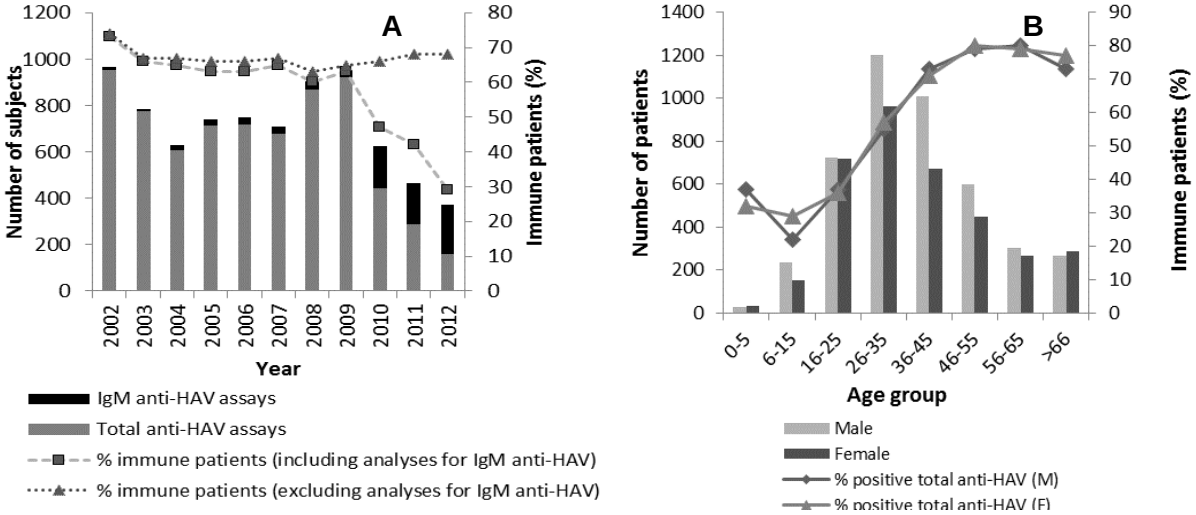

Figure 12 - Percentage of immune patients by year (A) Percentage of immune patients by genre and age group. M – Male; F – Female (B).

4.1.3. Characterization of infected patients

The age of infected patients with positive IgM anti-HAV ranged from 22 to 87 years, with a mean age of 44 years. The IgM anti-HAV was detected in 22 patients (8 males and 12 females). The higher incidence of HAV infection was observed in patients aged from 36 to 45 years. The incidence of infection was not significantly different among age groups neither between genres ($p>0.05$) (Figure 13A), but varied over the study period (Figure 13B).

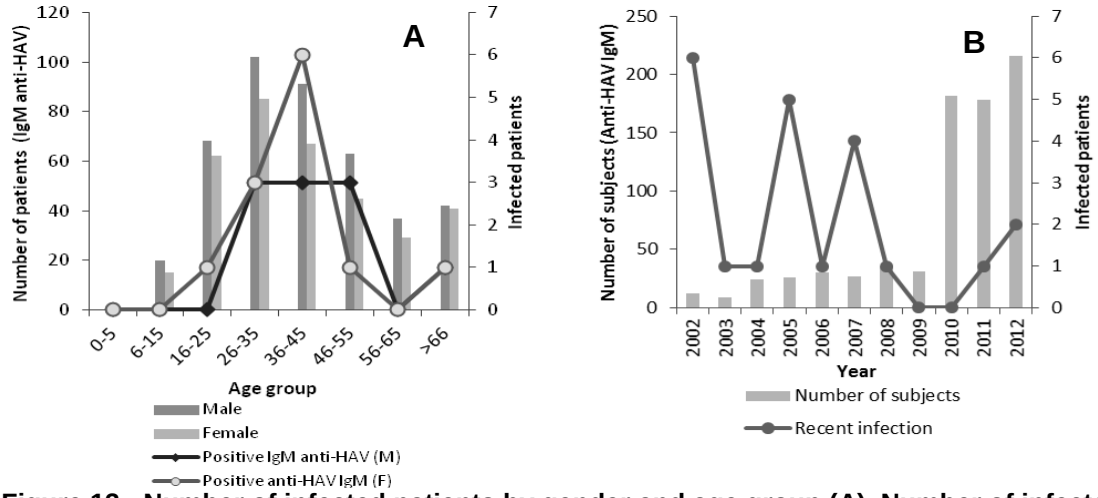

Figure 13 - Number of infected patients by gender and age group (A). Number of infected patients by year (B).

5. Discussion

When the statistical analysis was made for both analyses (IgM anti-HAV and total anti-HAV), the percentage of immunity was 73% (n=966) in 2002, 63% (n=953) in 2009 and 28.6% (n=374) in 2012. Since 2010, the number of analyzed samples decreased abruptly, and on the other hand, at this time a change in the analyses required was observed. Before 2010 more than 90% of the analyses were done for total anti-HAV and after that there was a big decrease on the number of samples analysed for the total anti-HAV (in 2012 only 42% of the samples were analysed for the total anti-HAV). This changing led to a doubtful result, since IgM anti-HAV does not allow us to survey the actual number of immune patients. Considering only total anti-HAV detection to evaluate the immunity, about 73.6% (n=954) of the population was found immune in 2002, 65.2% (n=922), in 2009 and 67.7% (n=158) in 2012, which indicate that no significant changes on immunity rate during the study period was observed in the District of Aveiro.

In 1984, according to Lacour et. al., 84.9% of the Portuguese population was immune to HAV and the prevalence of anti-HAV antibodies in patients aged less than 20 years old was 93.4% (Lecour, Ribeiro et al. 1984). In 2002, the immunity had decreased to 58%, being 62.4% in patients less than 20 years old (Rodrigues, 2004). In Aveiro District, the immunity rate was 22.1% for patients aged 6 to 15 years old and 37.2% for patients aged 16 to 25 years old. There was a great decrease in prevalence of anti-HAV antibodies throughout the last years for these age groups. According to World Health Organization (WHO), the estimated immunity rate in western Europe countries was 18% for 10-14 age group and 28% for 15-19 age group, values that are even lower than those observed in Aveiro District (WHO, 2009). The decrease in the immunity rate for the young patients can be explained by the improvement of sanitary conditions in developed countries.

Although, the rate of immunity increased with age, for the children groups the immunity was low, bellow 25%, which could lead to an increased risk of unpredictable outbreaks in the future (Lednar 1985). It is important to note that for children less than 5 years old the seroprevalence was higher than for the older children, this can be explained by a high number of vaccinated children relatively to the other groups, despite the HAV vaccine is not included in National Plan of Vaccination (DGS, 2012). For the elderly people (>66) the immunity was lower than that observed for people aged 55 to 66 years old, this can be explained by the gradual weakening of the immune system with age. In fact, unlike other forms of hepatitis, hepatitis A does not progress to a chronic disease, but an association between mortality and age has been observed. A mortality rate of

symptomatic disease of 1.5/1000 between the children under five years of age and of 27/1000 in people aged over 50 years old was observed. Thus, these results bring a new concern, since the prevalence curve tends to deviate to the more advanced age groups (CDC, 1999).

The increase of immunity with age, not followed by an increase in the fraction of recently infected patients, suggests that patients contact with the viruses, acquiring immunity without symptoms. However, although the incidence of recent infection was low (only 22 cases), it is important to notice that this study was made in ambulatory regime and normally people with symptoms of HAV infection are addressed directly to the hospital.

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Chapter 5 - Hepatitis C Infection in the District of Aveiro (Portugal): an Eleven-Year Surveillance Study (2002–2012)

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1. Abstract:

Hepatitis C virus (HCV) is a major cause of liver disease worldwide and causes substantial morbidity and mortality. The common absence of symptoms associated leads to uncertainty to the geographic distribution of this disease. In the absence of a vaccine and effective treatment, prevention is extremely important, especially for at risk groups. The hepatitis C infection rate was surveyed throughout an eleven-year period by sex and age group in Aveiro District. In this retrospective study, blood samples from patients of Aveiro District, in ambulatory regime, collected at the Clinical Analysis Laboratory Avelab between 2002 and 2012 were screened for the presence of antibodies against HCV antigen using a chemiluminescence immunoassay. Approximately 4% of the patients presented positive anti-HCV antibodies. The HCV infection was age-dependent and varied between sexes ($p < 0.05$). The number of infected patients decreased during the study period ($p < 0.05$). The results presented in this study indicated that middle-aged males are more affected than women which may indicate that this group is more prone to risky behaviors. Moreover, the decrease in positive cases during study period may indicate a decrease in exposure to risk factors.

Keywords: hepatitis C, infection, seroprevalence, epidemiology, surveillance study.

2. Introduction

HCV infection is associated with a wide spectrum of liver damage, which can range from minimal histological changes to liver cirrhosis, hepatocellular carcinoma or eventually death (Veldt et al., 2007). The development of cirrhosis is age-dependent and the risk is higher when the infection occurs after 40 years old. Approximately 20% of those

who develop cirrhosis will suffer hepatocellular carcinoma (Maasoumy and Wedemeyer 2012).

The acute hepatitis C is commonly asymptomatic. When symptomatic, it includes anorexia, vague abdominal discomfort, nausea and vomiting, fever and fatigue, progressing to jaundice in about 25% of patients (Marcellin 1999). Eventually, 60%-90% of infected people evolve to chronic disease. Chronic hepatitis is defined as a continuing disease without improvement for at least six months (Hsu, 1994).

In developed countries, before the introduction of routine testing of donated blood, the HCV main course of transmission were blood and blood products transfusions. Nowadays the illicit use of injectable drugs is the main source of HCV transmission. The prevalence of HCV in drug users is higher than that of human immunodeficiency virus (HIV) (Champion, Taylor et al. 2004). In developing countries the use of re-used and non-sterilized syringes and needles in medical procedures is the main cause of transmission (Craine, Hickman et al. 2009). Sexual transmission is less common than with Hepatitis B virus (HBV) and the risk of perinatal transmission is low (Noorali, Pace et al. 2010).

The main serological marker is anti-HCV, the presence of anti-HCV antibodies cannot be confirmed until 12-27 weeks after exposure, creating a window period of seronegativity and elevated risks of infectivity. Genotyping becomes essential in the clinical decision on the adoption of appropriate therapy (Saludes et al., 2014). There are 6 different genotypes numbered 1 to 6 and the sequence can vary 30% to 50% (Noorali, 2010). The genotype 1b is the most frequent genotype in Europe (WHO, 2014).

There are seven drugs licensed for the treatment of HCV, standard interferon (IFN) or Pegylated interferon alpha (PEG IFN- α), ribavirin (RBV), the protease inhibitors (PIs) boceprevir, simeprevir and telaprevir, the nucleotide analog polymerase inhibitor sofosbuvir and the most recently licensed treatment ABT-450/r–Ombitasvir and Dasabuvir with Ribavirin. The last one is the most effective treatment with a sustained virologic response in 90% of cases. The limitations of HCV treatment include high costs, the need for sophisticated laboratory tests and trained clinicians, as well as the limited efficacy and high toxicity of some of the medicines (Lawitz, Poordad et al. 2014, WHO 2014)

In Portugal, the combination of Pegylated interferon alpha (PEG IFN- α) and ribavirin (RBV) therapy is approved for the treatment of chronic HCV infection in individuals with HCV infection of non-1 genotypes (Anjo et al., 2014; Saludes et al., 2014). This combination therapy provides recovery in approximately 60% of patients (Velosa et al., 2012), however, has serious side effects such as anemia, granulocytopenia, and depression, and is associated with long-term treatment and high costs. The effectiveness

of the combined PEG IFN-α and RBV therapy is less than 50% for genotype 1, the most prevalent genotype (Zhu & Chen, 2013)

Recent estimates have shown that 185 million people around the world have been infected with HCV and 35.0000 die each year. The estimated prevalence of HCV infection is highest in Central and East Asia and in the North Africa/Middle East regions. (Figure 5) (WHO, 2014). In the European region, 1.1-1.3% of population is infected with HCV (Lavanchy 2011).

In Portugal it is estimated that only 30% of patients are currently diagnosed and that the real prevalence of HCV is 1-1.5% (100.000-150.000 individuals). The annual costs related to hepatitis C in Portugal are approximately 71 million euros. It is also estimated that 60% of infected people have chronic hepatitis C (Anjo et al., 2014). Similar to what happens globally, the genotype 1 was the most prevalent genotype in two epidemiological studies conducted in Portugal (2001 and 2009), being present in 50-60% of patients (VHPB, 2011; Velosa, 2011). According to Anjo et al. (2014) the estimated mortality rate in Portugal is 9 deaths per 100.000 inhabitants. In Portugal it is attributed to HCV 20% of the total number of deaths due to liver cirrhosis and 50% of the total number of deaths from hepatocellular carcinoma (HCC) (Anjo et al., 2014).

HCV is highly heterogeneous and such heterogeneity is hindering the development of an effective vaccine (Rosen and Gretch 1999).

Portugal lacks recent epidemiological studies on hepatitis C and it becomes important to update this information in order to know the evolution of the disease and compare it with other countries in order to decide the best treatment to be applied.

3. Material and Methods

3.1. Samples

In this retrospective study all serum samples analysed for anti-HCV antibodies from patients of the District of Aveiro, in ambulatory regime, collected at the Clinical Analysis Laboratory Avelab (Aveiro, Portugal) during the period 2002-2012, were analysed. The Clinical Analysis Laboratory Avelab comprises delegations located in 14 municipalities in the district of Aveiro and Coimbra.

The age and sex of each patient were registered. The study was approved by the Ethical Committee of the Clinical Analysis Laboratory Avelab, specifically by the Pharmacist Dr. António Ferreira Neves and Doctors Alberto Ferreira Neves, António Rodrigues and Maria Teresa Raposo.

3.2. Sampling

Samples were collected, using the Avelab Laboratory protocol. The venous blood of the patients was collected and reserved into a tube with separator spheres allowing clot formation. The samples were centrifuged at 1381 x g for 10 minutes. The blood samples were analyzed within one hour after collection. When this procedure was not possible the samples were stored at 2-8ºC and processed until 24 hours after collection. The samples were dismissed 7 days after sampling.

3.3. Antibodies Detection

The samples were analyzed in an automated Siemens ADVIA Centaur® XP immunoassay analyzer. It was used the acridinium ester (AE) as the chemiluminescent label. Samples were diluted in buffer and purified HCV antigen was added forming in the presence of specific antibodies immune complexes. AE labeled mouse monoclonal anti-HCV antibodies, biotinylated mouse monoclonal anti-HCV Fab fragment and streptavidin coated paramagnetic capture particles were incubated with the immune complexes. The biotinylated conjugate and acridinium labeled conjugate bind to antigen sites not occupied by sample HCV antibodies. A magnet is used to separate the microparticles and the unbound material is washed. The bound acridinium ester conjugate is then measured by a chemiluminescent reaction. The amount of light produced is inversely proportional to the antibodies concentration (Siemens, 2006).

Regarding hepatitis C, the most important screening marker is anti-HCV antibody that must be required systematically in individuals who have been exposed to any risk situation considered for this infection and is part of laboratory studies for pregnant woman. Positive result for anti-HCV antibodies correspond to current or past infection (prevalence of infection). It was not possible to distinguish current from past infection.

3.4. Statistical analysis

The data were treated using the Statistical Package for the Social Sciences (SPSS) 20.0 for Windows. To simplify the statistical analysis the patients were grouped by age ranges: 0-5, 6-15, 16-25, 26-35, 36-45, 46-55, 56-65 and >66. The absolute (n) and relative (%) frequencies were presented for qualitative variables. The normality of data was checked before analysis. As all the variables failed this statistical method assumption, the non-parametric Chi-square (X^2) test was used to check if the distribution of variables was similar in the different groups for immunity. The significance level established was 0.05.

4. Results

4.1. Characterization of the sample

The annual average of analyzed samples was 5403. It was observed a decrease of about 9% on the number of analysis performed between 2009 and 2012 (Figure 14A). From the total of 59440 serum samples analyzed between 2002 and 2012, 18092 (30.4%) were performed in male patients and 41348 (69.6%) in females. The age of the patients ranged between 0 and 99 years. Patients aged between 26-35 years represented the age group that carried out more analysis (Figure 14B).

From the total serum samples analyzed, 3.7% had positive anti-HCV antibodies (Figure 15).

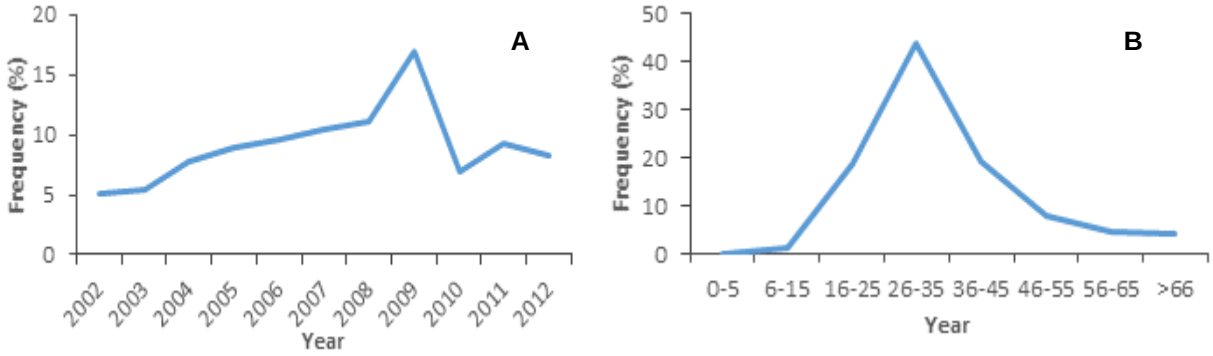

Figure 14 - Frequency (%) of the samples by year (A) and age group (B) during the study period.

Figure 15 - Samples classification during study period.

4.2. Characterization of infected or past infected patients

The age of patients with positive anti-HCV antibodies ranged from 0 to 99 years, with a mean age of 37 years. Anti-HCV antibodies were detected in 2203 patients (636 females and 1567 males).

The higher incidence of HCV infection was observed in male patients aged from 36 to 45 years and in female patients older than 66 years. The incidence of infection was significantly different ($p < 0.05$) among age groups and between genres (Figure 16A) and varied over the study period (Figure 16B).

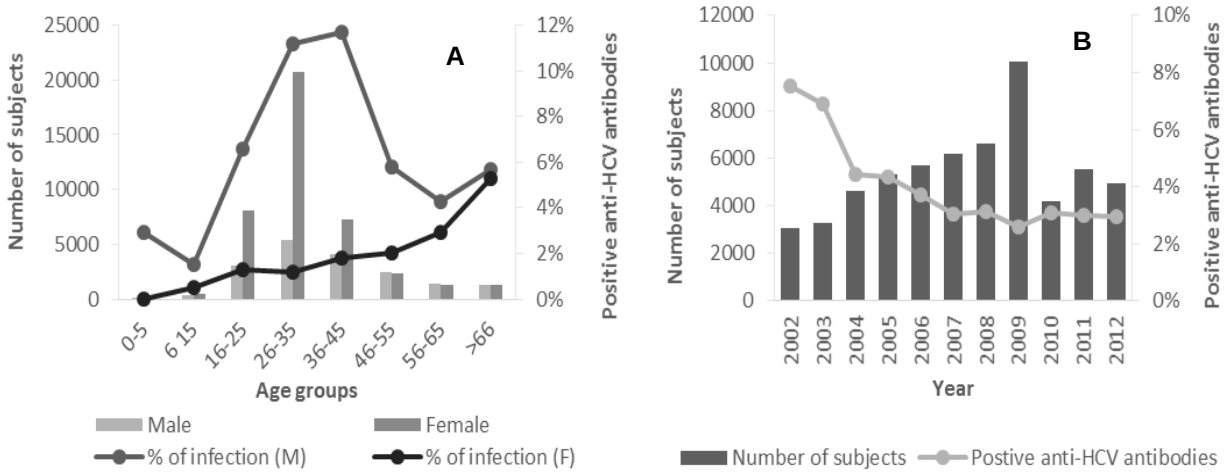

Figure 16 - Percentage of positive anti-HCV antibodies by genre and age group (A). Percentage of anti-HCV antibodies by year (B)

5. Discussion

During the study period there was a considerable decrease in the percentage of positive results for anti-HCV antibody. In 2002 7.5% of the subjects had been in contact with the virus, whereas in 2012 this value decreased to 3% but has stabilized since 2007. Anti-HCV antibodies do not distinguish acute from chronic or resolved infection thus is not possible to assess the chronic Hepatitis C in the district of Aveiro. The results show a decrease in the incidence of the virus during the study period. These results are consistent with what has been happening in other industrialized countries, presumably reflecting a decrease in percutaneous exposures (Group 2004).

In studies carried out in Portugal was estimated an incidence of 1-1.5%, which is significantly below to that found in Aveiro (Anjo, 2014).

The incidence rate in males and females was significantly different. Although the number of females presented in this study were higher than men, the percentage of

incidence of the virus was greater for males in all age groups. The fact that this analysis belongs to the general framework of analysis performed by pregnant women may explain this difference between male and female incidence. Nevertheless the rate of incidence in the 26-35 and 36-45 age groups was 11.2% (603 individuals) and 11.7% (478 individuals), respectively, which must not be ignored. The higher incidence in these age groups can be explained by the risk behaviors associated with younger people such as drug addiction. In United Stated, where the highest prevalence is also among middle aged people, injection drug use is responsible for 68% of infections (de Paula 2012, WHO 2014). About 40% of people infected older than 40 years old will develop cirrhosis in 20 years, whereas for younger patients this value decrease to 20% but it is possible that the fibrosis progression in someone infected younger than age 40 years could accelerate as that person ages and reaches the age group for which progression to cirrhosis after 20 years of infection is higher (Group 2004)(Global Burden of Disease (GBD) for Hepatitis C).

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Chapter 6 - Conclusions and future perspectives

This work reports two different themes, one depicts the inactivation efficiency of three phage of a highly relevant bacterium in nosocomial infections and the other characterizes the District of Aveiro regarding HAV immunity and HCV infection.

The main conclusions of this work are summarized in the following topics:

- The use of phage therapy with two phages increases the efficiency of phage therapy and delays the emergency of phage-resistance suggesting that phage therapy can be used to treat urinary tract infections caused by *E. cloacae.* High efficiency of isolated phages combined with long storage periods paves the way for deeper studies and possibly in vivo studies.

- The results of the study of HAV immunity in Aveiro suggest a positive impact of vaccination of children under 5 years old in the immunity pattern but the rate of immunity in the children groups are still quite low indicating that the impact of future infections and outbreaks will be substantially more problematic in children than in adults. The decrease in the immunity in older patients, associated to the increase in the mortality rate in people over 50 years old, suggests that the impact of future infections will be also a concern for the elderly group. In the future, cost effectiveness studies of hepatitis A vaccine will be important to the decision of introducing universal vaccination of the Portuguese population, taking in account that some reports have shown its value in other developed countries.

- In the future more accurate epidemiological data on hepatitis C not only in Portugal but across the world would be helpful in order to characterize the disease. Ideally these studies should be accompanied by more information regarding risk factors and other information relating to patients. Despite these difficulties, it can be conclude that there was a decline in the prevalence of the disease in the District of Aveiro throughout the study period, however, the prevalence is still high when compared with values observed in other European countries. It is important to invest more in prevention and public awareness as existing treatments, even though effective, are still very expensive and some of them have serious side effects.