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CHARACTERIZATION OF KLEBSIELLA PNEUMONIAE BACTERIOPHAGES WITH BIOTECHNOLOGICAL POTENTIAL.

CARACTERIZACIÓN DE LOS FAGOS DE KLEBSIELLA PNEUMONIAE CON POTENCIAL BIOTECNOLÓGICO.

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1 ABSTRACT.

The extensive use and misuse of antibiotics has led to an increased emergence of multidrug-resistant *Klebsiella pneumoniae* strains. They are a serious concern worldwide due to their propensity to spread and the scarce effective treatments left. Consequently, phage therapy is garnering renewed interest as an alternative method to defeat antibiotic resistant bacteria. Phages – natural pathogens of bacteria – have several properties: high capacity to replicate and host specificity that turns them into a great advantage over antibiotics.

Eight bacteriophages infecting *Klebsiella pneumoniae* were characterized according to their genetic material and morphology by performing endonuclease digestions and transmission electron microscopy imaging with 1% phosphotungstic acid or 2% uranyl acetate as staining dyes. Then, they were classified in agreement with their morphological characterization.

Seven phages (EKP3P1, EKP3P2, EKP3P4, EKP3P5, EKP8P2, EKP8P3 and EKP8P4) were classified into *Siphoviridae* family showing hexagonal heads with long non-contractile, sometimes flexible tails and closely related restriction patterns. EKP8P1 phage was classified into *Podoviridae* family showing an icosahedral head with a short non-contractile tail and a different restriction pattern. They all belong to *Caudovirales* order. Moreover, a prophage was found in EKP8P1 sample, and classified into *Siphoviridae* family according to its morphology.

The genome of EKP3P5 phage, a double stranded DNA of 47,622 bp long, was sequenced and annotated manually. EKP3P5 phage is a temperate phage encoding integrase, holin and endolysin proteins, among others. Therefore, EKP3P5 could not be used in phage therapy due to the risk of transferring virulence and resistance genes to the host bacteria.

For all the above reasons, this thesis provides detailed knowledge of the physical structure along with genomic qualities of eight bacteriophages infecting multidrug-resistant *Klebsiella pneumoniae* strains. This is important for determining the potential of phages as therapeutic agents and the first step to improve phage therapy.

RESUMEN

El uso inadecuado y excesivo de los antibióticos ha aumentado el número de cepas multiresistentes de *Klebsiella pneumoniae,* que constituyen un problema a nivel mundial por su gran capacidad de propagación y los pocos tratamientos disponibles. Por ello, se ha incrementado el interés hacia la fagoterapia como tratamiento alternativo en las enfermedades infecciosas. Los bacteriófagos – patógenos naturales de las bacterias – suponen una ventaja frente a los antibióticos por su alta capacidad de replicación y especificidad hacia el hospedador.

Se han caracterizado ocho bacteriófagos de *Klebsiella pneumoniae* de acuerdo a su material genético, mediante enzimas de restricción, y a su morfología, analizada por microscopía electrónica de transmisión con tinciones de ácido fosfotúngstico al 1% y acetato de uranio al 2%. Se han clasificado según su morfología.

Siete bacteriófagos (EKP3P1, EKP3P2, EKP3P4, EKP3P5, EKP8P2, EKP8P3 y EKP8P4) pertenecen a la familia *Siphoviridae*, caracterizados por cabezas hexagonales, colas largas no contráctiles y en algunos casos flexibles y patrones de restricción similares. El bacteriófago EKP8P1 pertenece a la familia *Podoviridae* caracterizado por una cabeza icosaédrica, una cola pequeña no contráctil y un patrón de restricción diferente. Todos ellos pertenecen al orden *Caudovirales*. Además, se encontró un profago en la muestra de EKP8P1, perteneciente a la familia *Siphoviridae.*

El bacteriófago EKP3P5 tiene un genoma de dsADN con una longitud de 47,622 pb que fue secuenciado y anotado manualmente. Es atemperado y en su genoma se codifican proteínas como la integrasa, holina o endolisina. No puede ser usado en fagoterapia por ser capaz de transferir genes de virulencia y resistencia a la bacteria hospedadora.

Este trabajo ha permitido obtener un conocimiento detallado sobre la morfología y las propiedades genómicas de ocho bacteriófagos; información valiosa para estudiar el potencial de estos fagos en su uso como agentes terapéuticos y el primer paso para avanzar en la fagoterapia.

2 INTRODUCTION.

Antibiotic resistance is one of the most worrying and challenging problems around the world. The excessive use and misuse of antibiotics selects resistant strains leading to their increased emergence; this is endangering the way infectious diseases are treated nowadays. As a consequence, new effective treatments, such as phage therapy, need to be investigated to prevent and control antibiotic resistant bacterial infections; without them, the quality of health will decrease drastically.

The World Health Organization classifies *Klebsiella pneumoniae* as a critical multidrug resistant bacterium. As a priority pathogen, there is a need to put much initiative and implication into research of new treatments. In this thesis, the main purpose is to characterize the physical structure and genomic qualities of *Klebsiella pneumoniae* infective phages and evaluate their potential in the phage therapy.

2.1 CURRENT SITUATION OF ANTIBIOTIC RESISTANCE

The World Health Organization (WHO) described antibiotic resistance as the most serious concern worldwide and claimed that it can imply the end of the antibiotic era. Antibiotic resistance is a natural phenomenon in bacteria, however, the selective pressure exerted by the use and misuse of antibiotics enhances it[1]. Up to 50% of all the antibiotics prescribed for people are not needed or are not optimally effective as prescribed[2].

Centers of Disease Control and Prevention (CDC) estimated in 2013, that there were at least 2,040,442 illnesses and 23,000 deaths per year in USA caused by antibiotic resistance. Recent publications estimate that in 2016 there were 700,000 deaths worldwide and that this number could rise to 10 million by the year 2050[2,3].

2.2 MULTIDRUG-RESISTANCE BACTERIA: *KLEBSIELLA PNEUMONIAE*

The WHO regards *Klebsiella pneumoniae* as a bacterium of main public health importance. Carl Friedländer discovered *K. pneumoniae* in 1883[4]. It is a Gram-negative and lactose-fermenting bacillus, an opportunistic pathogen member of the family *Enterobacteriaceae*[5]. In addition, it is a leading cause of nosocomial infections like

pneumonia, sepsis and new-borns infections[5], but it has the ability to cause urinary tract, respiratory tract and blood infections[1] in patients with a compromised immune system[5]. *K. pneumoniae* is related to high morbidity and mortality rates[6].

Several factors are associated with *K. pneumoniae* virulence such as: capsular antigens, fimbriae, biofilm formation, O antigens and siderophores[7]. The capsule is the major virulence factor and it is usually related to the intensity and seriousness of the infection[8]. The capsule and biofilm formation protect the bacteria from being killed by the immune system and they also impair the diffusion of antibiotics[5].

K. pneumoniae is a source of antibiotic resistance, an adaptive feature acquired by the bacterium after being in contact with antibiotics[9]. *K. pneumoniae* has acquired resistance genes by mutations and horizontal gene transfer (HGT)[10] *(Image 1).* Consequently, extended-spectrum β-lactamases (ESBLs) and carbapenemase-producing strains have appeared.

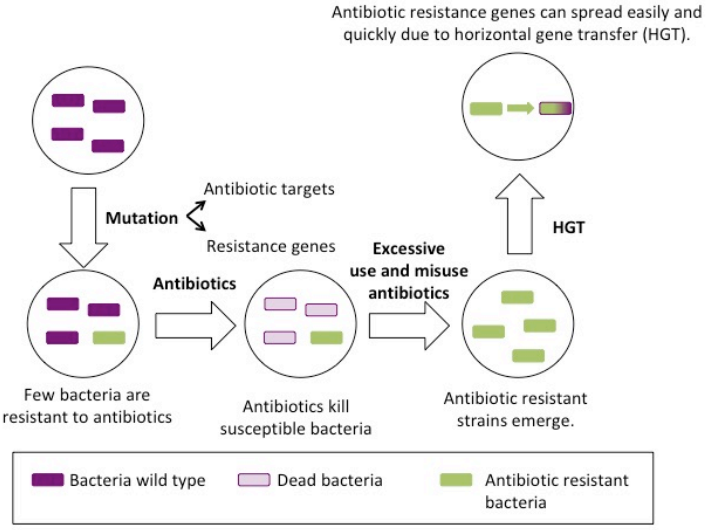

Image 1: General drawing of the acquisition and expansion of antibiotic resistance. Natural selection is the main responsible of the appearance of antibiotic resistance strains, however mutations increase this feature. The excessive use and misuse of antibiotic select resistance strains. Resistance strains will multiply and spread by means of horizontal gene transfer (HGT). Image made by the author.

2.2.1 Extended-spectrum β-lactamases (ESBLs) and carbapenem resistance

β-lactam antibiotics are the most used group of antibiotics (60% of worldwide antibiotic usage) due to their wide antibacterial spectrum and low toxicity; they were the first treatment for *K. pneumoniae*. However, their mechanism of action − blocking the cell wall biosynthesis − is impaired by β-lactamases such as TEM-1, TEM-2 and SHV-1[11].

β-lactamases underwent mutations in their active site, which increased their resistance to a wider repertoire of antibiotics, leading to the appearance of extended-spectrum β-lactamases (ESBLs). The first ESBL gene identified was SHV-2 followed by TEM-3[12], currently there are many versions of them for example: CTX, OXA, PER, GES; CTX-M being the most common[12,13]. ESBL genes are commonly encoded in plasmids, making them transmissible between bacterial hosts via HGT[12].

The appearance of ESBL and the horizontal transfer of ESBL-encoding plasmids and transposons narrow the available therapeutic options. It is worth mentioning that ESBL-producers sometimes also harbour genes that confer resistance to also other antibiotic classes, such as fluoroquinolones and aminoglycosides[13]. During the late 80s, a rise in the use of carbapenems, a broad-spectrum β-lactam, took place, as it was the only antibiotic left to treat ESBL-producing *K. pneumoniae*[14]. As a consequence, carbapenem-resistant phenotypes were selected and plasmid-mediated

carbapenemases started spreading among bacteria. The most common and prevalent carbapenemase is KPC, a β-lactamase able to hydrolase all known β-lactam antibiotics and to resist β-lactamase inhibitors[15]

Since the therapeutic options to treat *K. pneumoniae* are limited, phage therapy is gaining ground, being a promising alternative.

2.3 INTRODUCTION ON BACTERIOPHAGES

Two scientists, Frederick Twort and Félix d' Hérelle, discovered bacteriophages in the twentieth century. It was Félix d´Hérelle who named these new organisms as bacteriophages, which literally means bacteria-eaters[16], and published a report describing their ability to infect bacteria, replicate inside them and kill the cells. Since phages are the most abundant and genetically diverse organisms, it has be estimated that there are more than 10^{31} phages on Earth, they are known as the most successful life form in the world[17].

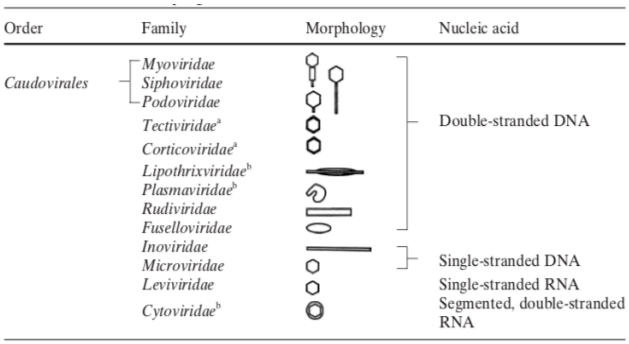

Image 2: Classification of phages according their morphology and nucleic acid. From: Shosuke Imai et al. Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. J. Infect Chemother, 2005.

The discovery of bacteriophages has allowed us to understand a wide variety of biological processes since bacteriophages constitute a reliable biological model[16]. Furthermore, they play an important role in ecology and evolution as they take part in biochemical cycles and bacterial diversity[18].

Phages are a heterogeneous group and they can be classified according to their genome in RNA or DNA phages and according their morphology in thirteen families *(Image 2).* Generally, phage morphology consists of a hexagonal head-like capsid and a tail. Most of the phages (96%) are tailed phages, belonging in the order *Caudoviriales,* whereon they belong in *Podoviridae, Myoviridae* or *Siphoviridae* family regarding their tail morphology. The rest of the phages, divided into ten families, are cubic, filamentous or pleomorphic; some of them can have lipid structures or an envelope. Moreover, their genome size is also very variable from 4 kb to up to 600 kb. Despite the genome size, all genomes encode genome packing, head, tail, DNA replication, RNA-dependent RNA polymerase, transcription regulation and lysis genes[19].

Phages are obligate parasites of bacteria. The host range of these viruses is defined by the interaction of the tail structures of the phage and specific receptors on the surface of the host bacteria[16]. There are evidences of phages being restricted to a unique host strain while there are others that can infect multiple strains that even belong in different species[20].

Phages can have lytic or lysogenic life cycle based on whether they are virulent or temperate, respectively. Independently of the life cycle, phages have to recognise receptors on the host bacteria to adsorb and infect it. Then, the viral genome is injected into the host cell. Subsequently, replication strategy will start. In a lytic process, the viral

genome is replicated and new viral proteins are produced. New phage particles are formed, and finally, the host is lysed and phage particles are released into the environment. On the contrary, lysogenic cycle implies the integration of the viral genome in the bacterial genome as a prophage. During this process, the viral genome will replicate in unison with the host. Stressful conditions can lead to the exclusion of the prophage and initiate the lytic cycle[21].

2.3.1 Importance of phages in bacterial evolution.

If the phages succeed in infecting bacteria, they can participate in bacterial evolution by several mechanisms: (1) phage release via cell lysis leads to the release of host DNA fragments, which can be acquired by other bacteria via transformation; (2) host DNA fragments or plasmids can be incorporated inside the viral capsids during the assembly of the viral particles. Other bacteria via generalized or specific transduction will acquire this DNA; (3) toxins or other pathogenic proteins (adhesion factors, superantigens, mitotic factors) encoded in the viral genome will be incorporated to the bacterial genome during lysogenic life cycle; (4) prophages can induce inversions and deletions in the host genome[21,16].

2.3.2 Phage therapy against *K. pneumoniae*.

Despite the fact that the term “phage therapy” may seem something novel, the use of phages as bactericidal agents has been used for more than ninety years[22]. Nowadays, this approach is gaining more and more attention due to ESBL and carbapenem resistance.

Phage therapy has several advantages over chemotherapy[23]: (1) during phage therapy, a natural bacteria-killer is used so it is effective against antibiotic-resistant strains because the mechanism of bacteriolysis differ from the one that uses antibiotics; (2) there is a great specificity between phages and bacteria, which implies that eukaryotic cells are not affected and, consequently, fewer side effects should be show up; (3) even though bacteria can develop resistance to phages, phages can also mutate to defeat bacterial resistance; (4) the cost associated with the development of phage therapy is cheaper than the discovery of new successful drugs; (5) phages can replicate as long as a host is present, leading to an exponential increase in their numbers and therefore become available in abundance[20].

There are two different ways to employ phage therapy. The first approach only comprises one phage, which is selected by testing the bacteria against a collection of phages. The second one consists of a cocktail of different phages. In this case, the spectrum of the action mechanism is wider than the activity of a single phage[24], since each phage may infect using different bacterial receptors and each one may have different phage-encoded proteins, such as depolymerases, which help killing bacteria[25].

There is not a common consensus about which approach is the best one. Some scientists defend cocktails of phages because it may slow down the evolution of phage-resistance since if a bacterium develops resistance to one phage it will still be sensitive to the other phages[26]. On the other hand, there are scientists that claim that at certain point bacteria will develop resistance to all phages questioning whether it is worthwhile to use phage cocktails or a highly specific phage[27,28,29].

To date, 34 genomes of *K. pneumoniae* infective phages have been deposited into the NCBI database. The *K. pneumoniae* infective phages described in previous publications belong in order *Caudoviriales* and more specifically, in *Podoviridae, Myoviridae* or *Siphoviridae* families[30,31,32].

Several research groups have performed controlled animal experiments and have provided encouraging data on the potential of phage therapy to treat *K. pneumonie*[33,34].

Nowadays, phage products are subjected to the antibiotic's legislation. There is not a framework regarding the legal status of phage products and the clinical trials to ensure their quality, safety and efficacy for their commercialization. As a consequence, the progression of phage therapy is held back[35].

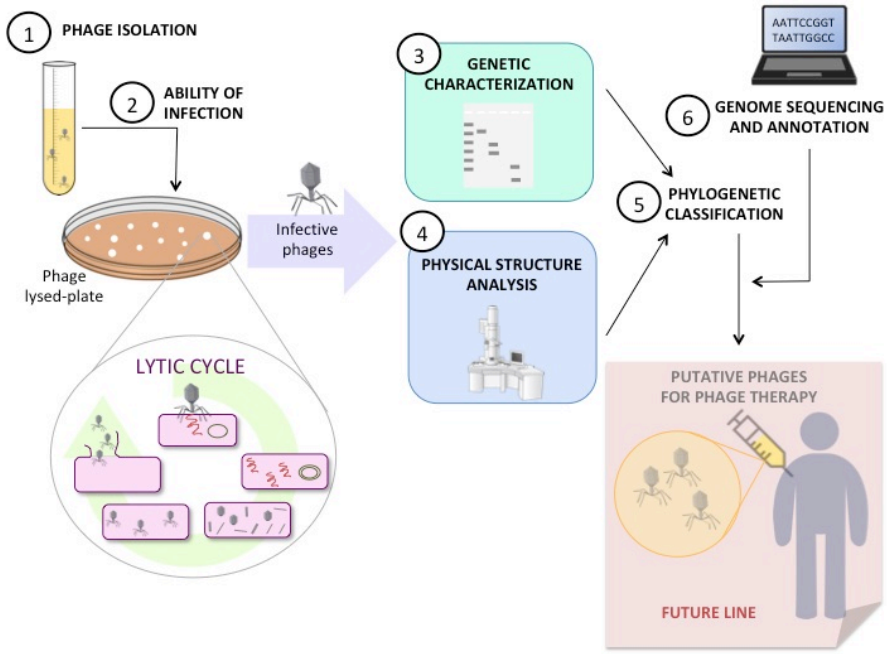

Image 3: Principal process in the study of putative phages for phage therapy. Steps one to six were followed throughout this thesis: infective phages were characterized genetically and physically and classified according to their characteristics. In addition, one viral genome was annotated. The future line is the application of phage therapy for which further studies need to be done. Image made by the author.

3 AIM

The aim of this project was to characterize the physical structure along with genomic qualities of bacteriophages infecting multidrug-resistant clinical *Klebsiella pneumoniae* isolates in order to determine their potential as therapeutic agents against infections.

1. Study the ability of each phage to infect *Klebsiella pneumoniae*.
2. Isolation of genomic material of bacteriophages along with a restriction enzyme analysis to identify each phage and determine their genome size.
3. Study the physical structure of each bacteriophage by electron microscope.
4. Determine the phylogenetic classification of each phage based on the first and second objectives.
5. Genome sequencing and manual annotation of one of the studied phages.

4 MATERIALS AND METHODS:

4.1 *K. PNEUMONIAE* HOST STRAINS.

The bacterial strains EKP3 and EKP8 were used as hosts of bacteriophages. Both strains are resistant to a wide variety of antibiotics *(Table 1)*.

Table 2: Description of *Klebsiella pneumoniae* strains used in this thesis.

	Isolate	ST* from database	Virulence genes	Capsule types	ANTIMICROBIAL RESISTANCE GENES				
					Aminoglycoside	Beta-lactam	Fluoroquinolone	Sulphonamide	Trimethoprim
EKP3	70165	14	*irp, fyu, ybt, kfu, mrk*	*K2*	*strB, strA*	*blaTEM-1A, blaSHV-28, blaKPC-3*	*QnrS 1,oqxB, oqxA,*	*sul1*	*dfrA14*
EKP8	2008025	11	*mrk*	*K13*	*aadA2, aadB, aac(6')-33*	*blaKPC-2, blaSHV-11*	*oqxB, oqxA*	*sul1 sul2*	

***ST: sequence type.**

4.2 PHAGE SAMPLES.

The phages studied in this thesis (*Table 2)* had been isolated from a water sample from Nenäinniemi wastewater treatment plant located in the city of Jyväskylä.

Table 3: Description of bacteriophages used in this thesis

PHAGE NAME	ISOLATION HOST	ISOLATION DATE	PHAGE STOCK TITER (pfu*/ml)
EKP3P1	EKP3	20.3.2017	1,56 x10^9
EKP3P2	EKP3	29.5.2017	2,42 x10^9
EKP3P4	EKP3	12.6.2017	Not determined
EKP3P5	EKP3	12.6.2017	4,67 x10^9
EKP8P1	EKP8	20.3.2017	Not determined
EKP8P2	EKP8	29.5.2017	8,2 x10^8
EKP8P3	EKP8	12.6.2017	2,2 x10^8
EKP8P4	EKP8	12.6.2017	1 x10^7

***Plaque-forming unit**

Phage stocks were provided after being prepared as follows: 100 µL from a previous phage stock (titre around 10^{8-10} pfu), 200 µL of bacteria strain EKP3 or EKP8 –

depending on the phage – grown overnight at 37°C, and 3 mL of L-soft agar (0.7% agar) were plated on a 1% L-agar plate and incubated at 37°C overnight.

The soft layer of half-infected plates was scraped into 50 mL tube with 5 mL of L-medium and incubated with 230 rpm agitation at 37°C for 4 hours. Afterwards, the mixture was centrifuged at 4700 rpm at room temperature for 12 min and the phage lysate (supernatant) was filtered through a 0.8/0.2 µm filter (32mm Syringe Filter with 0.8/0.2µm Supor® membrane). Finally, phage titer was assessed (*Table 3)* as it is previously explained. Phage stocks were stored at 4°C.

Titer of the stock was estimated by plating different concentrations of a phage stock with 100 µL of EKP3 or EKP8 and 3 mL of L-soft agar (0.7% agar). After growing overnight at 37°C, the number of plaques were counted.

$$\frac{number\ of\ plaques}{volume\ of\ stock\ (mL)} x 10^{dilution\ factor} = {}^{pfu}/_{ml}$$

Equation 1: equation to calculate a phage stock.

4.3 PHAGE LYSED PLATES.

The presence of infective phages was examined as follows: 200 µL of bacterial culture (EKP3 or EKP8) was mixed with 3 mL of 0.7% agar and the mixture was poured on 1 % agar plate to make double layer agar plates. 50 µL of bacteriophage lysate stock was spotted on the plate and incubated at 37°C overnight. By next day, the plates and the formed degraded spot were inspected.

4.4 CHARACTERIZATION OF PHAGE GENOMES

4.4.1 Genome isolation

At first, DNA isolation kits were used for phage genome extraction since, in general, phages are more likely to be DNA phages rather than RNA phages. RNA isolation kit was also tested for phage EKP8P1 genome since DNA isolation was not suitable for its genome isolation, as it will be discussed lately. Table 3 gathers the different protocols tested for the phages.

Table 4: Protocols for genomic material isolation are indicated according to each phage. Suitable and not suitable protocols are indicated. Titers of the phage stocks used throughout the thesis are mentioned.

PHAGE	TITER OF THE PHAGE STOCK (pfu/ml)	PROTOCOL	RESULT
EKP3P1	$3.3x10^9$ – $5.8x10^{10}$	Phage DNA Isolation Kit (Norgen Biotek Corp).	Suitable
EKP3P2	$2x10^9$	Phage DNA Isolation Kit (Norgen Biotek Corp).	Suitable
EKP3P4	$4.2x10^6$	DNA isolation with $ZnCl_2$ + DNeasy® Blood & Tissue Kit (QIAGEN)	Not Suitable
EKP3P5	$5.4x10^9$	Phage DNA Isolation Kit (Norgen Biotek Corp).	Suitable
EKP8P1	$3x10^9$	Phage DNA Isolation Kit (Norgen Biotek Corp).	Not suitable

	$7x10^9$	DNA isolation with $ZnCl_2$ + DNeasy® Blood & Tissue Kit (QIAGEN)	Not suitable
	$7x10^9$	QIAamp Viral RNA Mini Kit (QIAGEN)	Suitable for DNA but not suitable for RNA
	$7x10^9$	DNA isolation with $ZnCl_2$	Suitable
EKP8P2	$1x10^9$	DNA isolation kit- Norgen biotek. Corp.	Suitable
	$2x10^9$	DNA isolation with $ZnCl_2$ + DNeasy® Blood & Tissue Kit (QIAGEN)	Not suitable
EKP8P3	$1x10^8$	Phage DNA Isolation Kit (Norgen Biotek Corp).	Suitable
EKP8P4	$4,5x10^8$	Phage DNA Isolation Kit (Norgen Biotek Corp).	Suitable

4.4.1.1 DNA isolation with Phage DNA Isolation Kit (Norgen Biotek Corp).

The phage genome was isolated with Phage DNA Isolation Kit (Norgen Biotek Corp). DNase and RNase treatments were performed to eliminate host genomic DNA and RNA in the sample. 1 µL of DNase (1 mg/mL) and 10 µL of RNase (1 mg/mL) were added to 1 mL of the phage lysate (see titers in Table 3) and incubated for 45 minutes 37°C. Then, 500 µL of Lysis Solution, provided by the kit, were added to the solution and mixed by vortexing. 10 µL of proteinase K (20 mg/mL) (Thermo Scientific) were added to the mixture and incubated first at 55°C for 15 minutes and then at 65°C for another 15 minutes to inactivate it. Proteinase K degrades DNAse and RNAse enzymes and most importantly, it degrades the viral capsid, made of proteins, so that genome is released

After incubation time, 320 µL of isopropanol were added to the sample and mixed by vortexing.

Afterwards, recommendations of the supplier were followed. Finally, two sequential elutions were performed with 75 µL of PCR grade water.

4.4.1.2 DNA isolation with $ZnCl_2$ precipitation.

This protocol is based on protocol by Santos[36]. First, 1 mL of L-medium was added to 1mL of phage lysate in order to have 2 mL sample. As in the previous protocol, DNase and RNase treatment were performed in order to avoid host genomic DNA and RNA contamination. 2 µL of DNase (1 mg/mL) and 20 µL of RNase (1 mg/mL) were added to 2 mL of the phage lysate and incubated for 30 minutes at 37°C. 40 µL of freshly filtered 2 M $ZnCl_2$ were added to the lysate (40 µM final concentration of $ZnCl_2$) and incubated at 37°C for 5 minutes and centrifuged for 1 minute at 10000 rpm (9.6 x g). The pellet was suspended in 1 mL of TES buffer (0.1M Tris-HCl pH=8, 0.1M EDTA and 0.3% SDS), filtered with 32mm Syringe Filter with 0.8/0.2µm Supor® membrane (Pall Corporation), and incubated at 60°C for 15 minutes. 40 µL of proteinase K (20mg/ml) (Thermo Scientific) were added and incubated for 90 minutes at 37°C.

Right after this precipitation, a purification with DNeasy® Blood & Tissue Kit (QIAGEN) was performed to elute the DNA as follows: 1 mL of buffer AL was added and mixed by vortexing and then 1 mL of 96% ethanol was added and mixed thoroughly. The whole sample was pipetted using 600 µL each time into the column and centrifuged at 6,000 x g for 1 minute. 500 µL of buffer AW1 were added and centrifuged at 6,000xg for 1 minute. 500 µL of buffer AW2 were added and centrifuged twice at 20,000 x g for 3 minutes. Lastly, two elutions were performed with 75 µL of PCR grade water.

This protocol was also performed without using DNeasy® Blood & Tissue Kit (QIAGEN) for EKP8P1 as it is described below: after suspending in TES buffer, 120 µL of 3 M sodium-acetate (pH=5.2) were added and incubated on ice for 15 minutes. Following, sample was centrifuged for 1 minute at 12,000 rpm at 4°C.

Supernatant was retrieved and precipitated with 1 volume of ice-cold isopropanol and stored for 5 minutes on ice. Mixture was centrifuged for 30 minutes at 13,000 rpm and 4°C. DNA pellet was washed with ice-cold 70% ethanol and centrifuged for 15 minutes at 13,000 rpm and 4°C. Pellet was dried at room temperature and dissolved in 20 µL of PCR grade water (VWR).

4.4.1.3 RNA purification.

Neither of the previous protocols suited EKP8P1 and there was not any TEM image available to infer its viral family. Regarding to these facts, an attempt to isolate its genome with QIAamp Viral RNA Mini Kit (QIAGEN) was done. As a control, ϕNN Host range mutant 2 phage, an RNA phage of *Pseudomonas syringae* pv. phaseolicola HB10Y, was used. The instructions given by the provider were followed. Finally, two elutions were performed with 40 µL of PCR grade water by centrifuging at 6,000 x g for 1 minute.

4.4.1.4 Measuring the DNA/RNA concentration.

Certain isolated DNA samples were precipitated with ethanol in order to concentrate them following this method. 1/10 volume of 3 M sodium-acetate (pH=4,7) and 2,5 x volume of 96% ethanol were added to the samples and incubated for 1 hour in the freezer. After that, samples were centrifuged at 13,000 rpm for 30 minutes at 4°C. The pellet was washed with 900 µL of 75% ethanol and centrifuged again for 15 minutes at 4°C. Pellet was dried at room temperature and eluted in 25 µL of PCR grade water and incubated at 50°C for 10 minutes.

DNA samples were measured with Qubit™ dsDNA and RNA HS Assay Kit (Invitrogen) following the supplier instructions.

4.4.2 Restriction enzyme analysis.

Restriction enzyme analysis was designed to identify each phage and estimate their genome size. Different enzymes were tested in this experiment. For phages EKP3P2 and EKP3P5: NcoI (Fermentas Fast Digest), NotI (Fermentas Fast Digest), NdeI (Fermentas Fast Digest), SphI (Fermentas Fast Digest), XhoI (Fermentas Fast Digest), PstI (Fermentas), SalI (Fermentas) and EcoRV (Fermentas). For T4, which was used as a control, NcoI (Fermentas Fast Digestion), XhoI (Fermentas) and BglII (Fermentas) were utilized. Enzymes were chosen according to literature[37]. For phages EKP3P1, EKP8P2, EKP8P3 and EKP8P4 only three enzymes were used according to the first results: NdeI (Fermentas Fast Digestion), EcoRV (Fermentas) and SalI (Fermentas).

Enzyme reactions were performed to 300 ng of DNA in a final volume of 20 µL according to manufacturer's instructions. Each reaction, but NdeI, were performed overnight at 37°C. Only NdeI reaction was conducted at 37°C for 2 hours.

After the enzyme digestion, the DNA fragments were separated by electrophoresis in a 1% agarose gel containing 0,5 µg/mL ethidium bromide in TAE-buffer for 90 minutes at 90 V and 180 mA. 10 µL of GeneRuler™ 1Kb Plus DNA ladder (Thermo Scientific) was used as a size marker.

4.4.3 DNase and RNase treatments to purified a sample.

DNase and RNase treatments were performed to EKP8P1 phage in order to test whether it was a DNA or RNA phage. As a control, a previously isolated DNA phage (EKP8P3) was used.

The DNase treatment was performed in 10 µL with: RNase-free DNase I 1 U/µL (#ENO521) (Thermo Scientific), 1X reaction buffer with $MgCl_2$ and isolated-DNA from EKP8P1 (13.1 ng/µL) and EKP8P3 (16.73 ng/µL). The sample was incubated for 30 minutes at 37°C. Afterwards, 1 µL of 50 mM EDTA, provided by the supplier, was added and incubated for 10 minutes at 65°C. For RNase treatment: 2 µL of RNase A (20 mg/ml) (Invitrogen by Thermo Fisher Scientific) were added to the sample and incubated for 45 minutes at 37°C.

Mixtures were loaded in 1% agarose gel containing 0.5 µg/mL ethidium bromide in TAE buffer and run for 1 hour at 90 V and 180 mA.

4.5 IMAGE PHAGE MORPHOLOGY

Phage morphology was imaged with transmission electron microscopy (TEM). The morphology was determined and the capsid size was measured using a Jeol Jem 1400 electron microscope. The size of phage particles were determined from the average of eight independent viral particles to get a reliable diameter.

4.5.1 Phage purification from lysate for tem.

A high-titre (see Table 3) phage lysate (5 mL) was centrifuged at 25,000 x g (14,500 rpm) for 2 hours at 4°C. Phage pellet was suspended in 5 mL of filtered (32mm Syringe Filter with 0.8/0.2µm Supor® membrane) 0.1M ammonium acetate (pH=7). Two consecutive centrifugations were performed at at 25,000 x g for 2 hours at 4°C.The phage pellet was suspended in 70 µL of sterile 0.02M potassium phosphate (pH=7.5). Samples were stored at 4°C until being analysed.

4.5.2 Staining for tem.

TEM is a useful technique that allows analysing the structure of a virus easily and quickly. Different dyes and dying times were tested in order to get the best contrast and conditions for each phage. Three negative stains were tested: phosphotungstic acid (PTA), uranyl acetate and ammonium molibdate stain (the detailed dying protocols are given below).

5 µL of purified phage sample from step 4.5.1 was deposited on Formvar carbon-coated 200-mesh grid and let dry for 2 minutes. Excess suspension was dried with filter paper. The staining was performed with 5 µL of 1% PTA (pH=7) and dried for 2 minutes or 10 µL of 5% ammonium molibdate (pH=7,5) and dried for 1 minute. In case of uranyl acetate, the staining was performed twice with 2 µL of 2% uranyl acetate and dried for 2 minutes.

4.6 *IN SILICO* ANALYSIS OF PHAGE GENOME

The genome of EKP3P5 was sequenced by using Illumina HiSeq™ 2500 technology and the resulted reads were assembled with De Novo Assembly tool of software Geneious v. R11 to produce a single contig representing the whole genome of the phage. The EKP3P5 genome was scanned for possible open reading frames (ORF)

using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) according to the bacterial, archaeal and plant plastid genetic code and a minimal ORF length of 150 nucleotides.

In addition, the genome was analysed using GeneMark.hmm[38] (http://opal.biology.gatech.edu/GeneMark/gmhmmp.cgi) for prokaryotes with *Klebsiella pneumoniae 322* as reference genome and with fgenesV (http://www.softberry.com/berry.phtml?topic=virus&group=programs&subgroup=gfindv). BPROM(http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) was used to find promoters. Predicted ORF sequences were compared with known proteins using BLAST[39] programs: blastn and blastp (after translation) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

5 RESULTS.

5.1 PHAGE LYSED PLATES.

The eight studied phages are virulent based on their ability to produce clear lysate plates; therefore, they are able to infect their original isolation host strains EKP3 and EKP8 *K. pneumoniae (Image 4)*.

Presence of halos is usually related to proteins that can damage the bacteria by affecting the matrix or biofilms. EKP3P4, EKP8P2, EKP8P3 and EKP8P4 phages produce clear zones surrounded by small halos. EKP3P1 phage produces a turbid zone surrounded by a small halo. EKP3P2 and EKP3P5 phages stand out because they have a turbid zone surrounded by a large halo. EKP8P1 phage produces a turbid zone with small halos *(Image 4).* Morphology of the halos depends on the properties of each phage as they have been studied using the same medium and conditions.

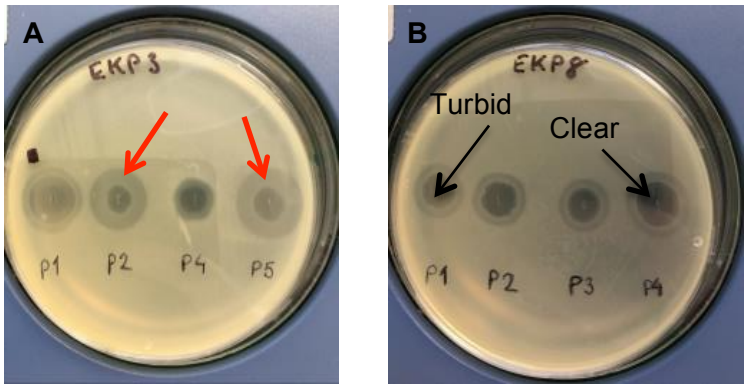

Image 4: Phage lysed plates. A) Infective plates of phages isolated with *K. pneumoniae* EKP3 as a host. Phages from left to right: EKP3P1, EKP3P2, EKP3P4 and EKP3P5. B) Infective plates of phages isolated with *K. pneumoniae* EKP8 as a host. Phages from left to right: EKP8P1, EKP8P2, EKP8P3 and EKP8P4. Red arrows indicate a prominent second halo of infection.

5.2 GENOME ISOLATION EFFICIENCY

Genome isolation using Phage DNA Isolation Kit was suitable for most phages. DNA isolation with $ZnCl_2$ precipitation and DNeasy® Blood & Tissue Kit was performed with phages EKP8P1 and EKP3P4 using EKP8P2 as a control, since it had been previously isolated with Phage DNA Isolation Kit and resulted to be a DNA phage. This protocol was suitable for EKP3P4 but not suitable for EKP8P1.

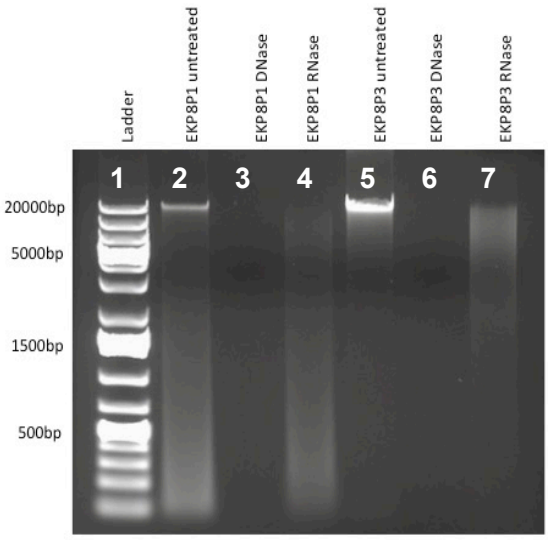

Image 5: Agarose gel electrophoresis for DNase and RNase-treated EKP8P1 and EKP8P3. Ladder: GeneRuler^{TM} 1Kb Plus DNA ladder (Thermo Scientific).

QIAamp Viral RNA Mini Kit was performed due to neither of the DNA isolation protocols suited EKP8P1. Neither RNA nor DNA were isolated from the control (ϕNN Host range mutant 2 phage), however 2.86 ng/µL of RNA and 12.25 ng/µL of DNA were isolated from EKP8P1.

Considering that at this stage no tested nucleic acid extraction methods were successful to EKP8P1, a DNase and RNase treatment was performed (*Image 5*) to see whether it was a DNA or RNA phage. EKP8P3 was used as a DNA phage control; however, no RNA phage control was used because the RNA isolation protocol did not suit the only RNA phage available in the laboratory at that time.

DNase degraded EKP8P1 and EKP8P3 genome since tracks three and six are completely clear (*Image 5)*. RNase eliminated the brightest band but not the smear *(Image 5, tracks four and seven)* in both samples.

DNase and RNase treatments for EKP8P1 and EKP8P3 show a pattern that differs from the untreated tracks *(Image 5, tracks two and five).* This result will be analysed afterwards in the discussion. EKP8P1 genome was finally isolated with $ZnCl_2$ precipitation without using the spin columns.

5.3 RESTRICTION ENZYME ANALYSIS OF PHAGE DNA

Phage genomes were subjected to restriction enzyme analysis to determine the restriction patterns, estimate their genome size and to see if and how different the phages are from each other. This analysis also proves whether the genomes constitute of single-stranded or double-stranded DNA.

First results, carried out with EKP3P2 and EKP3P5 *(Image 6A),* showed that both phages were sensitive to *EcoR*V, *Sal*I and *Nde*I but were insensitive to *Pst*I and *Nco*I. As the last two enzymes did not work, they were not tested again in the following analyses.

EKP3P1, EKP8P2, EKP8P3 and EKP8P4 genomes were sensitive to *Nde*I, *Sal*I and *Eco*RV *(Image 6B and 6C).* EKP8P1 was insensitive to *Hind*III and, unlike the other phages, to *NdeI (Image 6D).*

Recapitulating, EKP3P2, EKP3P5, EKP3P1, EKP8P2, EKP8P3 and EKP8P4 are sensitive to the same enzymes and have a similar pattern, which might indicate that they are closely related; however, EKP8P1 is insensitive to those enzymes, which might indicate that it is not related to them.

Getting a restriction pattern means those phages have dsDNA genome as the enzymes have cut the samples. Even though the undigested samples only show one patterns, we cannot confirm that there is just one type of phage in each stock. It might happen that there were two genomes of the same size.

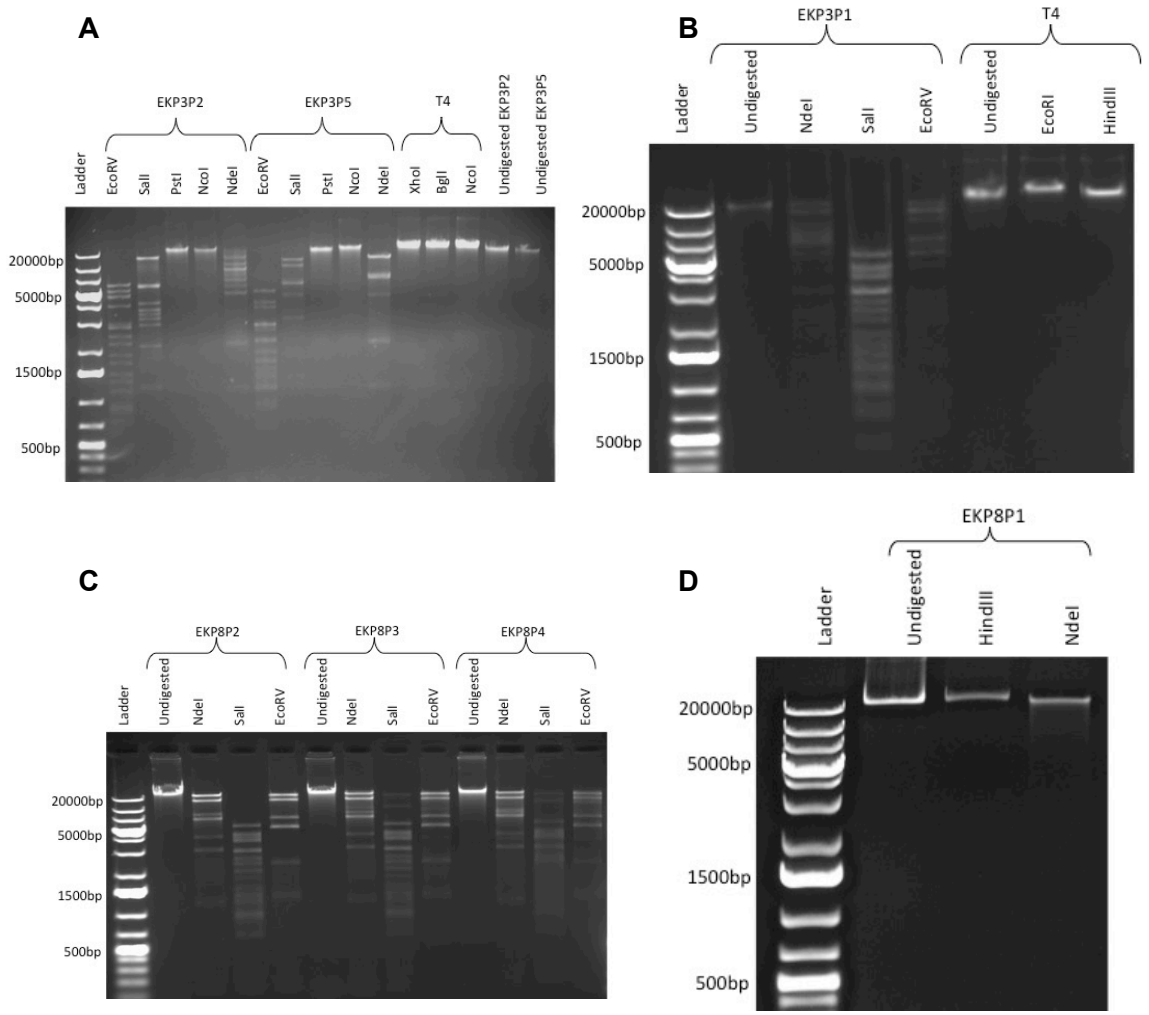

Image 6: Agarose gel electrophoresis. DNA restriction endonuclease digestion of EKP3P2 (A), EKP3P5 (A), T4 (A, B), EKP3P1 (B), EKP8P2 (C), EKP8P3 (C), EKP8P4 (C), EKP8P1 (D). Labels on top of the gels show the restriction enzymes used. Ladder: GeneRuler^{TM} 1Kb Plus DNA ladder (Thermo Scientific).

Phage T4 was used as a control. It was insensitive to *Xho*I, *Bgl*I, *Nco*I *(Image 6A), EcoR*I and *Hind*III *(Image 6B).* These enzymes were chosen because they were supposed to cut T4 genome according to literature[40] and REBASE[41] (http://rebase.neb.com/rebase/rebase.html).

On the basis of digestion pattern produced after treating the DNA with *Sal*I, the approximate genome size of each phage was estimated as follows: *Sal*I cuts the DNA resulting into production of DNA fragments of varying sizes, the addition of those fragments is approximately the size of the genome. The estimated genome sizes are: 60 kb for EKP3P1, 43.2 kb for EKP3P2, 57 kb for EKP3P5, 70 kb for EKP8P2, 65 kb for EKP8P3 and 72.5 kb for EKP8P4.

Genome size of EKP8P1 and EKP3P4 could not be estimated due to none of the enzymes used for EKP8P1 were able to cut its genome (*Image 6D*) and the restriction analysis for EKP3P4 did not work successfully (*Image 7*). Electrophoresis for EKP3P4 restriction (*Image 7*) shows blurry patterns because there is a background of DNA or protein that impairs its visualization.

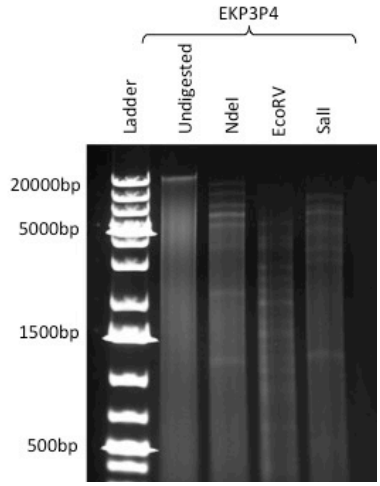

Image 7: Agarose gel electrophoresis for restriction enzyme analysis of EKP3P4. Labels on top of the gel show the respective restriction enzymes used. Ladder: GeneRuler™ 1Kb Plus DNA ladder (Thermo Scientific).

5.4 MORPHOLOGICAL CHARACTERIZATION

All bacteriophages were examined by transmission electron microscope. The best stain for phages EKP3P1, EKP3P2 and EKP3P5 was 1% PTA, however, for the rest of the phages the best stain was 2% uranyl acetate. Staining with ammonium molibdate was not accurate as the background was too light, complicating the focus of the image.

Electron microscope reports revealed that seven phages (EKP3P1, EKP3P2, EKP3P4, EKP3P5, EKP8P2, EKP8P3 and EKP8P4) have hexagonal heads with non-contractile tubular tails and the eighth one, EKP8P1, has an icosahedral head with a small non-contractile tail *(Images 8 and 9)*.

According to the morphological characterization *(Images 8 and 9),* seven out of eight phages were classified to virus families in *Siphoviridae* and, the eight one, in *Podoviridae* (T7-like); all belonging to the order *Caudoviriales (Table 4)*.

An unexpected phage was discovered while imaging EKP8P1 sample. Phages in the sample seemed to have a small non-contractile tail but a long non-contractile-tailed phage was found *(Image 11).* According to its morphological features, it can be classified in the order of *Caudoviriales* and its virus family *Siphoviridae.* This phage has icosahedral head of 81 nm of diameter and an inflexible tail of 135 nm length.

As there was just one *Siphoviridae* phage among others *Podoviridae* phages in the sample, it seemed that the new phage could be a prophage. In addition, *K. pneumoniae* strain EKP8 has viral elements in its genome *(Table 1).*

Table 4: Morphological characteristics of each phage. The estimated head and tail sizes are shown along with genome size. A classification according to morphological criteria is indicated. ND: not determined.

PHAGES	ORDER	FAMILY	GENOME	HEAD	TAIL
EKP3P1	*Caudoviriales*	*Siphoviridae*	60 kb	72 nm	160 nm
EKP3P2	*Caudoviriales*	*Siphoviridae*	43,2 kb	73,7 nm	190 nm
EKP3P4	*Caudoviriales*	*Siphoviridae*	ND	80 nm	200 nm
EKP3P5	*Caudoviriales*	*Siphoviridae*	57 kb	88 nm	206 nm
EKP8P1	*Caudoviriales*	*Podoviridae*	ND	61,5 nm	23 nm
EKP8P2	*Caudoviriales*	*Siphoviridae*	70 kb	69,4 nm	180,5 nm
EKP8P3	*Caudoviriales*	*Siphoviridae*	65 kb	67,6 nm	202,7 nm
EKP8P4	*Caudoviriales*	*Siphoviridae*	72,5 kb	74 nm	185 nm

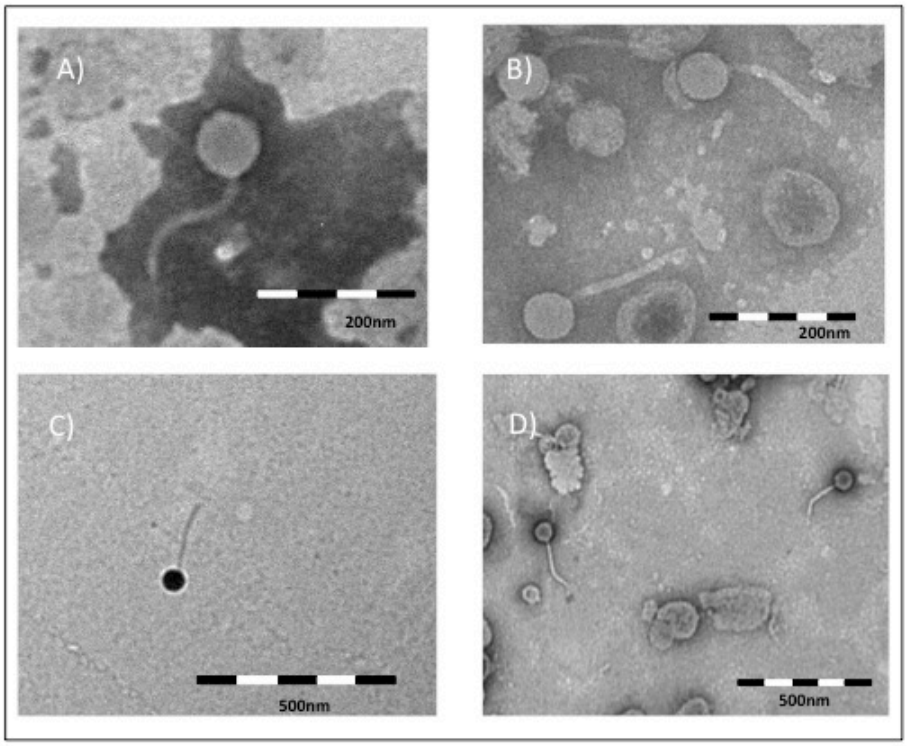

Image 8: Phages isolated with *K. pneumonia*e strain EKP3 as a host. A) EKP3P1 (*Siphoviridae)* stained with 1% phosphotungstate, Magnification 30000X. B) EKP3P2 (*Siphoviridae)* stained with 1% phosphotungstate, Magnification 30000X. C) EKP3P4 (*Siphoviridae)* stained with 2% uranyl acetate. Magnification 15000X. D) EKP3P5 (*Siphoviridae)* stained with 1% phosphotungstate, Magnification 15000X

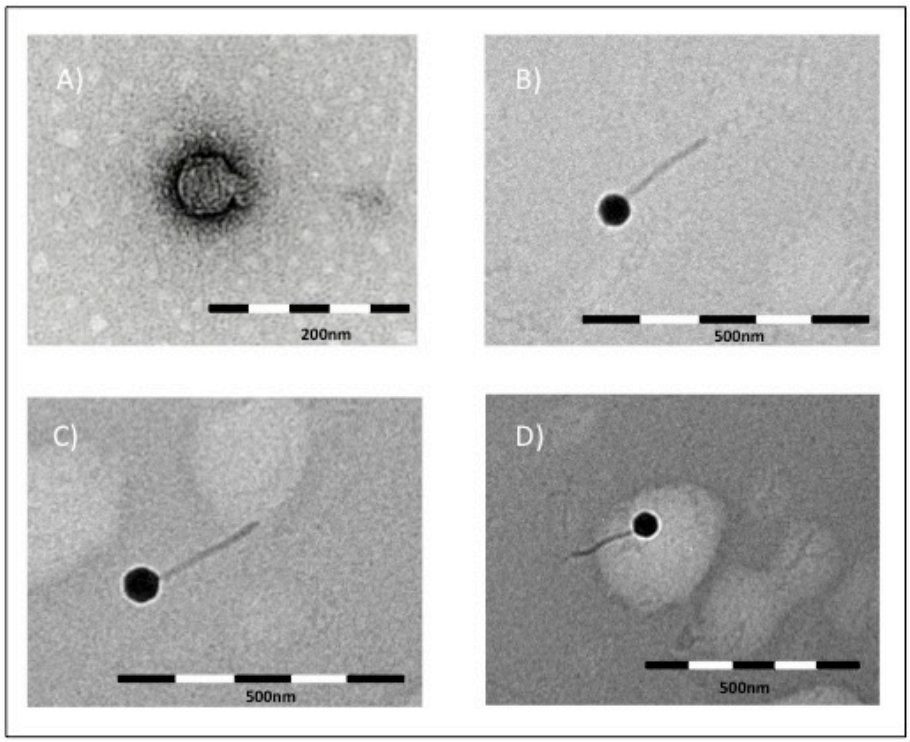

Image 9: Phages isolated with *K. pneumoniae* EKP8 as a host. A) EKP8P1 (*Podoviridae)* stained with 2% uranyl acetate. Magnification 40000X. B) EKP8P2 (*Siphoviridae)* stained with 2% uranyl acetate. Magnification 25000X. C) EKP8P3 (*Siphoviridae)* stained with 2% uranyl acetate. Magnification 25000X. D) EKP8P4 (*Siphoviridae)* stained with 2% uranyl acetate. Magnification 25000X.

5.5 *IN SILICO* ANALYSIS OF PHAGE EKP3P5 GENOME

EKP3P5 has a DNA genome of 47,622 bp with a 52,47% of G+C content. The servers ORF finder, GeneMark.hmm and fgenesV were used to find possible open reading frames; as a result, 189, 65 and 77 ORFs were found, respectively. 102 promoters were found with BPROM. Putative proteins are presented in the image below (see Table S1 in supplementary material for more information):

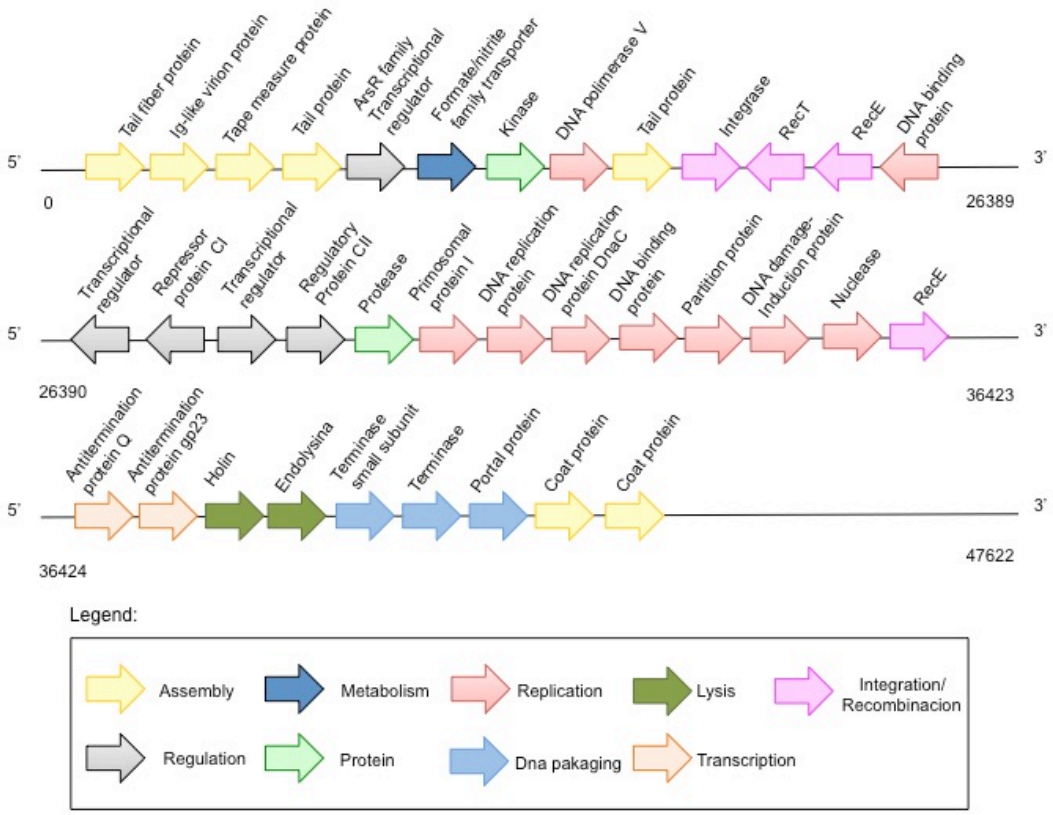

Image 10: Representation of manual annotation of EKP3P5 genome. Genome is presented in 5'-3' direction. Arrows indicate the direction of the gene but are not related to the gene length. Same colour of the arrows indicate that proteins share related functions. Numbers below the line represent the coordinates in the EKP3P5 genome. Image made by the author.

6 DISCUSSION

Klebsiella pneumoniae is considered as one of the major species of clinical relevance. It can be treated with antibiotics, a historically successful tool to treat infectious diseases, however, the appearance of antibiotic resistant strains complicates the treatment. As a consequence, high rates of mortality and morbidity are associated with this pathogen. The failure of new drug discovery programs has resulted in that many research groups had turned their attention to phage therapy. The major condition to move forwards in phage therapy is the availability of well-characterised phage libraries. Hence, an attempt to characterize bacteriophages in order to assess their potential for their use in phage therapy has been made in this thesis.

The studied phages have the ability to infect *K. pneumoniae;* they are virulent phages according to their ability to produce clear lysate plates *(Image 4)*. Two examples of enhanced lytic activity are phages EKP3P2 and EKP3P5 since they have a second halo surrounding the halo of infection. This halo suggests the ability to produce additional

molecules that damage the bacteria by affecting the matrix of *K. pneumoniae* allowing a better approach to infect the cells, and also, as the cell is less protected, these molecules enhance the activity of antibiotics and the immune system. The other phages only form the zone of infection, which means that those phages are effective in infecting the bacteria but not effectively producing other molecules[28,25].

After confirming that the phages could infect the bacterium of interest, the next step was the characterization of the genetic and physical structure. Starting with the genetic analysis, many protocols were used to isolate the genomic material, as there was not a single protocol that suited all the phages. Commercial Phage DNA Isolation Kit was suitable for most of the phages *(Table 3)*.

EKP8P1 behaved differently from the other phages, since it could not be imaged with the TEM and its genome could not be isolated with the same DNA isolation protocols used for other phages. In addition to that, both RNA and DNA were isolated from the sample with the QIAamp Viral RNA Mini Kit. In order to clear matters up, DNase and RNase treatments *(Image 5)* were performed to confirm whether EKP8P1 had a DNA or RNA genome.

DNase treatment degraded the genomic material entirely in EKP8P1, and also, as it was expected, in EKP8P3 since it was used as a positive control. RNase, in both phages, eliminated the brightest band but not the smear. According to these results, it was certainly sure that the RNase was contaminated with some DNase. The RNA isolated from EKP8P1 sample could be a false positive result or a contamination from the host. At this point, even though DNA was isolated with a modified $ZnCl_2$ precipitation protocol, no statement can be done about the genomic material of EKP8P1. Further studies need to be performed to confirm whether it is a DNA or RNA phage, for example, the DNase/RNase treatment should be repeated with uncontaminated reagents.

The electrophoresis of EKP3P4 genome revealed a white smearing background. This background can be degraded DNA from the host or the phage or a contamination of proteins *(Image 7).* The DNase/RNase treatment may have degraded the phage genome or have not degraded the host genome. The same conditions were performed with all phages indicating that this phage may be more sensitive to this treatment or the amount of host genome in the sample was bigger than in the other ones. As the background impairs to see the digestion pattern to estimate the genome, further research is needed to determine the genome size.

For phages EKP3P1, EKP3P2, EKP3P4, EKP8P2, EKP8P3 and EKP8P4 the isolation and enzyme digestion of DNA was performed successfully *(Images 6A, 6B, 6C)*. Restriction profile confirmed that the genomes of all these seven phages comprised dsDNA molecules of approximately 40-70 kb in size *(Table 4).*

DNA samples of *Siphoviridae* phages (EKP3P1, EKP3P2, EKP3P4, EKP3P5, EKP8P2, EKP8P3 and EKP8P4) were sensitive to *Nde*I, *Sal*I and *EcoR*V and showed a different but closely related restriction pattern. This provides evidences of being closely related, hence it seemed that they all belong to the same viral family.

Siphoviridae Klebsiella phages KP16 and KP36, isolated by Kesik-Szeloch et al, are also sensitive to *EcoR*V and insensitive to *Nco*I[37]. The restriction pattern of *EcoR*V for KP16 and KP36 is closely related to the pattern of *EcoR*V for the studied phages in this thesis. This supports the hypothesis that similar restriction patterns can be an evidence of family relation.

Podoviridae phage EKP8P1 was insensitive to *Hind*III *and Nde*I *(Image 6D). Podoviridae Klebsiella* phages KP34 and KP32, isolated by Kesik-Szeloch et al, are sensitive and insensitive to *Hind*III[37], respectively. Differences in sensitivity to restriction endonucleases among phages belonging to the same family might show the ability of these phages to face with the bacterial defences. This agree with the fact that T7-like virus, which belong to *Podoviridae* family*,* have a wide variety of strategies to overcome restriction modification system[42]. As none of the endonucleases provided a digestion pattern to estimate the genome, further research is needed to determine the genome size of EKP8P1.

T4 phage was used as a positive control during the whole thesis, however, it is worth mentioning that it has not been a suitable positive control for DNA phages, as the restriction enzymes did not work. There was not another known DNA phage available at that moment in the laboratory, so further experiments must be performed with another phage.

Bacteriophages are usually classified according to morphological criteria rather than molecular data[43] because there are not satisfying genetic markers and there are slight differences among the genomes of tailed-phages[44].

Considering that 96% of bacteriophages belong to *Caudoviriales* order[44] characterized by dsDNA genomes inside of an icosahedral capsid and the presence of contractile or non-contractile tails, it was not a surprise that TEM revealed that the phages studied in this thesis belonged to it *(Image 8 and 9). Caudoviriales* order is subdivided into three families: *Siphoviridae*, *Myoviridae* and *Podoviridae*.

According to ICTV (International Committee on Taxonomy of Virus), *Siphoviridae* family have a non-contractile, long, thin, 65-570x7-10 nm tails that can be flexible or non-flexible and icosahedral heads of 60 nm in diameter. *Myoviridae* family have contractile, rigid, long, thick, 80-455x16-20 nm tails with variable size and shape of heads. Lastly, *Podoviridae* family have a non-contractile tail about 20x8 nm and an icosahedral capsid of 60 nm in diameter.

All phages were examined using a negative stain in which the specimen, in this case the phages, appears light against the dark surrounding background. This technique is based on the ability of the stain to scatter electrons strongly and adsorb to biological sample.

Best TEM images for phages EKP3P1, EKP3P2 and EKP3P5 *(Image 8A, 8B and 8D)* were obtained with 1% PTA. This anion comprises twelve tungsten atoms linked by oxygen atoms and the phosphorus atoms in the centre of the molecule. Its dense electron density due to the presence of 12 tungsten atoms with an atomic number of 74 is the base of its use as a negative stain. The rest of phages were stained with 2% uranyl acetate *(Image 8C, 9A, 9B, 9C and 9D).* This compound produces the highest

electron density due to the atomic weight of 238 Da of uranium. It can bind to proteins, lipids with sialic acid carboxyl groups and nucleic acid phosphate groups of DNA and RNA. Our images with 2% uranyl acetate seem to have reacted as a positive staining as the capsids are stained in black, but the reason could be that the phages had a tiny break in their capsids so uranyl acetate was able to enter inside the capsid and interact with the proteins or the phosphate groups of DNA. Staining with 5% was not accurate for the phages studied in this thesis. This result can be explained with the electron density of ammonium molibdate: as this compound has a lower electron density than the other tested stains, the image contrast is lower.

Comparing the TEM images and DNA isolation results with the classification provided by ICTV it was assumed that phages EKP3P1, EKP3P2, EKP3P4, EKP3P5, EKP8P2, EKP8P3 and EKP8P4 belong to *Siphoviridae* family and phage EKP8P1 to *Podoviridae* family. All the viruses could be classified into the order *Caudoviriales.*

Only one long-tailed phage was found while imaging EKP8P1 sample *(Image 11),* which is likely to be a prophage, a phage that was integrated in the host genome but released eventually. This hypothesis is supported by the fact that there are bacteriophages inserted in the genome of the strain EKP8 and the rest of the imaged viral particles were *Podoviridae* phages. It is not likely to be a contamination because there should have been more tailed-phages and several plaque isolations steps were performed to purify the phage stock. Taking this into consideration, new phages should be characterised using bacteria unable to release prophages. In this way, there would be no hesitation between a prophage and the studied phage.

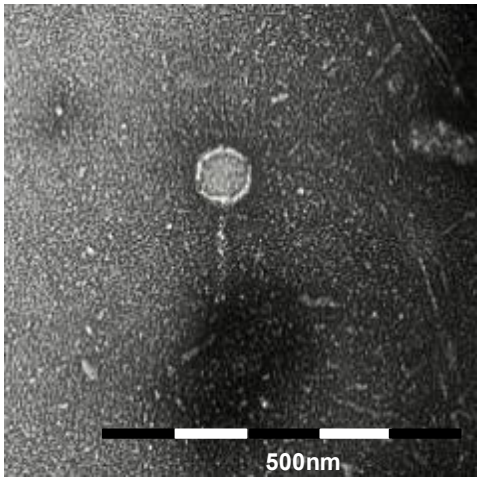

Image 11: Prophage found while analysing EKP8P1 sample. Stained with 2% uranyl acetate. Magnification 15000X.

The genome of EKP3P5 was analysed further after sequencing. Sequencing revealed that EKP3P5 has a genome of 47,6 kb in size with a G+C content of 52,47%. The genome size is comparable with the values estimated by ICTV for *Siphoviridae* family.

Siphoviridae bacteriophages organize their genome in a way that related functions cluster together[45] as it can be appreciated in image 11. The genome has been divided into nine different parts regarding the pathway in which the predicted proteins are involved. The most important putative proteins encoded in EKP3P5 genome are discussed below.

The hypothetical presence of terminase proteins, tape measure proteins and portal proteins along with tail and coat proteins gives us a hint about the assembly of the phage. According to the literature, the assembly process could be performed as follows: coat proteins may start forming the icosahedral capsid and join the portal protein, which will interact lately with the terminase protein. These two last proteins are involved in the recognition of the viral DNA and form a powerful molecular motor that helps

translocating the DNA inside the head. Once DNA packaging is complete, tail proteins are recruited. Tape measure protein indicates the tail length and facilitates DNA transit to the cell cytoplasm during infection [44,45].

An Ig-like virion protein was found in the genome of EKP3P5, which may be involved in the recognition of the host cell surface receptors. As it has been previously described, these proteins act in the first step of infection and limit the host range.

A putative integrase was found in EKP3P5 genome. The integrase is an enzyme that recognises a site-specific sequence in both the viral and bacterial genomes and allows the phage to integrate inside the host chromosome[46]. According to that, it seems that this phage may be a temperate phage, which enters in lysogenic cycle. There are several reasons why to avoid using temperate phages in phage therapy: (1) temperate phages may encode toxins and virulence factor, therefore, when the prophage is established the bacteria can turn into a more pathogenic strain; (2) the bacteria become immune to the superinfection for related phages once the lysogen is established; (3) temperate phages are capable of transduction, which could lead in the antibiotic resistance dissemination[26].

The ability of EKP3P5 to produce a second halo of infection has been previously discussed. The annotation of its genome revealed, that this phage encoded two putative proteins related with the degradation of the capsule of *K. pneumoniae*: holin and endolysin, two peptidoglycan hydrolases. Holin acts by creating holes in the host cell membrane allowing endolysin to reach the outlayer of peptidoglycan and degrade it. Endolysin has been proposed to be an alternative to antibiotics. While β-lactam antibiotics inhibit the synthesis of peptidoglycan, endolysin degraded the bacterial cell directly independently the bacterium is growing or not[23].

Despite the fact that phage therapy is a promising alternative to face infectious diseases, it is necessary to be aware of its limitations. Bacteria can develop resistance to phages by means of different mechanisms such as adsorption resistance, restriction-modification and CRISPR-Cas system[27,47]. Taking into account that the massive use of antibiotics has led to the emergence of antibiotic resistance strains, the massively used of phages can also develop resistance. However, phages have several mechanisms to defeat bacterial defences and develop resistance that are summarized below.

To achieve replication, phages have to attach to the host cell. Phages can modify their receptors to recognise the bacteria. They also encode enzymes, such as endosialidases, which degrade the bacterial wall and gain access when the bacteria shield its receptor to reduce the susceptibility to phage infection. Phages have developed many anti-restriction mechanisms to face restriction-modification systems. Some of these anti-restriction mechanisms are: inhibitor proteins (Ocr, DarA and DarB) of the restriction system, reduction of the restriction sites and incorporation of modified nucleotides in the viral genome. Finally, phages can encode anti-CRISPR proteins that impair the CRISPR interference[48]*(Image 12)*.

The use of bacteriophages in biotechnological processes requires more knowledge of phage characteristics such us host range, latent period, growth time and resistance to stress conditions such as different temperature or pH. Further experiments need to be performed for a clinical use of phages[26].

Phage therapy has many challenges that need to be overcome. Phages can trigger immune and allergic reactions; in addition, antibodies can neutralize the phages leading to a decrease therapeutic effect. Phage therapy rarely if ever results in side effects as for example, intensification of the pain[24]. The use of phages as a therapeutic approach can lead to the emergence of resistant strains. As it has been previously discussed, bacteria and phages are a clear example of coevolution; therefore, phages can mutate to adapt the new host. The most serious concern about phage therapy is transfer of toxin genes via transduction; this can modify the host in a way that the bacteria become more pathogenic. This problem can be overcome by the selection of phages without the ability of transduction or by using genetically modified phages[23].

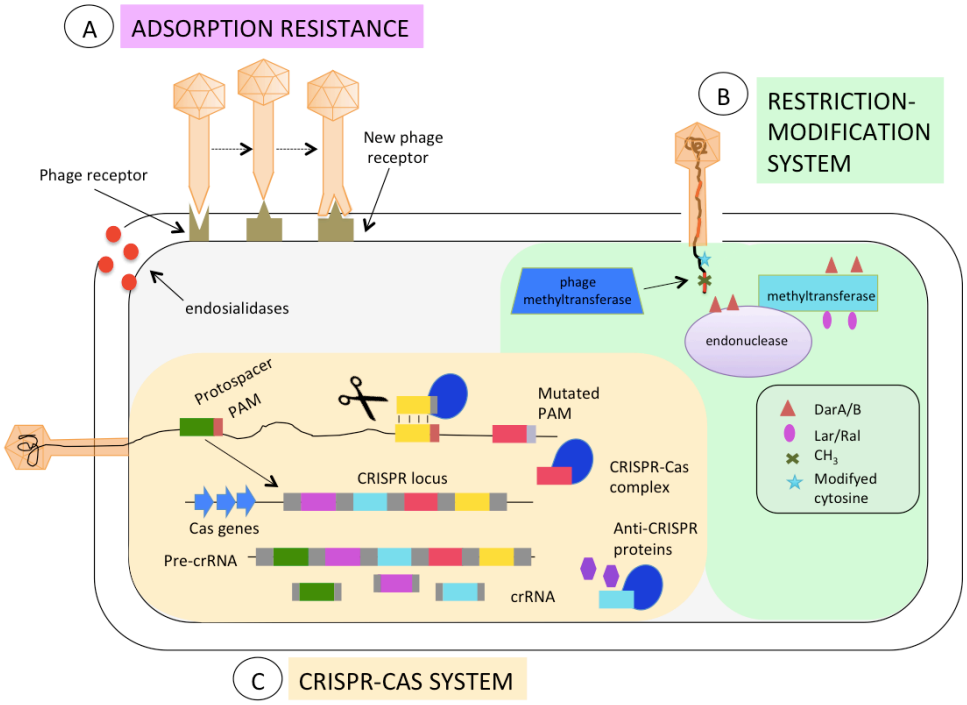

Image 12: Overview of the viral mechanisms to face bacterial defences. A: Adsorption resistance. Bacteria can alter their cell surface receptors to reduce the susceptibility to phage infection. Nevertheless, phages can alter their receptor binding structures to match the new bacterial receptor. **B: Restriction-modification system (RMS).** This system consists of an endonuclease that recognises specific sites in foreign DNA sequences and cuts them and a methyltransferase that modifies the host DNA by transferring a methyl group and prevents its cut. It blocks new viral infections by degrading the viral DNA. There are four major groups of RMS based on their structure, recognition site, cofactor requirements and cleavage position[42]. Phages can synthetize proteins (DarA/B, Lar/Ral) to block the methyltrasnferase and endonuclease activities. **C: Representation of the different steps in the CRISPR-Cas system.** CRISPR-Cas system is considered the immune system of bacteria as it can target and cleave viral DNA in a sequence-specific manner. The CRISPR interference takes place when the sequence of the protospacer is identical to the spacer hence the Cas proteins cleave the plasmid or viral DNA avoiding the replication. Phages encode anti-CRISPR proteins, which impair the formation of the CRISPR-Cas complex and the interference. Image made by the author.

7 CONCLUSIONS.

- EKP3P1, EKP3P2, EKP3P4 and EKP3P5 infect EKP3 *K. pneumoniae* strain while EKP8P1, EKP8P2, EKP8P3 and EKP8P4 infect EKP8 *K. pneumoniae* strain.
- There is not a single DNA isolation or staining protocol that suits all phages. Protocols need to be optimized for each phage.
- Seven phages (EKP3P1, EKP3P2, EKP3P4, EKP3P5, EKP8P2, EKP8P3 and EKP8P4) have a dsDNA genome. No statement can be made about the genetic material of EKP8P1. Further experiments must be performed to verify the genetic material of EKP8P1. Genome sizes vary from 40 kb to 73 kb.
- All bacteriophages belong to *Caudoviriales* order and were classified in *Siphoviridae* (EKP3P1, EKP3P2, EKP3P4, EKP3P5, EKP8P2, EKP8P3 and EKP8P4) and *Podoviridae* (EKP8P1) families regarding their physical structure.
- The analysis of the phage genome provides essential information about the life cycle of the virus. This allows identifying temperate phages (EKP3P5) and dismissing them for phage therapy.

CONCLUSIONES

- Los fagos EKP3P1, EKP3P2, EKP3P4 y EKP3P5 infectan la cepa de *K. pneumoniae* EKP3, mientras que los fagos EKP8P1, EKP8P2, EKP8P3 y EKP8P4 infectan la cepa de *K. pneumoniae* EKP8.
- No hay un protocolo de aislamiento de material genético o tinción óptimo para todos los fagos. Los protocolos tienen que ser optimizados de manera individual para cada virus.
- Siete fagos (EKP3P1, EKP3P2, EKP3P4, EKP3P5, EKP8P2, EKP8P3 y EKP8P4) tienen un genoma caracterizado por una doble cadena de ADN. En el caso de EKP8P1 no se puede asegurar la naturaleza de su genoma. Se debe realizar un estudio mas exhaustivo de EKP8P1 para determinar la naturaliza de su genoma. El tamaño de los genomas varía de 40 kb a 73 kb.
- Todos los fagos pertenecen al reino *Caudoviriales* y a las familias *Siphoviridae* (EKP3P1, EKP3P2, EKP3P4, EKP3P5, EKP8P2, EKP8P3 y EKP8P4) y *Podoviridae* (EKP8P1).
- El estudio del genoma permite conocer información importante sobre el ciclo de vida de los fagos, permitiendo identificar a los fagos atemperados (EKP3P5), los cuales serán rechazados para la fagoterapia.

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