# ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE AGAINST MDR *Klebsiella pneumoniae* FROM HOSPITAL SEWAGE

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# ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE AGAINST MDR *Klebsiella pneumoniae* FROM HOSPITAL SEWAGE

**by**

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## LIST OF SYMBOLS

| | |
|---|---|
| + | Plus |
| - | Minus |
| × | Multiplication |
| ÷ | Division |
| ± | Plus-minus |
| % | Percentage |
| < | Less than |
| > | More than |
| ≤ | Less than or equal |
| ≥ | More than or equal |
| ℃ | Degree Celcius |
| β | Beta |
| μ | Micro sign |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| dsDNA | Duple strain DNA |
| ssDNA | Single strain DNA |
| ESBL | Extended Spectrum Beta Lactamases |
| CRE | Carbapenem-resistant *Enterobacteriaceae* |
| AmpC | AmpC beta-lactamases |
| KP | *Klebsiella pneumoniae* |
| PC | Phage cocktail |
| MDR | Multi-Drug Resistance |
| XDR | Extensively Drug Resistance |
| PDR | Pan Drug Resistance |
| HWW | Hospital Wastewater |
| PFGE | Pulse- Field Gel Electrophoresis |
| MLST | Multilocus Sequence Typing |
| µL | Microliter |
| mL | Millilitre |
| µm | Micromole |
| nm | Nanometre |
| Min | Minute |
| mg | Milligram |
| L | Liter |
| g | Gram |
| kV | Acceleration Voltage |
| x | Magnification |
| HRTEM | High Resolution Transmission Electron Microscope |

| | |
|---|---|
| TEM | Transmission Electron Microscope |
| SEM | Scanning ELECTROM Microscope |
| STEM | Scanning Transmission Electron Microscope |

## LIST OF APPENDICES

**PENGASINGAN DAN PENCIRIAN BAKTERIOFAJ TERHADAP MDR *Klebsiella pneumoniae* DARI KUMPULAN HOSPITAL**

## ABSTRAK

*Klebsiella pneumoniae* yang rintang terhadap pelbagai jenis ubat (MDR-KP) semakin meningkat dalam persekitaran penjagaan kesihatan. Bakteriofaj telah dikenal pasti sebagai agen terapi yang berpotensi untuk melawan bakteria MDR dan ia boleh dipencilkan daripada air kumbahan serta sumber persekitaran lain. Selain itu, bakteriofaj juga dianggap sebagai agen penting dalam memerangi jangkitan MDR-KP. Kandungan bahan organik dan anorganik yang tinggi dalam loji air kumbahan hospital (HWW) mewujudkan persekitaran yang sesuai untuk bakteriofaj, yang dapat dijelaskan melalui proses pengasingannya dalam kajian ini, kajian ini menggunakan sampel air kumbahan yang diambil dari Hospital Pakar Universiti Sains Malaysia (HPUSM) yang kemudiannya akan dianalisis secara terperinci melalui beberapa prosedur penapisan dan pemekatan untuk mengasingkan faj yang boleh bertindak melawan MDR-KP. Ciri-ciri bakteriofaj yang dipencilkan kemudiannya dikaji berdasarkan kestabilannya terhadap suhu, pH, kloroform, dan julat hosnya. Selain itu, mikroskop elektron transmisi (TEM) digunakan untuk menentukan morfologi bakteriofaj. Bakteriofaj yang dipencilkan telah menunjukkan julat hos yang terhad dengan keutamaan spesifik terhadap strain MDR-KP. Analisis morfologi menunjukkan faj yang dipencilkan tergolong dalam keluarga *Siphoviridae* dan *Podoviridae*. Tambahan pula, faj ini menunjukkan kesan litik yang baik walaupun dalam keadaan yang mencabar, seperti pH tinggi, suhu ekstrem, dan kehadiran kloroform. Penemuan ini mengukuhkan lagi potensi bakteriofaj sebagai agen biokawalan yang khusus dan berkesan terhadap jangkitan MDR-KP, sekali gus membuka peluang untuk mengaplikasikan terapi faj dalam penjagaan Kesihatan.

**ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE AGAINST MDR *Klebsiella pneumoniae* FROM HOSPITAL SEWAGE**

## ABSTRACT

The prevalence of multidrug-resistant *Klebsiella pneumoniae* (MDR-KP) in healthcare settings has been increasing recently. Bacteriophages are a potential therapy against MDR bacteria and can be isolated from effluent water and other environmental sources. Additionally, bacteriophages are recognized as critical agents in the battle against MDR-KP infections. The high concentration of inorganic and organic compounds in hospital wastewater (HWW) provides a favourable environment for organisms, including phages, which supports the successful isolation of bacteriophages. Therefore, this study utilized wastewater samples collected from Hospital Pakar Universiti Sains Malaysia (HPUSM) and subjected them to a series of filtration and enrichment procedures to isolate phages that target MDR-KP. The isolated phages were subsequently characterized by their temperature stability, pH stability, chloroform stability, and host range. A high-resolution transmission electron microscope (HRTEM) was used to determine the morphology of the bacteriophages. The isolated phages exhibited a confined host range and showed high specificity for MDR-KP strains. Morphological analysis revealed that the phages belonged to the *Siphoviridae* and *Podoviridae* families. Furthermore, the phages demonstrated lytic activity under various undesirable conditions, including high pH, extreme temperature, and chloroform. These findings highlight the bacteriophages' potential as specific and effective biocontrol agents against MDR-KP infections, providing an opportunity for phage therapy in healthcare settings.

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

Bacteriophages, often known as phages, are the most prevalent viruses that infect bacteria worldwide. As they coexist with the microbes that serve as hosts, they are ubiquitous in the environment. In various environments, bacteriophages recovered from wastewater used for therapeutic purposes play a significant role in regulating bacterial populations because of their innate ability to target multidrug-resistant (MDR) bacteria (Naureen *et al.*, 2020) .

There is a tremendous variety of phages, all with simple structures, and they are effective at killing MDR bacteria. Bacteriophages attach to bacterial surface receptors and inject genetic material, infecting bacteria through either a lytic or lysogenic cycle. In a lytic infection, the replicating bacteriophage targets other bacteria and destroys their cells (Khorshidtalab *et al.,* 2022; Peng *et al.,* 2023). A lysogenic infection occurs when the DNA of a bacteriophage integrates into the bacterial genome and is transmitted to the next generation of bacteria. Under certain conditions, the DNA of the phage may excise from the bacterial chromosome, producing lytic phage particles (Soressa Bakala and Motuma, 2022).

In the past ten years, there has been a correlation between the alarming increase in MDR bacteria and a decline in the development of new antibacterial solutions. The challenges in treating numerous potentially fatal MDR bacterial infections have refocused scientific efforts on bacteriophages (Principi et al., 2019). Globally, MDR infections are caused by ESKAPE bacteria, which are recognized as a major contributor to MDR infections. These include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species. MDR bacteria have increased globally**,** resulting in significant economic and health consequences (Mancuso et al., 2021).

The World Health Organisation (WHO) estimates that by 2050, the worldwide cost of treating illnesses caused by bacteria resistant to multiple drugs will exceed USD 100 trillion and that the number of fatalities caused by these infections could reach 10 million, surpassing even deaths from cancer and heart disease combined (Alharbi and Ziadi, 2021). In 2017, the WHO published a list of "priority pathogens" that have developed resistance to antibiotics. The greatest threat comes from bacteria resistant to more than one antibiotic. This is especially true in healthcare facilities, nursing homes, and among patients who require invasive medical equipment like blood catheters and ventilators as part of their treatment. Some bacteria, including *Klebsiella pneumoniae,* develop beta-lactamases resistant to carbapenem, carbapenem-resistant *Enterobacterales* (CRE), and AmpC beta-lactamases (Lépesová *et al.*, 2020). In 2020, MDR was

listed by the WHO as one of the top thirteen global health concerns (Pires *et al.*, 2023).

Recently, bacteriophages have been regarded as a novel, advanced, and risk-free alternative to conventional treatments. With this approach, the dissemination of MDR bacteria could be controlled by utilizing bacteriophages. Phages isolated from hospital wastewater (HWW) have been shown to be effective against MDR bacterial infections (Soressa Bakala and Motuma, 2022).

This study aims to comprehensively isolate, characterize, and identify bacteriophages targeting MDR *Klebsiella pneumoniae* (MDR-KP) strains (CRE, ESBL, AmpC) from hospital sewage.

## 1.2 Problem statement & Study rationale

Multidrug-resistant (MDR) *Klebsiella pneumoniae*, particularly strains producing carbapenemases (CRE), extended-spectrum beta-lactamases (ESBL), and AmpC beta-lactamases, poses a significant public health threat. These pathogens are associated with high morbidity and mortality rates due to their resistance to most antibiotics, leaving treatment options limited and often ineffective (Sharma *et al.*, 2023a). The lack of viable therapeutic alternatives makes managing infections caused by MDR *K. pneumoniae* particularly challenging. Bacteriophages, viruses that infect and lyse bacteria offer a promising biotherapeutic approach to combat MDR *K. pneumoniae* infections (Loh *et al.*, 2021). However, there is a considerable knowledge gap regarding the efficacy and host specificity of bacteriophages that target MDR *K. pneumoniae.* (Hesse *et al.*, 2021).

This study aims to detect and characterize phages specific to MDR *K. pneumoniae* from hospital sewage, which could potentially enhance phage therapy for patients suffering from these infections. Bacteriophages are highly specific to their bacterial hosts, making their isolation critical for targeting and lysing antibiotic-resistant strains. Hospital sewage is an ideal source for isolating phages against MDR bacteria, as it frequently contains high concentrations of antibiotic-resistant microorganisms due to the extensive use of antibiotics in healthcare settings (Łusiak-Szelachowska *et al.*, 2022). The hospital wastewater frequently contains significant levels of antibiotic-resistant microorganisms. Making hospital sewage an excellent resource for investigating and isolating phages that attack MDR bacteria. By understanding the interaction between bacteriophages and MDR *K. pneumoniae*, this research aims to pave the way for developing effective phage-based therapies to treat these challenging infections (Samir *et al.*, 2022).

## 1.3 Objectives

### 1.3.1 General Objectives

To isolate and characterize bacteriophages against MDR -*Klebsiella pneumoniae* (CRE, ESBL, AMPC) strains from hospital sewage.

### 1.3.2 Specific Objective

1. To isolate bacteriophages against MDR -*Klebsiella pneumoniae* strains (CRE, ESBL, -AMPC) from HPUSM sewage.

2. To characterize bacteriophages against MDR - *Klebsiella pneumonia* strains (CRE, ESBL, AMPC) from HPUSM sewage.

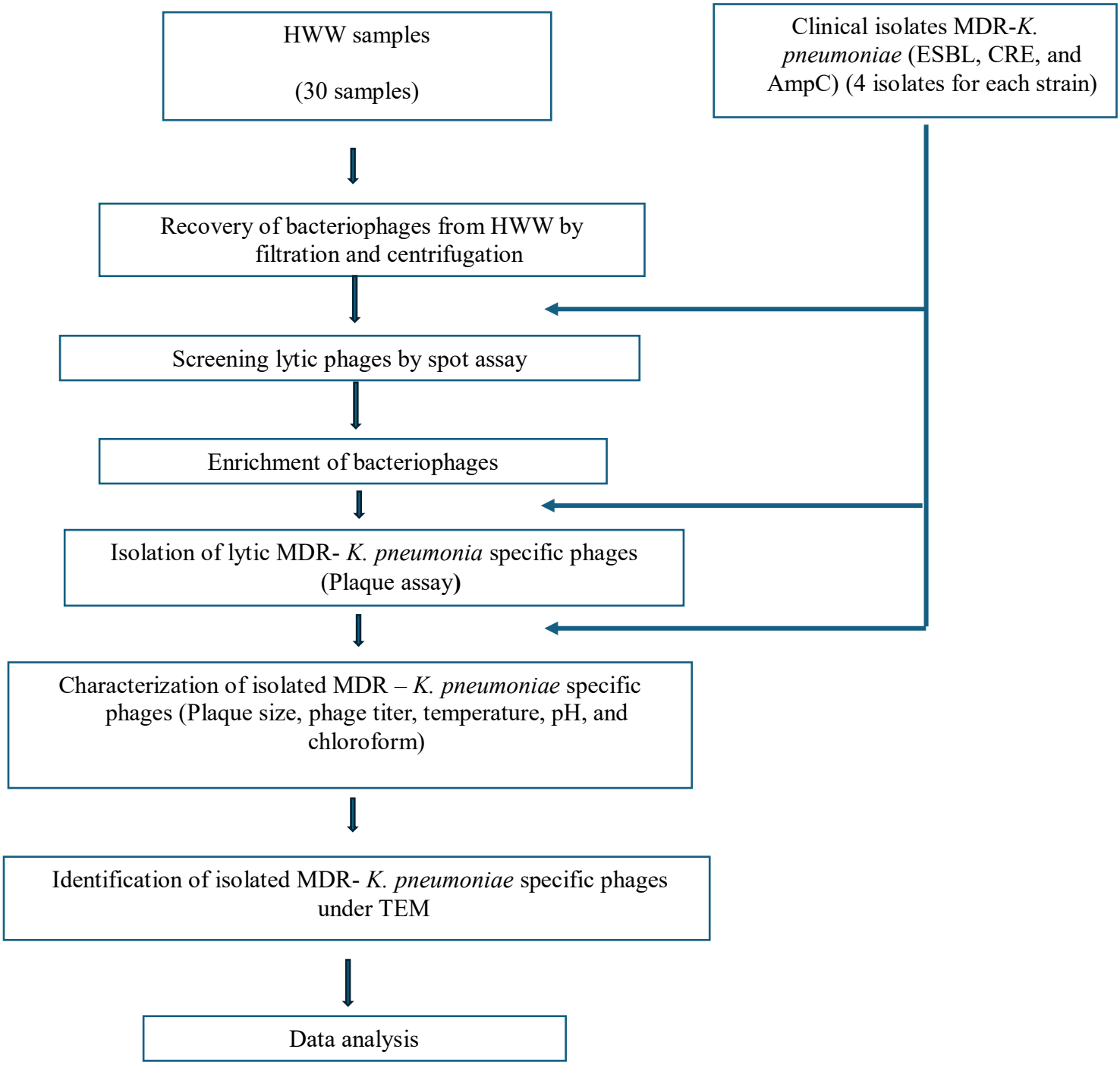
3. To identify the isolated bacteriophages against MDR - *Klebsiella pneumoniae* using a High – Resolution Transmission Electron Microscope (HRTEM).

## 1.4 Research hypothesis

1. Bacteriophages is abundance in HWW and Multidrug-Resistant (MDR) *Klebsiella pneumoniae* strains, specifically (ESBL, CRE, and AmpC) can be isolated using the clinical isolates.

2. The isolated bacteriophages will have specific characteristic when testing at different growth and environmental conditions such different pH, temperature and chloroform concentration.

### 1.5 The overview of the study



# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Viruses

The word for the virus in Greek, *ios*, comes from the verb *iimi*, which means "to move, to cause movement, to put something into something else, to throw the poison, the toxin, or the arrow" (Mammas *et al.*, 2020). Even though the phrase was chosen based on how poison was translated into ancient Greek, certain activities of this verb have unique qualities that define some aspects of viruses (Mammas *et al.*, 2020). The actual origin and emergence of viruses remain unknown due to the absence of historical evidence, such as fossils, which would provide clues. Viruses were initially identified following the development of a porcelain filter known as the Chamberland-Pasteur filter (Pilot *et al.*, 2023). This filter could eliminate all microscopic bacteria from any given liquid sample. Adolph Meyer demonstrated in 1886 that a tobacco plant disease known as tobacco mosaic disease could spread from a diseased plant to a healthy plant using liquid plant extracts. Dmitri Ivanovsky, a Russian botanist, demonstrated in 1892 that this disease could spread even after the Chamberland-Pasteur filter had removed all living microbes from the extract (Pilot *et al.*, 2023). Despite this, it took a considerable amount of time before it was established that the infectious agents referred to as "filterable" were not merely extremely small bacteria but rather a novel category of very minute particles that caused disease (Pilot *et al.*, 2023).

Modern viruses consist of a combination of nucleic acid fragments acquired from various sources during their evolutionary development. Viral particles, known as virions, are extremely small, measuring only 20–250 nanometers in diameter (Pilot *et al.*, 2023). Therefore, Light microscopy cannot reveal viruses, unlike bacteria (which are around 100 times bigger) (Figure 2.1).

Viruses are non-cellular, parasitic organisms that do not belong to any one kingdom (Pilot *et al.*, 2023). The structure of viruses is identical; they all include proteins, nucleic acids, and lipid membranous envelopes (Figure 2.2) (Fenner *et al.*, 1987). The most characteristic associated with viruses is their shape, which can be used to group them into a few different categories (Louten, 2016). There are four main categories for viral shapes: filamentous, isometric (or icosahedral), enveloped, and head and tail (Figure 2.3) (Pilot *et al.*, 2023). A helical or icosahedral structure characterizes most viruses.

Nevertheless, some viruses' complex structures deviate significantly from the more common helical or icosahedral forms. Complexly structured viruses include several bacteriophages, poxviruses, and Gemini viruses (Louten, 2016).

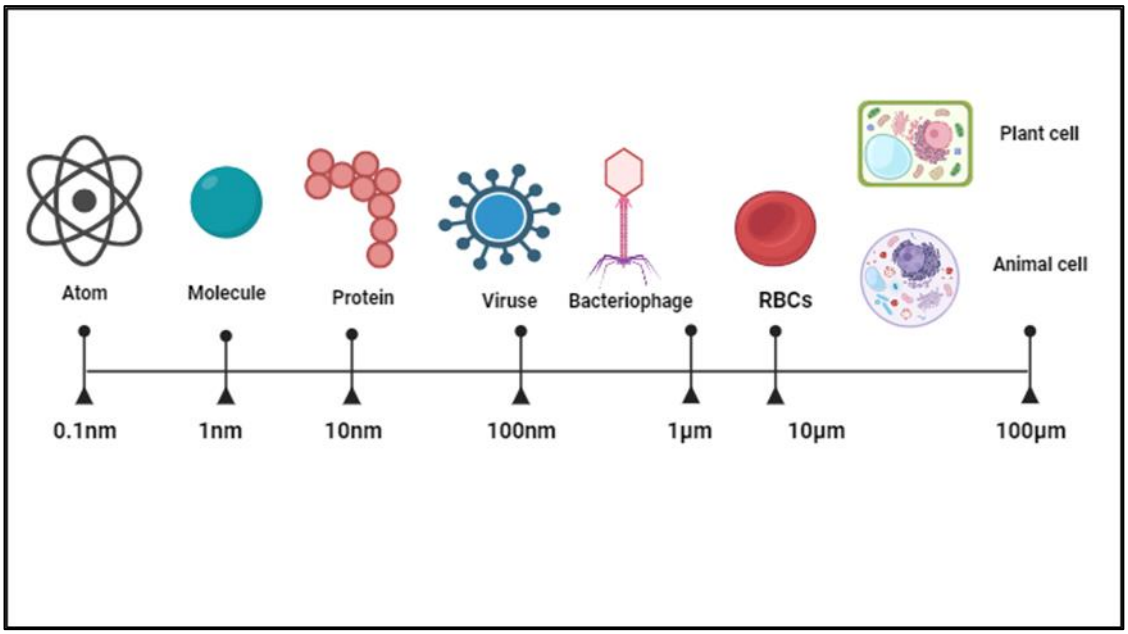



Figure 2.1: The Measurement units used to make comparison between viruses and other entities based on size.

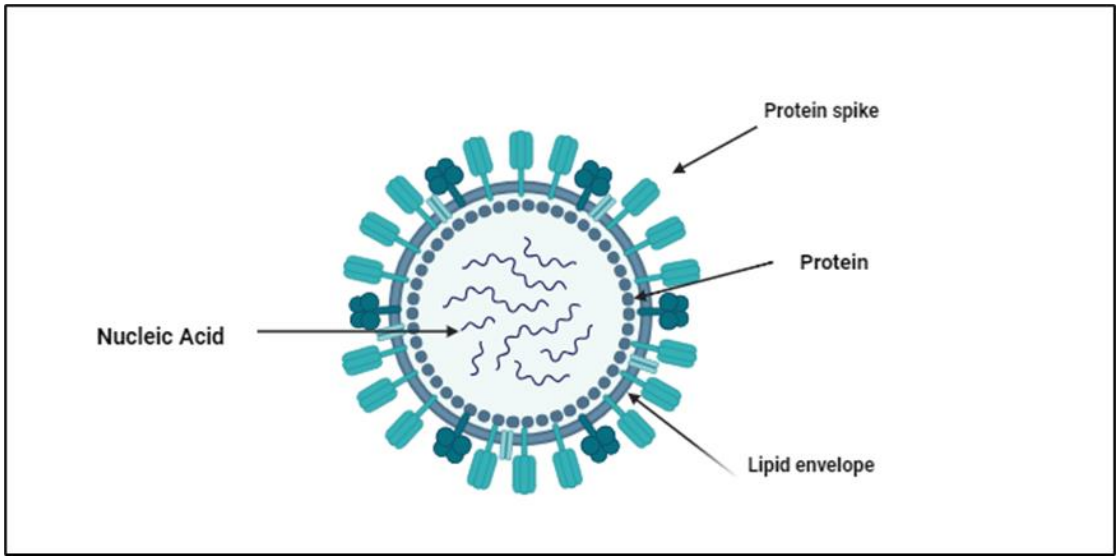



Figure 2.2: The basic structure of viruses includes the protein, which protects the nucleic acid genetic material, and the lipid envelope, which contains the protein spick for attachment to several host cells and provides additional protection.

Another method of categorizing viruses is determining the presence of an envelope (Louten, 2016). The lipid envelope of a virus originates from one of the cell's membranes; the plasma membrane is the most common source, but it can also originate from the endoplasmic reticulum, the Golgi complex, or the nuclear membrane, depending on the virus. Proteins known as matrix proteins help bind the viral envelope to the capsid within. A virus is considered non-enveloped or naked if it does not have an envelope (Louten, 2016).

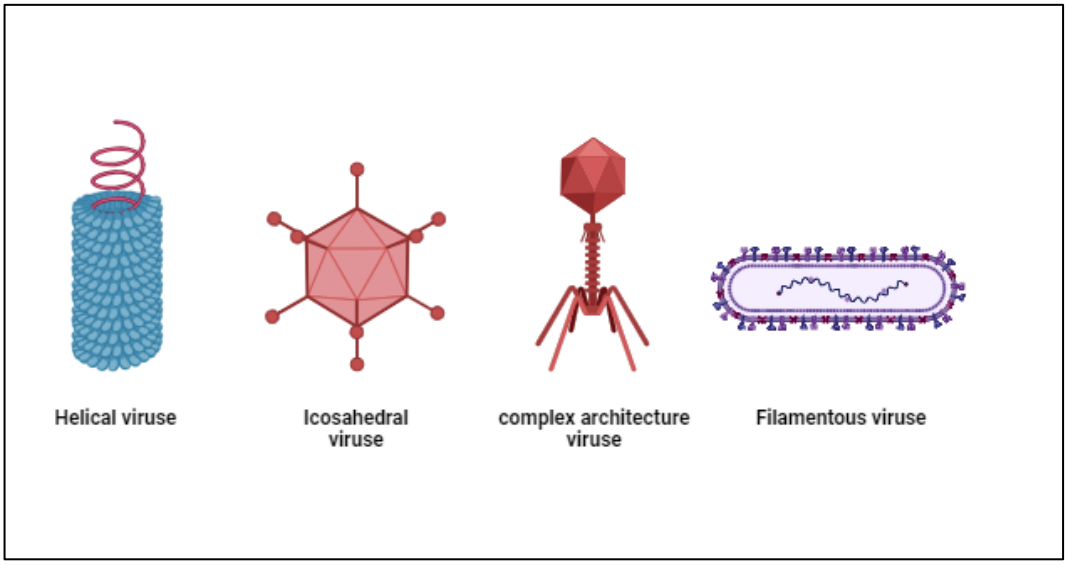



Figure 2.3: The difference between the four main orders of viruses is based on shape.

The replication mechanism is not in the traditional binary fashion seen in most organisms but rather in a quick flash of thousands of viral particles released by a single virus. Viruses can replicate in cell cultures or blood at a rate of tens of millions /ml (Taylor, 2014).

Viral uniqueness is based only on their reproduction method. Viruses are parasitic because they lack ribosomes, mitochondria, and other cellular organelles (Taylor, 2014). Additionally, they can infect a wide range of organisms, including bacteria, plants, and animals. They reside in an intermediate realm between a living thing and an inanimate entity (Pilot *et al.*, 2023). Living organisms demonstrate the phenomena of growth, metabolism, and reproduction. Viruses undergo replication, but they are entirely dependent on their host cells to carry out this process. Additionally, Viruses lack metabolic activity and cannot demonstrate growth in size; instead, viruses form in a fully mature state (Pilot *et al.*, 2023). The genetic substance of all other living things is DNA, and the messenger or building block for proteins and other structures is RNA. Another thing that makes viruses special is that their genetic material might be DNA or RNA (Taylor, 2014). Although both forms of nucleic acid are used by viruses during cell replication, no virus has been found so far that incorporates both types as genetic material (Taylor, 2014).

In virus classification, there are a lot of physical and chemical aspects of viruses that are considered, including the type of nucleic acid they contain and the amount of protein they encode. Modern DNA sequencing methods make it easy and fast to sequence viral genomes, which in turn lets scientists compare the nucleic acid sequences of different viruses to find their degree of relatedness (Louten, 2016). Additional features of virions are also considered, such as virion size, capsid shape, and the presence or absence of an envelope. The International Committee on Taxonomy of Viruses (ICTV) in 1966 uses several criteria to group viruses together based on their similarities and differences (Louten, 2016).

At present, there are seven orders of viruses recognized by the ICTV, with 103 families included within each order. Notable viruses, including retroviruses, papillomaviruses, and poxviruses, are among the 77 virus families that have not been placed in any order yet (Louten, 2016). Nobel laureate David Baltimore created one system of classification in the 1970s. The genome type and replication technique of a virus are the two main factors that the Baltimore classification system employs to group viruses into different classes (Louten, 2016).

## 2.2 Bacteriophage (phage)

“Phage like the Ninja” (Panosian Dunavan, 2020)

The term "bacteriophage" comes from the Greek words "phagein," meaning "to eat" or "destroy," and "bacterio," meaning "virus" that infects bacteria (Essa et al., 2020). Alexander Sulakvelidze calls them "the most ubiquitous organisms on Earth", they populate every conceivable habitat where bacteria flourish, including fresh water, sewage water, soil, and air. The number of phages in water systems is estimated to be between 104 and 108 virions /ml, while in the soil it is approximately 109 virions per gram, and globally, the estimated number of phages is 1031- 1032 (Essa et al., 2020). Sewage has the highest recorded number at 1010 (Aghaee et al., 2021). Phage populations can effectively treat bacterial infections and control the bacterial population in the environment and the human body (Ranveer et al., 2024).

Additionally, bacteriophages derived from wastewater and employed for therapeutic purposes. Numerous varieties of phages are characterized by their basic structure. Since bacteriophages are biological enemies of the bacteria that host them, they help humans defeat bacterial illnesses (Khorshidtalab *et al.,* 2022). Phage treatment does not harm beneficial microorganisms since phages are very specific, unlike antibiotics (Aghaee *et al.,* 2021). Phages can lyse bacteria, but at the same time, most of these phages have not been applied in vivo studies (Azam *et al.,* 2021).

As a result of the fact that the mechanisms of resistance to phages are distinct from those of antibiotics, phages have been extensively used in the treatment of multidrug-resistant bacteria (Shariati *et al.,* 2023). Additionally, phage-antibiotic combination therapy has the potential to resensitize bacteria that are resistant to antibiotics. Noteworthy is the fact that phages can damage the structure of biofilms and enhance the ability of antibiotics to penetrate deeper layers of biofilms. This happens by triggering the production of enzymes such as polysaccharide depolymerase (Shariati *et al.,* 2023). This enzyme can specifically degrade the macromolecule carbohydrates that are present in the envelope of the bacterial host. Additionally, it assists the phage in attaching itself to the bacterial cells, penetrating them, and lysing them (Shariati *et al.,* 2023). The inhibition of bacterial attachment, interference with quorum sensing, and degradation of the exopolysaccharide matrix are all additional ways in which phages have the potential to impede the formation of bacterial biofilm. As a result, phages cannot only eliminate bacteria but also eradicate the biofilm community that these

microbes inhabit (Shariati *et al.,* 2023). The phages have a wide range of size and shapes (Karczewska *et al.,* 2023).

The global classification is based on genetic type determination. Phage particles have a protective protein covering their genetic material and one kind of nucleic acid, which can be DNA or RNA (Moineau, 2013). Most phages also include a protein tail that allows them to specifically recognize a surface receptor on the host bacterium. In addition to their important responsibilities in maintaining microbial ecological balance, phages have recently been acknowledged as the most abundant microbes on Earth (Moineau, 2013).

### 2.2.1 The history of bacteriophage

In 1896, the first observation of a bacteriophage was reported. The British chemist Ernest H. Hankin was the first scientist to report the occurrence of antimicrobial activity in the Yamuna and Ganges rivers, which are in India (Essa *et al.,* 2020). He identified the Ganga and Yamuna rivers as a source of an unidentified chemical with great action against *Vibrio cholerae,* limiting the expansion of the cholera pandemic (Silva *et al.,* 2022). The scientific community, on the other hand, did not effectively research phages until thirty years had passed. In 1915, the first scientist, Frederick Twort, hypothesized that the clear zones he noticed in bacterial culture were caused by non-pathogenic viruses that were growing on bacteria (Essa *et al.,* 2020). Nevertheless, in 1917, the French-Canadian Félix d'Herelle is officially credited with the discovery of phages. He was the one who noticed the identical phenomenon of bacterial lysis and coined

the term "bacteriophages" (Essa *et al.*, 2020). Unlike Twort, who showed that lysis was triggered by an enzyme secreted by the bacteria itself, d'Herelle was completely certain that the phenomenon he observed was caused by a virus capable of parasitizing bacteria (Essa *et al.*, 2020). In recent years, phages capable of lysing pathogenic bacteria such as *Salmonella typhi*, *Escherichia coli*, *Pasteurella multocida*, *Vibrio cholerae*, *Yersinia pestis*, *Streptococcus* species, *Pseudomonas aeruginosa*, and *Neisseria meningitidis* have been isolated (Silva *et al.*, 2022). He had to wait until 1939, when the electron microscope, which had just been developed at the time, revealed the viral nature of the phage (Essa *et al.*, 2020). The Eliava Institute (EIBMV) was established in Georgia in 1923 by d'Herelle and Georgi Elliava. During World War II, many regions of the Soviet Union and Eastern Europe had limited access to antibiotics, leading to the development of phage therapy (Panosian Dunavan, 2020). Phage therapy was extensively supported in the Soviet Union and has been widely used in Russia and Eastern European countries for more than 80 years, particularly in Tbilisi, Georgia (Panosian Dunavan, 2020). A program to treat phage patients with suppurative infections was established at the Hirszfeld Institute in 1952. Controlled animal experiments were first published in the English scientific literature in the 1980s. Some Western European countries have begun to employ it for therapeutic purposes in recent years (Panosian Dunavan, 2020). In 2015, Dr. Steffanie Strathdee of UC San Diego's (UCSD) Associate Dean of Global Health Sciences played a significant role in expanding the field of phage therapy, which has seen an increase in interest in the United States due to rising concerns about MDR (Panosian Dunavan, 2020). In 2016, Paul Turner and colleagues isolated a phage that could restore antibiotic susceptibility in MDR *P. aeruginosa* (Silva *et*

*al.*, 2022). This phage was later used to treat a patient with a long-standing aortic graft infection who had not responded to repeated surgical operations and rigorous antibiotic therapy with a single application of phage (Silva *et al.*, 2022).

In recent years, renewed interest in phage therapy for the treatment of MDR organisms has resulted in outstanding breakthroughs. Several recent studies have highlighted the advancements in phage therapy (Carascal *et al.*, 2022).

### 2.2.2 Bacteriophage life- cycle

The life cycle of a phage includes the lytic cycle, a lysogenic cycle, pseudolysogenic cycle, and a chronic cycle (Zhang *et al.*, 2022), depending on the specific phage and the physiological condition of the bacteria. If the phage is virulent, it triggers the lytic cycle, resulting in cell lysis (Silva *et al.*, 2022). Temperate phages possess genes that control two distinct cycles, and the occurrence of a specific cycle can be controlled through various factors (Silva *et al.*, 2022). During the lytic cycle, the phage initiates the formation of new viral offspring promptly following infection and releases them, causing the host cell to undergo lysis (Zhang *et al.*, 2022). During the lysogenic cycle, the genetic material of the phage, called a prophage, replicates alongside the host DNA. This can happen by integrating into the host's chromosome or plasmid (Zhang *et al.*, 2022).

Prophages transition from the lysogenic state to the lytic cycle, which occurs when the prophage is exposed to high-stress conditions such as UV, starvation, or chemicals, as shown in Figure 2.4 (Zhang *et al.*, 2022). Pseudolysogeny occurs when the host cell is under stress conditions, like starvation, but transitions into the lysogenic or lytic cycles as soon as the condition improves (Zhang *et al.*, 2022).

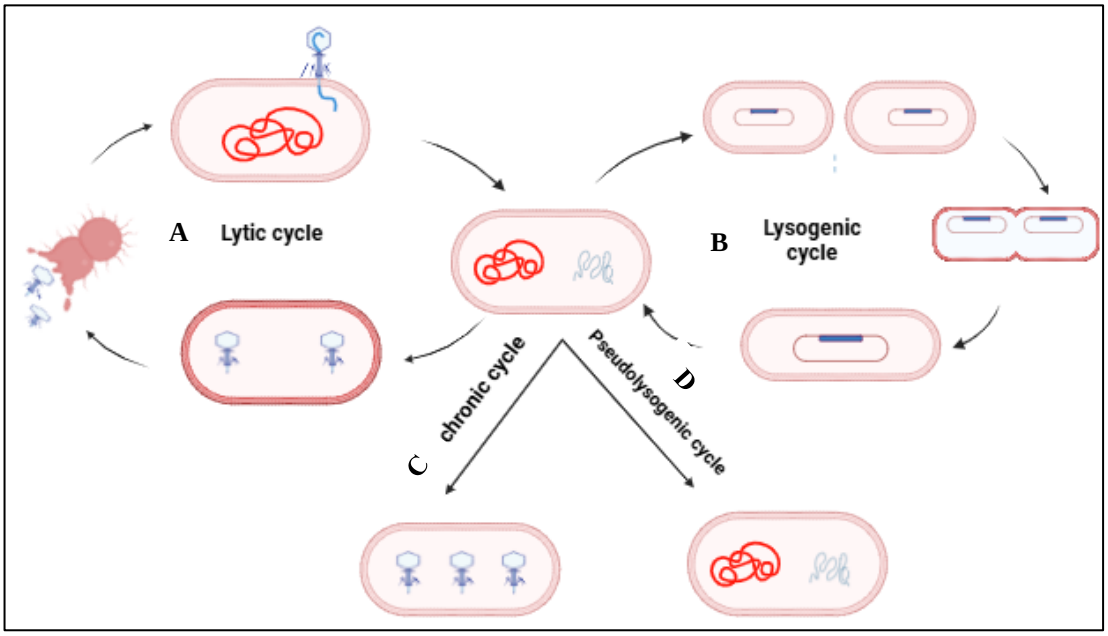



Figure 2.4: The diagram demonstrates the life cycle of bacteriophages: (A) Lytic cycle, (B) Lysogenic cycle, (C) Chronic cycle, (D) Pseudolysogenic cycle.

Phage pseudolysogeny is a non-classical life cycle in which the phages do not lyse the host or integrate into the genome to form a long-term, stable relationship (Zhang *et al.*, 2022). In 1971, Baess and colleagues discovered pseudolysogeny, which was initially defined as an unproductive, unstable contact that develops into pathogenic growth upon further investigation. Using starving, slowly developing cells, Los (2003) showed that the T4 phage of *E. coli* can produce pseudolysogens (Los *et al.*, 2003). Additionally, in the chronic cycle, phages continue multiplying in the host and leave the cell through budding instead of lysis, which protects the host and causes phage production to remain constant (Chung *et al.*, 2023).

The phage's ability to proliferate in the host after reaching the attachment stage is dependent on the genetic composition and regulatory mechanisms of the phage (Chung *et al.*, 2023). Furthermore, the presence of various proteins and the adaptations of host receptor-binding proteins (RBPs) may significantly impact host range regulation (Chung *et al.*, 2023).

### 2.2.3 Bacteriophage morphology

Many bacteriophages have a helical symmetry protein tail connected to an icosahedral head. Encasing the nucleic acid, the capsid is a complex structure of

repetitive structural protein subunits (Silva *et al.*, 2022 It forms the head of the phage. The capsid protects the nucleic acid, which also contains proteins that make the phages specific to certain bacteria. The hetero-oligomeric tail, formed by several proteins, ensures genome release when the virion is attached to the host cell. The neck links the head to the tail (Silva *et al.*, 2022). Numerous phages possess supplementary morphological characteristics, such as tails and spikes; certain ones may even contain lipids. Phages exhibit significant variation in the nature and features of nucleic acid, the structure and content of viral particles, and their size. The International Committee on Virus Taxonomy classified phages into 11 families (Jofre and Muniesa, 2014). The characteristics of bacteriophages from the families commonly found in sewage water, soil, and foods are listed. The structure of phages can be as simple as that of *Leviviridae*, which has a single RNA molecule and an accompanying RNA polymerase, both enclosed within an icosahedral capsid (Jofre and Muniesa, 2014). Phage morphology can exhibit complexity, such as *Myoviridae*, which includes a head and a double-stranded DNA molecule (Jofre and Muniesa, 2014). This DNA molecule is attached to a collar connected to a contractile tail. A base plate with pins and fibers can be found at the end of the tail. Bacteriophages possessing a tail are commonly observed and reported (Jofre and Muniesa, 2014).

Of all the phages that have been described, the *Siphoviridae* make up 50%. The sizes of phages vary, with the *Leviviridae* measuring 20 nm and the elongated head of the *Myoviridae* measuring 110 × 20 nm, while the tail of the *Myoviridae* can exceed 100 nm as shown in Figure2.5 (Jofre and Muniesa, 2014).

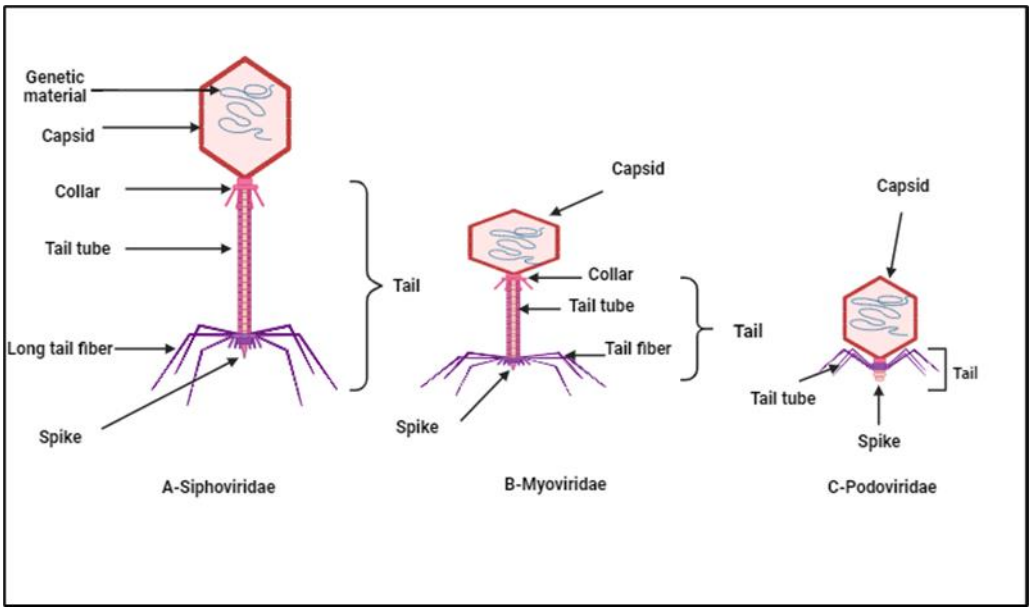



Figure 2.5: Main bacteriophage types: (A) *Siphoviridae* have a long, noncontractile tail; (B) *Myoviridea* have a long, contractile tail; and (C) *Podoviridae* have a short, noncontractile tail. All types have an icosahedral head, collar, and spike.

### 2.2.4 Bacteriophage classification

The main criteria used to classify bacteriophages are the type of nucleic acid they contain (genetic information), the shape of their capsids (particularly whether they have a tail), and whether they have an envelope. Phage DNA or RNA can be single-stranded (ss) or double-stranded (ds) (Sausset *et al.*, 2020). Phage genomes range in size from around 3.5 kb to approximately 540 kb. Phage diversity is significant, although non-enveloped-tailed dsDNA phages, or *Caudovirales*, account for 95% of all phages. The traditional classification of this group is into the *Siphoviridae*, *Myoviridae*, and *Podoviridae* families (Sausset *et al.*, 2020). Phages can be classified according to their morphological characteristics, which include tails (found in 96% of phages), polyhedral, filamentous, or pleomorphic structures, and some of them include lipid or

lipoprotein envelopes (Essa *et al.*, 2020). Most phages that have been characterized are classified under the *Caudovirales* order, which is characterized by a tailed morphology. This order is divided into three families: *Myoviridae*, which have a contractile tail (for instance, phage T4); *Siphoviridae*, which have a non-contractile tail (for instance, phage λ); and *Podoviridae*, which have a very short tail (for instance, phage T7) (Table 2.1) (Essa *et al.*, 2020). ICTV has recently updated the phage classification system, which launched in August 2022 (Zhu *et al.*, 2023). The several significant families previously included in the ICTV system have been deleted (Zhu *et al.*, 2023). These families include *Siphoviridae*, *Podoviridae*, and *Myoviridae*, whereas the new families that were updated are *Autographiviridae*, *Straboviridae*, *Herelleviridae*, and *Drexlerviridae*.

A recent study conducted by Zhu (2022) focused on the higher average similarity, which indicates that the updated families are more preserved, which increases the feasibility of family-level classification (Zhu *et al.*, 2022).

Table 2.1: The phages classification based on nucleic acid and morphology.

| Family | Nucleic acid | Morphology |
|---|---|---|
| *Siphoviridae* | Linear dsDNA | Long non-contractile tail, non-enveloped |
| *Podoviridae* | Linear dsDNA | Short non-contractile tail, non-enveloped |
| *Myoviridae* | Linear dsDNA | contractile tail, non-enveloped |
| *Tectiviridae* | Linear dsDNA | Isometric, non-enveloped |
| *Corticoviridae* | Circular dsDNA | Isometric, non-enveloped |
| *Lipothrixviridae* | Linear dsDNA | rod-shaped, enveloped |
| *Rudiviridae* | Linear dsDNA | Rod-shaped, non- enveloped |
| *Leviviridae* | Linear ssRNA | Isometric, non-enveloped |
| *Inovirida* | single-stranded (ss)DNA | rod-shaped or filamentous, nonenveloped |

### 2.2.5 Bacteriophage distribution

The different environments are habitats for a wide range of bacteriophages, each of which appears in a unique form. Regarding both temporal and spatial distribution, the spread of bacteria and phages is contingent upon the limits of their respective ranges as well as the areas where their ranges overlap (Naureen *et al.*, 2020). Phages are found in all areas wherever their hosts exist, including hypersaline habitats, polar regions, deserts, and within animals other than bacteria, freshwater, seawater, sewage water, and soil. It is well known that bacteria may be found practically anywhere and in any environment. Phages, on the other hand, can be found in all locations (Naureen *et al.*, 2020). Phages are

also present on the surfaces of the body, such as the skin, oral cavity, lungs, intestines, and urinary tract (Batinovic *et al.*, 2019). They are a natural predator of the extensive microbiome that exists within the body, surpassing bacteria, and they play significant roles in determining the composition of the bacterial communities that are found within different parts of the body (Batinovic *et al.*, 2019). Furthermore, it has been established that phages can penetrate the epithelial lining of these structures by a process known as fast-directional transcytosis. This allows them to gain access to the cytosolic and vesicular compartments of eukaryotic cells (Batinovic *et al.*, 2019).

Every day, it is estimated that 31 billion bacteriophage particles enter the human body through the process of transcytosis, which involves passing through the epithelial cells of the stomach (Batinovic *et al.*, 2019). Based on the information recorded in the Gut Phage Database, it has been shown that the human gut contains over 142,000 non-redundant viral genomes, most of which are phages (Ballesté *et al.*, 2022). Bacteriophages are as abundant as bacteria in the raw sewage that passes through sewage systems, which is the primary habitat of a complex microbial community whose primary source is the human gut (Ballesté et al., 2022).

### 2.2.6 Bacteriophage therapy

In 1919, Felix d'Hérelle made the first clinical trial of bacteriophages; in 1922, the first recorded usage in the US occurred (Aswani and Shukla, 2021). He focused on harnessing the ability of phages to specifically target harmful bacteria and ensuring their safety for human host cells. D'Hérelle established the

Bacteriophage Laboratory in France and initiated the manufacturing of the initial commercially available phage mixtures, eventually leading to the establishment of the renowned French firm L'Oréal (Essa *et al.*, 2020).

Simultaneously, bacteriophages were employed for therapeutic applications in the United States. Following the 1940 discovery of penicillin, Western European countries and North America dropped phage therapy and started the era of antibiotics (Essa *et al.*, 2020). However, phages have continued to be used for therapeutic purposes in Eastern Europe and the former Soviet Union countries, including Poland and Georgia. Various nations have created distinct organizations focused on the research and manufacturing of medicinal bacteriophages (Essa *et al.*, 2020). The Eliava Institute of Bacteriophages, Microbiology, and Virology (EIBMV) is in Tbilisi, Georgia, and the Institute Hirszfeld of Immunology and Experimental Therapy (HIIET) is in Poland (Essa *et al.*, 2020).

In the previous study, Merril (2003) reported that the utilization of phage, as shown in the Soviet Union and Poland, had undergone thorough evaluation. According to a review paper from 1998, only 27 studies on bacteriophage therapy were published between 1966 and 1996 (Merril *et al.*, 2003). The issue of antibiotic resistance, which has become an important issue in the 21st century, has led to a renewed interest in phage therapy in the Western world (Aswani and Shukla, 2021). In 2012, the worldwide distribution of MDR- bacteria inspired the World Health organization (WHO) to issue a global emergency, alerting about the possible beginning of an era in which antibiotics would become ineffective against bacterial diseases (Aswani and Shukla, 2021). In 2017, the World Health