# Evaluation of Phage-Antibiotic synergy against *Klebsiella pneumonaie* and encapsulation of phages to improve their stability under gastronomic conditions.


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## DEDICATION

I dedicate the outcome of my efforts to:

- My father is an inspiration to me, and his advice and motivation kept me going during my studies.
- My supervisor and co-supervisor, whose guidance laid the way for an incredible research journey.

**Declaration**

I, Muqaddas Shahzadi, declare that the material and information in this thesis are my original work. I have not previously presented any part of this work elsewhere to any other degree.

**Muqaddas Shahzadi**

**Certificate:**

This thesis is submitted by **Muqaddas Shahzadi** and accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, and Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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**Evaluation of Phage-Antibiotic synergy against *Klebsiella pneumonaie* and encapsulation of phages to improve their stability under gastronomic conditions.**

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**List of tables:**

**List of abbreviations:**

| Abbreviations | Full form |
|---|---|
| AMR | Antimicrobial Resistance |
| CDC | Centers for Disease Control |
| ESKAPE | *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. |
| FDA | Food and Drug Administration |
| h | Hours |
| LBA | Luria Bertani agar |
| MDR | Multi Drug Resistant |
| mg | Milligram |
| MHA | Mueller Hinton agar |
| MIC | minimum inhibitory concentrations |
| min | Minutes |
| ml | Milli liter |
| NA | Nutrient agar |
| PFU/ml | Plaque forming units per milliliters |
| TSA | Tryptic Soya agar |
| WHO | World Health Organization |

| | |
|---|---|
| CLSI | Clinical Laboratory Standard Institute |
| PG | Peptidoglycan |
| EPS | Extracellular Polymeric Substance |
| PAS | Phage-Antibiotic Synergy |
| eIND | Emergency Investigational New Drug |
| SCWP | Secondary Cell Wall Polymer |
| TTPA | Tail Tubular Protein A |
| CPS | Capsular Polysaccharide |

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**ABSTRACT:**

The rise of multidrug-resistant *Klebsiella pneumoniae* in recent years has heightened global public health considerations. The capacity of this disease to produce hypervirulent strains with enhanced virulence features complicates control. *Klebsiella pneumoniae*, a member of the *Klebsiella* genus, causes a variety of infections, including respiratory, urinary tract, and septicemia, primarily affecting susceptible people in both community and hospital environments. *Klebsiella pneumoniae*, an ESKAPE pathogen known for producing nosocomial infections, uses virulence features such as capsules, fimbriae, lipopolysaccharides, adhesins, and iron absorption pathways for pathogenesis. However, the bacterium's increasing antibiotic resistance jeopardizes treatment efficacy, allowing it to bypass the immune system and cause serious infections. The investigation of *Klebsiella*-specific bacteriophages was prompted by the urgent need to combat the rise of virulent and antibiotic-resistant *K. pneumoniae* strains. These natural viruses are possible bacterial growth inhibitors and antibacterial sources against *Klebsiella* infections. Antibiotics, previously the gold standard for treating bacterial illnesses, are becoming less effective due to the prevalence of antibiotic-resistant bacteria. Antibiotic misuse and overuse have promoted resistance. Innovative treatments for illnesses caused by multi-drug resistance bacteria are desperately needed to combat this. Phage therapy has emerged as an attractive option for therapy for such illnesses. Studies on phage-antibiotic synergy against multidrug-resistant bacterial pathogens are gaining increasing attention worldwide. In this study, two previously isolated and characterized bacteriophages KPA and KPM, and their cocktail were used to evaluate their synergistic effect with subinhibitory concentrations of cefepime, gentamicin, and meropenem against multidrug-resistant, biofilm-forming, uropathogenic *Klebsiella pneumoniae* isolated from urine samples from catheterized patients. In this study, a significant reduction in host bacterial counts was observed when treated with KPA, KPM, and their cocktail with the subinhibitory concentrations of cefepime and meropenem, and no synergistic effect of phages was observed in host bacterial eradication when used with gentamicin,

but a cocktail of both phages worked synergistically with gentamicin. Cefepime at a concentration of 8 µg and 4 µg with KPA at MOI 0.1 and a cocktail of KPA and KPM caused a 100% reduction in the host bacterium and meropenem at a concentration of 1µg, and 0.5µg with KPA, KPM and their cocktail caused a 100% reduction in the host bacterium and the cocktail of KPA and KPM at 0.25 µg Meropenem killed 100% of the host bacterial cells. All antibiotics tested with KPA, KPM, and their cocktail inhibited biofilm formation by host bacteria, but the biofilm inhibition potential of both phage and their cocktail was higher with meropenem, followed by cefepime and gentamicin. A cocktail of KPA and KPM completely destroyed the biofilm formed on glass slides, followed by KPA and KPM. Throughout the study, the antibacterial and antibiofilm activity of the phage cocktail was higher, followed by KPA and KPM. Controlling gastrointestinal flora is an effective technique for treating illnesses caused by microbiome abnormalities. While traditional probiotics have been used in the past, recent attention has shifted to phage delivery for changing gut bacteria and gene regulation. However, phage proteins might be damaged during storage and delivery, reducing their effectiveness. This research focuses on improvements in phage encapsulation and distribution investigation, emphasizing carrier systems' protective capacities and focused colon delivery. Encapsulation techniques enhance phage stability and targeted delivery within the body. These strategies could reshape phage therapy and contribute to fighting antibiotic-resistant infections.

This research delves into the resilience of phage KPA and KPM within simulated gastrointestinal conditions. It uncovers that the encapsulation of these phages utilizing polymers such as alginate + agarose is a successful approach to safeguard them from the harsh acidic surroundings of the stomach. This encapsulation strategy ensures phage survival by mitigating the impact of low pH settings, a critical factor for their successful oral administration. The study demonstrates remarkable loading efficiencies of 99.3% and 99.6% of the phages KPA and KPM respectively, within matrices like alginate + agarose indicating the effectiveness of encapsulation.

Furthermore, the research underlines the significance of gastric emptying times during the phages' transit through the stomach. It suggests that the encapsulated phages' fate is intricately tied to factors such as formulation and particle size when introduced alongside a solid meal. The findings emphasize that the encapsulation process not only preserves phage viability in extreme gastric conditions but also allows for controlled release in the intestine's more favorable pH environment. In our study, the maximum phage entrapment was reported in the case of alginate + agarose at pH 2, followed by the remaining pH values, whereas maximum KPA and KPM phage releases have been observed in case of alginate + gelatin because at the intestinal pH of 7.4 alginate + gelatin capsules swell and release the encapsulated phages in a pH-dependent manner. However, the stability and release of phages in the simulated gastric fluid (pepsin) and simulated intestinal fluid (pancreatin) has also been determined. Maximum phage entrapment in the case of pepsin has been observed in alginate + agarose while no release has been reported in the case of pepsin that indicates the successful encapsulation of phages because the free phages are highly sensitive to extreme gastric conditions of the stomach, however, the highest phage entrapment in case of pancreatin has been observed in case of alginate + agarose and maximum release has been reported in case of alginate + gelatin. This work contributes to the advancement of microencapsulation techniques for phage-based therapies and highlights the potential of using encapsulated phages to navigate the challenges of oral delivery and targeted release within the gastrointestinal tract

# CHAPTER 1: INTRODUCTION

## 1. Introduction

In the past few years, multi-drug resistant *Klebsiella pneumoniae* has emerged as a major worldwide public health concern. (Hayder et al., 2019). It is one of the most challenging species to deal with because it creates hypervirulent mutants with expanded virulence determinants. The *Klebsiella* genus causes respiratory infections, urinary tract infections, and septicemia, especially among susceptible individuals, and frequently causes subsequent infections in ventilated or catheterized patients in community and hospital-based environments. (Townsend et al., 2021). *Klebsiella pneumoniae* is the "K" among the ESKAPE pathogens that are significantly responsible for nosocomial infections. The major virulence factors in its pathogenesis are the capsule, fimbriae, lipopolysaccharides, adhesins, and iron absorption mechanisms of *Klebsiella pneumoniae* (Lee et al., 2017). Because of the high level of resistance to once-effective treatments, this bacterium may evade the immune system, colonize, invade, and infect the human body. The development of hypervirulent pathotypes, their ability to escape the immune system, and their rising antibiotic resistance have all become significant challenges in the medical community. Multiple-drug-resistant *Klebsiella pneumoniae* has become a significant global public health issue in current years. One of the most prevalent human pathogens has a high level of resistance to several once-useful medications. This opportunistic bacterial infection, *Klebsiella pneumoniae* is well-known for having a huge variety and frequency of antimicrobial resistance (AMR) genes. The term bacterial resistance is referred to as the ability of microorganisms to withstand the bactericidal or bacteriostatic effect of antibiotics (Hasan et al., 2020). Bacteria grew resistant over time because of the widespread and inadvertent uptake of antibiotics, and challenges with these resistant germs have arisen for the treatment of several ailments (Hayder et al., 2019). Conventional approaches for discovering new antibiotics are insufficient, however, different approaches have been used to combat antibiotic resistance such as antibiotics cocktails or their mixture with other compounds, such as antimicrobial peptides, offering interesting alternatives, but

may eventually suffer from some of the same problems as single molecules (Worthington et al., 2013). The widespread dissemination of exceptionally pathogenic and antimicrobial-resistant *K. pneumoniae* strains in healthcare facilities and natural settings necessitates a better understanding of *Klebsiella*-specific bacteriophages as natural competitors of these bacteria as well as a potent source of antimicrobials for combating *Klebsiella* infections. (Maciejewska et al., 2017). We are still looking for the "silver bullet" that will limit the emergence of bacterial resistance. Phage therapy provides a potential technique for releasing harmful bacteria without diminishing other microbiome species, hence preventing dysbiosis. However, their great specificity complicates the creation of a curative approach since quick bacterial genotyping (which is not always possible) is required to attack a wide range of bacteria. (Domingo-Calap et al., 2020). By contrast, bacteriophages offer incredible potential as an antibiotic alternative not just owing to inherent variations in their methods of action, but also due to the practically endless diversity of phages, their capacity to swiftly "trained", and their propensity to develop *in situ* to circumvent the resistance mechanisms (Betts et al., 2013). Phage therapy distinguishes itself by allowing for low-dose phage treatments, host-specificity, less disturbance of natural flora, low intrinsic toxicity, and a lower likelihood for resistance induction (Luong et al., 2020). Bacteriophage and bacteriophage-encoded endolysin have been evaluated as possibilities for controlling infections caused by resistant pathogens due to their unique ability to kill the bacterial pathogens in a relatively brief disintegration time. Phages are promising weapons for battling microbial resistance. Bacteriophage injects its genome into a bacterial cell, changing the organism's genetic code in its favor so that the host cell eventually dies and releases roughly 100 phage particles. This process appears to be quite straightforward but is actually very effective. Phage treatment has several benefits over antibiotics, including a more precise killing spectrum, interaction with bacteria to minimize the establishment of phage resistance, and a greater richness and variety of phage components than antibiotics.

Meanwhile, developing a phage mixture from phage isolates with distinct genomic origins may help to reduce the emergence of phage resistance during practical use. Several bacterial illnesses, including those caused by multidrug-resistant microorganisms that are ineffective when treated with therapeutic agents, have been recuperated by phages, highlighting the benefits of phage treatment. Several phages have been identified to combat *K. pneumoniae* infections due to their strong drug resistance. (Peng et al., 2020). Antibiotics are therapeutic drugs that have been used to treat bacterial illnesses clinically from the 1940s. However, the advantages provided by these golden weapons have been drastically reduced in the days since the widespread advancement and proliferation of antibiotic-resistant bacteria. Non-antibiotic alternative therapies are desperately required to tackle the growing frequency of illnesses caused by multidrug-resistant bacteria. One of the causes of antibiotic resistance in bacteria is diagnostic incompetence and inappropriate prescribing of antibiotics, as well as imprudent usage. (Sengupta et al., 2013). Every year, tens of thousands of people die because of antibiotic-resistant illnesses. Due to a shortage of therapeutic choices, phage therapy has resurfaced as a possible therapeutic approach for treating MDR (multidrug-resistant) infections, with promising therapeutic benefits. (Pires et al., 2020). A combination of antibiotics and phages is a potential technique for lowering antibiotic doses and preventing antibiotic resistance during therapy. Combinations of antibiotics and phages have demonstrated Phage-Antibiotic Synergy (PAS) approach, wherein drugs enhance phage proliferation and remarkable antibacterial efficiency with decreased bacterial resistance emergence. PAS (Phage-antibiotic synergy) refers to the phenomenon in which phage progeny increases in the presence of a sublethal concentration of certain antibiotics. When phages infect bacteria cultivated in the presence of sublethal antibiotic dosages, the diameters of the phage plaques rise considerably. (Kim et al., 2018). It has been demonstrated that phage-antibiotic treatment may minimize the development of phage and antibiotics-resistant strains. One frequently cited instance is the fact that phage-caused cell surface receptor degeneration

reinstates antibiotic sensitivity, as these receptors are important for antibiotic efflux (Chan et al., 2016). Biofilms are microorganism communities that adhere to both biotic and abiotic substrates. It may play a role in the aetiology of debilitating diseases, including infections caused using drains, catheters, and implants insertion. Bacterial cells are encased in an extracellular matrix of polymeric substances EPS (lipids, proteins, polysaccharides, and nucleic acids). Antibiotic resistance is a feature of these structures (Akanda et al., 2018). Antibiotic resistance in biofilms may be caused by increased proliferation of cells and physiological modifications. Additionally, biofilm exhibits decreased metabolic activity, slower development, and a better possibility for antibiotic resistance gene exchange, which is also linked to antibiotic resistance stimulation. Because antibiotic therapy for biofilms is unsuccessful, there is increased curiosity regarding phages as a tool for avoiding biofilm development and eradication. Some investigations on the use of phages as a different approach for preventing and controlling biofilm appear promising. Conversely, in a lack of oxygen and nutrients, cells in the denser layers of biofilm exhibit reduced metabolic activity, which inhibits antibiotic action and phage replication. Phage resistance is also acquired within biofilm. The following mechanisms are known for acquiring phage resistance in bacteria: inhibiting phage DNA integration into bacterial DNA; deterioration of phage DNA by **(clustered regularly interspaces short palindromic repeats)** CRISPR/Cas; restricting phage process of replication, transcription, and translation; and shielding of phage attachment by alterations to the structure of bacterial receptors or limitations of binding receptors molecules due to the biofilm network accumulation (Pires et al., 2017). The phages can be used individually or as part of a phage mixtures. Phage combinations broaden the range of phage action while decreasing the emergence of phage-resistant mutants. Phage mixtures have been shown to be effective in avoiding the development of biofilm and destroying biofilm. To boost the effectiveness of biofilm eradication, phages are able to be combined with other antimicrobial substances such as antibiotics (Abedon et al., 2017) Bacteriophages,

because of their capacity to infiltrate biofilms, can be used in conjunction with or as a alternative for antibiotics in biofilm eradication. Depolymerases (phage-encoded matrix-degrading enzymes) can also be utilized to avoid and disseminate biofilm (Phage-derived lysins are bacteriophage enzymes that degrade peptidoglycan, the primary structural element of Gram-positive and Gram-negative bacteria's cell walls, causing bacterial cell death. According to recent findings, the use of phages and phage-derived endolysins in the prevention and removal of biofilm-associated illnesses is a viable treatment strategy. It is crucial to highlight phage cocktails rather than single phages as a positive factor in boosting biofilm killing and reducing the generation of phage-resistant bacteria, especially in biofilm-associated urinary tract infections (UTIs). (Gray et al., 2018). Antibiofilm properties are improved when phages are combined with antibiotics. When coupled with phage, several antibiotics were more potent at smaller doses. Controlling gastrointestinal flora is an effective technique for preventing and treating illnesses caused by microbiome imbalance. Aside from typical probiotic medicines, phage administration has recently shown significant potential in regulating gut microflora mixture and impacting gut bacteria gene regulation. The phage protein structure, on the other hand, is vulnerable to surrounding stressors during preservation and the delivery process, leading to a reduction of pathogenicity potential and flora competence in management. The procedure of confining or packaging phages, which are small viruses that invade and proliferate within the bacterial host, into a protective outer covering or casing is referred to as phage encapsulation. Encapsulation can be accomplished by employing a variety of procedures and polymers to improve phage stabilization, preservation, and administration. Encapsulation is an excellent method for improving phage stability while carefully redesigning the delivery dosage. To shield phages from extreme circumstances and free them in the colon, encapsulation carrier’s molecules comprised of diverse functional materials, especially enzymes-responsive and pH-responsive polymer substances, were employed. Simultaneously, different types of carriers revealed

varied structural and functional features that influenced their degree. Here are a few common ways for phage encapsulation: Liposomes encapsulation, microencapsulation using various polymeric substances such as sodium alginate, gelatin, agarose, polyethylene glycol (PEG), chitosan, nanoparticles, and various capsids, or recombinant protein-based encapsulation. They shield phages from adverse conditions in the environment including pH fluctuations and enzymatic breakdown, allowing for regulated release and targeted delivery to specific areas within the body. We will discuss structural analyses, the physio-chemical requirements for bacteriophage active replication, and the phage's stability and activity in human blood. The synergistic effect of phages with antibiotics on turbidity reduction, biofilm reduction, and inhibition will be discussed. Furthermore, to improve phage stability under gastronomic settings, we will investigate the stability of free and encapsulated phages in various polymeric substances, simulated intestinal fluid, simulated gastric fluid, and at various pH of the GIT tract to use them as therapeutic alternatives.

**Aims and Objectives:**

**Aims:**

Evaluation of Phage-Antibiotic Synergy against *Klebsiella pneumoanie* and encapsulation of phages to improve their stability under gastronomic conditions.

**Objectives:**

- Isolation and characterization of the host bacterium and antibiotic resistance determination.
- Minimum Inhibitory Concentration (MIC) determination.
- Bacteriophages against *Klebsiella Pneumonaie*.
- Stability of KPA and KPM phage at sub-inhibitory concentration of antibiotics
- Effect of different MOIs of KPA and KPM alone and in combination on turbidity reduction and CFU reduction of the host bacterium.
- Synergistic effect of KPA and KPM and their cocktail with antibiotics on host bacterium.
- Synergistic Effect of KPA and KPM and their cocktail with antibiotics on Biofilm Formation Inhibition.
- Biofilm Reduction by KPA, KPM, and their cocktail.
- Stability of KPA and KPM at various Gastronomic pH.
- Microencapsulation of phages in various polymeric substances.
- Phage Encapsulation efficiency in different polymeric substances.
- Characterization of Dried capsules.
- KPA and KPM phage entrapment and Release from different polymeric substances.
- KPA and KPM phage entrapment and release in simulated gastric fluid(pepsin) and simulated intestinal fluid(pancreatin)

# CHAPTER 2:

# LITERATURE REVIEW

## 2: Literature Review:

### 2.1. Antibiotic resistance:

The allegedly inevitable development of antibiotic-resistance genes among microbial pathogens now jeopardizes the long-term sustainability of our present antimicrobial treatment for treating severe bacterial illnesses like sepsis. Antibiotic resistance has approached a tipping point in several bacterial infections, with few treatment options available and pan-resistant forms becoming increasingly common. Non-antibiotic therapy for bacterial illnesses is being extensively considered, with one viable approach being the therapeutic use of phages that target pathogenic bacteria. Antimicrobial resistance in pathogenic bacteria poses a substantial threat to infectious disease management. Despite an urgent need for innovative antimicrobial medications, the research of new types of antibiotics has halted in recent years due to economic and market revenue generation constraints. In most areas of the globe, antibiotics were viewed as a panacea, resulting in decades of complacency about infectious illness.

Substances with antibiotic properties produced by several microscopic organisms were discovered long before the world acknowledged their utility in preventing and treating bacteriological infections. In 1945, Selman Waksman coined the term “antibiotic” which is defined as a chemical substance with antibacterial potential that worked by inhibiting or killing the growth of microbes responsible for the disease or the disease germ and having microbial origin that is prejudicial to other microorganisms. (Hutchings et al., 2019). It is an antibacterial agent that combats bacterial infection and is the pivotal line of defense against bacterial infections. With the discovery of Penicillin in 1928, a dramatic and far-reaching change occurred, which was recognized as the beginning of the antibiotic era. From the 1950s to the 1970s many new antibiotics were discovered. After this period, no new antibiotics were discovered. Later, the only approach was the modifications to the existing antibiotics (Fair et al., 2014). Before the 20$^{th}$ century, infectious diseases were widespread and resulted in high mortality and morbidity. As a result of the

discovery of penicillin, antibiotics gained worldwide importance, and methods of treating infectious diseases were reformed. Since the peak years of the golden era, continuous decrease in the discovery of new antibiotics, improper stewardship of antimicrobial agents, as well as poor infection management and containment resulted in the existing antimicrobial resistance catastrophe (Pancu et al., 2021). The advent of antibiotic resistance is putting the achievements of modern medicine at risk, increasing the consequences of infectious illnesses in terms of infection counts as well as medical expenses. Bacteria have developed resistance to antibiotics that they were initially sensitive to. Different resistance mechanisms such as reduced uptake of the drug, elevated export of efflux pumps, alteration of drug target, hydrolysis, and modification of antibiotics have developed over the past few years (Munita et al., 2016). Antibiotics resistance genes emerge in tandem with naturally occurring antibiotics. As a result of bacterial genetic mutations, genes are inherited by mobile genetic elements. (Peterson et al., 2018). Bacterial gene transfer can occur via numerous processes, including conjugation (transfer via mobile genetic components), and transformation (direct transfer of DNA). transduction (transmission of DNA by phage). (Schneider & C.L, 2017). Antibiotic resistance genes have a very high propagation efficiency in different species. Different mechanisms of resistance exist for different classes of antibiotics, and these mechanisms differ for different bacterial species, including genetic makeup. (Von Wintersdorff et al., 2016). β-lactam medicines are the most often employed class of antibacterial agents. MRSA (methicillin-resistant *Staphylococcus aureus*) emerged in the hospital environment in the early 1960s and has evolved to become resistant to many antibiotic classes. So, antibiotic resistance accelerated with time. Antibiotic resistance genes have a very high propagation efficiency in different species. Different mechanisms of resistance exist for different classes of antibiotics, and these mechanisms differ for different bacterial species, including genetic makeup. The resistance to antibiotics dilemma has been linked to the excessive and improper use of these drugs, as well as the pharmaceutical industry's lack of innovative drug research due to lower profits and difficult compliance with

regulations. Only two classes of antibiotics have been developed and are now in clinical use in the last 20 years. Oxazolidinones and lipopeptides are the new antibiotics. Most new antibiotics are not created entirely by new mechanisms, but rather are modifications of existing drugs. Lipopeptides include daptomycin, which was discovered many years ago but was not used commercially due to antagonistic effects. Gram-positive bacteria are resistant to oxazolidinones and lipopeptides. (Lewis, 2020). The resistance to antibiotics uncovers that ESKAPE pathogens are linked to the 12 most dangerous bacteria because of high mortality for which antibiotics are required immediately. ESKAPE pathogens include Gram-positive and Gram-negative bacteria such as ***Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.*** They are the leading cause of life-threatening infections, including nosocomial infections, particularly in immunocompromised patients. (De Oliveira et al., 2020)**.**

Pathogens are classified into three categories based on their resistance pattern: critical, high, and medium priority. The ones included in critical priority are Carbapenem-resistant *A. baumanni* and *Pseudomonas aeruginosa* including extended-spectrum β- lactamase (ESBL) or Carbapenem-resistant *K. pneumonia* and *Enterobacter spp.* In high priority class, vancomycin-resistant *E. faecium (VRE)* and methicillin and vancomycin-resistant *S. aureus* (MRSA and VRSA) are added. There are three types of mechanisms by which ESKAPE pathogens exhibit resistance. First, an enzyme inactivates the drug. Second, target site modification, and finally, decreased drug accumulation. Drug accumulation is reduced by two processes: decreased permeability to the drug and increased efflux of the drug. Aside from these resistance mechanisms, ESKAPE pathogens also form biofilms that inhibit the host's immune response and develop antibiotic resistance. (Mancuso et al., 2021).

Lipopeptides, fluoroquinolones, oxazolidinones, macrolides, tetracyclines, β-lactams, β-lactamase inhibitors, carbapenems, glycopeptides, and polymyxins are the ultimate line of protection for ESKAPE pathogens against which they also

showed resistance. Because of the acquisition of antibiotic-resistance genes, ESKAPE pathogens are accountable for reducing the number of treatment options. (Liu et al., 2022). Of the ESKAPE pathogens, *Klebsiella pneumoniae* is the cause of healthcare-associated infection. It is a Gram-negative, rod-like, non-motile and facultative anaerobic bacterium. Different types of infections are caused by *Klebsiella pneumoniae* that includes pneumonia, wound, and bloodstream infections as well as meningitis. Mostly, they are present in the human intestine and feces. *Klebsiella pneumoniae* is a highly pathogenic bacterium that causes nosocomial, community-acquired, and urinary tract infections. (UTIs). Patients who are exposed to ventilators and vein catheters are more vulnerable to *Klebsiella pneumonia* infection. Carl Friedlander identified *Klebsiella pneumoniae* as an enveloped bacillus in 1882 after collecting the bacteria from the lungs of pneumonia sufferers. It penetrates the oropharynx and intestinal tract's mucosal surfaces, and when it gets inside the body, it multiplies., *Klebsiella* develops resistance to various antibiotics and an increased virulence rate. These days it reports about 3% to 9% of the total nosocomial infections (Effah et al., 2020). Bacterial pathogenicity is determined by a polysaccharide capsule composed of complicated acidic polysaccharides. Capsule protects against serum bactericidal proteins and phagocytosis, as the host's defense against infection by bacteria is mostly dependent on these two processes, phagocytosis and serum complement protein activation. According to one study, *Klebsiella* without virulence factor(capsule) is considered less virulent than *Klebsiella* with virulence factor. Aside from the polysaccharide capsule, lipopolysaccharide is another virulence factor found on the outer covering of Gram-negative bacteria. Other types of virulent factors found in bacteria include fimbriae and siderophores. (Ballén et al., 2021).

*Klebsiella pneumoniae* is rapidly developing multidrug-resistant strains and frequently poses a serious threat to the community due to the ineffectiveness of therapy options. When *Klebsiella pneumoniae* resistance to β-lactam antibiotics was first reported, Gram-negative bacteria was studied thoroughly, and it demonstrated how beta-lactamase is responsible for the hydrolysis of the beta-

lactam ring. They hydrolyze the cephalosporins in the same way, rendering them ineffective for treatment. Carbapenem was used after cephalosporin resistance developed, but it also showed resistance, with *Klebsiella pneumoniae* accounting for 80% of carbapenems resistance. *Klebsiella pneumoniae* produces an enzyme called carbapenemase, which inhibits the effect of the antibiotic carbapenem by using a mode of action in which bacteria kill the antibiotic. For the Gram-negative bacteria, carbapenem antibiotics were used as the last line of defense which is now resistant to bacteria (Garbati et al., 2013). Because of the rising frequency of antimicrobial resistance (also known as AMR) and the lack of novel antibiotics, there has been renewed interest in using bacteriophages (phages) to combat AMR.

### 2.2. Bacteriophages:

Phages, or bacteria-infecting viruses, attack their bacterial hosts. They have every trait which renders viruses what they are. Bacteriophages are small (50–200 nm) and contain the genetic information for rapid and effective replication, but they are unable to replicate independently and need a bacterial host to do so.

Bacteriophages were initially reported by **Felix d'Herrelle** and **Frederick Twort** a little more than a decade ago, and for the most part, the treatment application of phages has been investigated and discussed with varying degrees of passion. In the early twentieth century, designated phage treatment was immensely appealing to d'Herrelle and others since it provided the first viable option for treating bacterial diseases. Such infections were prevalent and often severe and played a significant role in determining normal human lifespan. When bacteriophages (phages) were initially characterized 100 years ago, their potential uses in infectious illness therapy were instantly apparent. (Wittebole et al., 2014). The development of a self-replicating antimicrobial efficient of eliminating harmful bacteria might have far-reaching implications for human health. Bacteriophages (phages) are bacteria-eating viruses. They encompass every attribute of viruses. Phages are unable to replicate independently and must be replicated by a bacterial host; they are tiny (50-200 nm) and contain genetic codes for quick and effective replication. They are

generally specialized for a certain bacterial host, as accomplished by most viruses; a single phage might infect many distinct species within a genus and most strains within a species, but occasionally just one or very few strains within species, but occasionally just one or very fewer specific strains of a species. The host range of a specific phage is its bacterial predilection, which can be very restricted (just a few bacterial isolates allow replication) or very broad (infecting many distinct species as well as different genera). host spectrum or "preference" is important in evaluating phage therapeutic efficacy. Phages are ubiquitous and prolific in the environment; ocean water, for example, has $10^7$ phage particles per milliliter, and there are estimated to be $10^{31}$ overall phage particles in the earth's atmosphere, surpassing bacteria by a factor of ten. The population is simultaneously old and evolving, with continuous infections resulting in total phage population fluctuations daily. Unexpectedly, phages have significant genetic variability, and it is uncommon to distinguish between two individuals that are genetically similar, however, the level of difference fluctuates based on the host. The genomes, on the other hand, can differ in every possible manner known. (Knowles et al., 2016). Despite their small size, phages can be easily visualized using electron microscopy, which exposes a wide range of fascinating forms and sizes. The greatest number of phages in nature, however, belong to the order Caudovirales, which possess double-stranded DNA (dsDNA) and tails. These phages all include a head that includes the dsDNA and a tail that has receptor recognition capabilities at the point of attachment. These phages are classified into three morphological types based on their tails (Figure 1): siphoviridae (long length, noncontractile tails), myoviridae (contractile tails), and podoviridae (short length, pointy tails). Irrespective of their form, these dsDNA tailed phages can be classed as either lytic or temperate, which is an essential factor when contemplating therapeutic application. Lytic phages have only one infection outcome: phage replication, lysis of the bacterial host, and the expulsion of phage progeny. Temperate phages can also proliferate lytically, but this is not the only result of infection. The development of lysogeny is an alternate consequence. The phage DNA, termed prophage, is retained within the bacterium (often through

incorporation into the host organism's genome); the genes required for lytic development are turned off; and the lysogenic cell keeps on developing, splitting, and prospering. (Hatfull et al., 2022).

### 2.2.1. Phages Effective against *Klebsiella Pneumonaie*:

Increased cases of *Klebsiella pneumoniae* nosocomial infections resulted in higher fatality rates. As a result, many phages have been discovered in the post-antibiotic period that have proven to be particularly efficient against *Klebsiella* infections. Several new bacteriophages that are efficient against the *Klebsiella pneumoniae* strain have been identified in various research. A new phage IME268 was isolated from sewage in a recent investigation. Usually, hospital sewage is regarded as the primary source of sample for phage isolation. The goal of isolating more and more new phages is to create resistance by repeatedly using a single phage. As a result, more new bacteriophages are required to overcome the resistance created by *Klebsiella pneumoniae* strains (Nazir et al., 2022). Phage therapy was used in an animal model to test the efficacy of the phage against *Klebsiella pneumonia*e. The animal model for this investigation was mice afflicted with lobar pneumonia. The phage SS was isolated from the sewage sample. The phage SS was studied and shown to have potent lytic activity as well as being highly specific to *K. pneumoniae* (Chibber et al., 2008). Another lytic phage from the *Siphoviridae* family was identified against K. pneumoniae. A single dosage of phage was delivered intravenously. The findings were astounding, with animals treated with this phage showing a decreased level of *K. pneumoniae* infection in the lungs. Because of phage efficacy in vitro and in vivo, it is utilized as a substitute to antibiotic therapy (Cao et al., 2015). Another Siphoviridae phage was isolated from drinking water. Vb MK54 phage was shown to be highly selective for lytic host bacteria. Two endolysins with higher lytic activity were discovered because of whole genome sequencing. In vitro studies were carried out, and the results revealed that both endolysins killed the bacteria within a few hours. To test the in vivo effect, a mouse model was infected with K. pneumoniae, and both endolysins displayed therapeutic effects, as well as a significant reduction in bacterial burden. Another encouraging

aspect is that it is highly adaptable to its surroundings (Lu et al., 2022). From a hospital wastewater sample, a lytic bacteriophage vB KP13 was identified. Because phage has a short latent period, it can eliminate biofilm in just one or two days at a rate of more than fifty percent. As a result, it paves the path for phage therapies to become more accessible in years to come (Horvath et al., 2020). Two distinct phages, P1 and P2, were recovered from another sewage sample. Transmission electron microscopy and whole genome sequencing were used, and the sequencing revealed that P1 and P2 phages are lytic phages. However, phages do not develop any poisons or virulence factors, making them particularly effective for therapeutic purposes (Hesse et al., 2020). Phage ZCKP1 from the Mycoviridae family was isolated from freshwater. It could lyse microorganisms other than Klebsiella spp. Phage exhibited the ability to lower the number of host bacteria and the creation of biofilms. The in vitro results are extremely promising for application as a medicinal agent. Biofilms have a significantly lower bacterial count (Taha et al., 2018). Another phage, KPO1K2, was identified from Klebsiella pneumoniae strain B5055. Transmission electron microscopy (TEM) revealed an icosahedral head. This lytic bacteriophage was a member of the Podoviridae family. It also had an extensive spectrum of p when injected to mice; phage titer rose, hence it was suggested to employ this phage as a therapeutic agent to cure *Klebsiella* infections connected with urinary tract infections (Vivek Verma, 2019). Another investigation identified five bacteriophages from sewage that were highly targeted towards strain B5055. Different studies, such as one-step growth and adsorption rate, revealed differences between these five phages. According to the morphology of the phage's head and tail, TEM studies indicated that it belonged to the Podoviridae family. When administered to mice, it demonstrated that it was nontoxic to mice, implying that it is effective for phage therapy.

### 2.2.2. Phages Proteins effective against *Klebsiella Pneumonaie*:

Progress in molecular biology has opened the path for different tactics to engineer bacteriophages as well as phage-derived proteins to improve their effectiveness.

Depolymerases, endolysins, and holins are among the phage enzymes that are efficient against *K. pneumoniae*.

### 2.2.2.1. Depolymerases (Polysaccharide Degrading Enzymes):

Phage enzymes known as depolymerases, which are involved in the earliest stage of infection, offer highly promising outcomes. In addition to depolymerases, there are peptidoglycan hydrolases, endosialidases, lyases, and endorhamnosidases that have potential applications. Proteins encoded by lysis cassette genes, such as holins and endolysins, are also a big concern. Lysis cassette genes produce offspring during the lytic phage cycle. Endolysins, exopolysaccharides, and various phage encoded proteins are currently being studied as natural antibacterial agents in several sections of the food business for maintaining food quality and in the plant sector for the cultivation of plants (O'Flaherty et al., 2009). Phages are employed as diagnostic tools and to detect potential foodborne infections since they are highly tailored to host and host receptor identification. Polysaccharide degrading enzymes are exactly depolymerases are. These are virion-associated proteins that phages use to enzymatically degrade the polysaccharide of the host. There are two types of polysaccharides that are destroyed enzymatically in this process: capsular and structural. Capsular components include alginate, polysialic acid, and hyaluronan, whereas structural components include lipopolysaccharide and peptidoglycan. On the other hand, enzymes that descend into two major groups, hydrolases and lyases, are involved in the destruction of carbohydrate-containing polymers that are primarily found on the surface of bacteria. Along with the O antigen of LPS, hydrolases are involved in the destruction of peptidoglycan or capsular polysaccharide. The latter, lyases, oversee the β-elimination mechanism. Lyases include alginate and hyaluronan lyases (F. Brading et al., 1999)

### 2.2.2.2. Endolysins (Murein Hydrolysaes):

Phages have evolved two fundamental methods for liberating their progeny from cells of bacteria (Borysowski et al., 2006). Filamentous phages continue to emerge from bacterial cells without destroying them, while non-filamentous phages cause

host bacterium lysis. Phage lytic enzymes are exceptionally well-developed murein hydrolases that rapidly dissolve the host bacterium's cell wall to enable the release of the progeny. Lysis is caused by specific proteins causing rapid disruption to the bacterial cell wall.

It can be performed in three manners:

(i) suppression of peptidoglycan (PG) production by a single protein or

(ii) (ii) enzyme-mediated destruction of PG by lysin(s)

(iii) or holing-lysin complexes. Endolysin is a key enzyme in the bacteriophage lysis structure.

Lysins (also known as endolysins) are enzymes expressed by phage genomes that are generated during the late stage of the lytic cycle to dissolve the bacterial cell wall, allowing virions to be released. Lysins must be separated from all other phage-encoded lytic enzymes that are an intrinsic component of the virion tail that selectively breakdown the cell wall to permit phage genome insertion into the host cell (Loessner et al., 2005). Murein hydrolases of this type appear to be common in phage virions targeting Gram-negative or Gram-positive bacteria. The Peptidoglycan lytic potential related to tailed phage virions relates to the tail configuration or an inner head protein that constitutes part of the extended tail, if known. In other instances, the paucity of genetic and structural research, as well as the proteolytic digestion of structural proteins in some of these phages, make comprehensive detection of the protein with enzymatic activity problematic. A well-studied method is the coliphage T4 tail tube, that utilizes a lysozyme incorporated into a baseplate protein at the leading edge of the tube (Moak et al., 2004). Most lysins are made up of a single polypeptide with two functional domains in action: a catalytic domain that cleaves Peptidoglycan bonds and a cell wall binding module (CWBM) that can attach a species-specific carbohydrate receptor in the cell wall. PlyC, the lysin from C1 streptococcal phage, has recently been found to be a multimeric enzyme made up of eight cell wall connecting subunits corresponding to every catalytic subunit (Nelson et al., 2006). Lysins are

categorized as, N-acetylmuramoyl-alanine amidases (NAM-amidases), N-acetylmuramidases (lysozymes or muramidases) endo-N-acetylglucosaminidases (glucosaminidases), endopeptidases, and lytic transglycosylases based on their catalytic activity. Glycosidases operate on the sugar moiety, while endopeptidases hydrolyze the peptide cross-bridge and NAM-amidases break the amide bond joining the sugar and peptide parts of PG. Endopeptidases are additionally classified into l-alanoyl-d-glutamate endopeptidases, d d-glutamyl-meso-DAP endopeptidases, and inter-peptide bridge-specific endopeptidases, but this latter function has yet to be discovered in a phage endolysin (Scheurwater et al., 2008)

Endolysin is produced late in the infection process, after the phage has infected the bacteria and the progeny phages have been discharged. Endolysins have a variety of properties, including low molecular weight, potent bactericidal activity, and enhanced safety. According to current research, natural endolysin is an effective antibacterial medicine of choice for Gram-negative bacterial infections. However, there is a barrier in the outer membrane of Gram-negative bacteria that restricts endolysin, reducing its usefulness as a therapeutic agent. Endolysin research was done, and two endolysins, LysCA and LysG24, were expressed against *K. pneumoniae.* In vitro testing revealed that both endolysins have a modest ability to lyse *Klebsiella*. However, adding EDTA significantly boosted activity and demonstrated great lysis ability. Cecropin A residues combined with EDTA exhibited a synergistic impact on Gram-negative bacteria's outer membrane disruption. As a result, endolysin lysis is improved (Lu et al., 2022).

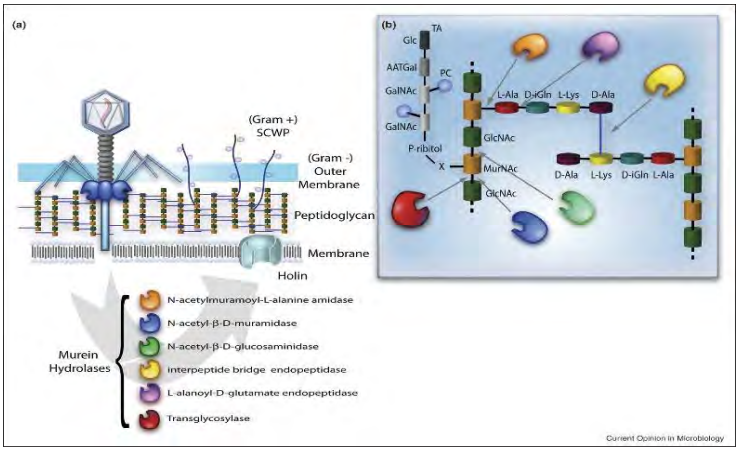


**Fig1.** Represents the Bacterial cell wall configuration and murein hydrolases.

(a) The cell wall of bacteria is depicted schematically. The primary polymer found around the cytoplasmic membrane is peptidoglycan, which is crucial to cell structure and osmotic stabilization. Furthermore, the outer membrane is found in Gram-negative bacteria, whereas in Gram-positive bacteria, cell wall polymers (all of which are referred to as '**secondary cell wall polymers'**, SCWP) such as teichuronic acids, teichoic, or other neutral or acidic polysaccharides are attached to the peptidoglycan chains. Murein hydrolases expressed by phage genomes are synthesized in the final stages of the lytic cycle after bacteriophage infection. Endolysins get accessibility to their substrate, the cell wall of bacteria, when holin, a tiny phage-encoded membrane protein, breaks the membrane at a genetically specified point.

(b) Endolysin targets and the precise arrangement of peptidoglycan in *S. pneumoniae*. Murein glycan strands are made up of GlcNAc (N-acetyl-d-glucosamine) and MurNAc (N-acetyl muramic acid) residues that alternate. Short peptides connect the glycan strands. Pentasaccharide units of teichoic acid (TA) are most likely connected to MurNAc repeats via linkage elements (X). Through GalNAc residues, phosphorylcholine (PC) moieties are connected to teichoic acid. The cleavage of bonds by the various murein hydrolases is represented by arrows (Hermoso et al., 2007)

### 2.2.2.3. Tail Tubular Protein A (TTPA):

The structural protein **Tail tubular protein A (TTPA)** is found in Klebsiella bacteriophage KP32. TTPA has previously been shown to have lytic activity against the exopolysaccharide (EPS) of a multidrug resistant strain of Klebsiella phage PCM2713. It's a multifunctional macromolecule with structural and enzymatic activities. The presence of a peptidoglycan hydrolase domain in the -helical region was discovered in the enzymatic activity of TTPA (Pyra et l., 2017). Polysaccharide is a significant virulence component of *K. pneumoniae* that wraps around the cell and is subsequently structured as capsular polysaccharide (CPS). CPS is made up of three to six sugar subunits that repeat. CPS has a negative impact on *K. pneumoniae* resistance (Keynan et al., 2007). Phage encoded enzymes were formed because of the cohabitation of phages and host bacteria. Their role is to depolymerize polysaccharide, allowing it to enter the outer membrane and introduce DNA into bacteria. They also decrease the density of CPS and eliminate it from encapsulated bacteria. Klebsiella enzymes mostly destroy glycosidic connections in CPS, resulting in the release of polymer units. When bacteria lack the protective layer of capsule due to enzyme loss, a change in capsule composition occurs, and bacteria become sensitive. This crucial involvement of CPS in the treatment of *Klebsiella*-associated illnesses is astounding. Capsule stripped strains are produced by phage encoded proteins with polysaccharide depolymerization ability. Bacteria become vulnerable to host immunological responses and antimicrobials. Another advantage of these enzymes is that resistance is less likely, and the negative effects on microbiota are less severe. A phage KP36 from the Siphoviridae family was isolated in a study, and a novel depolymerase enzyme was developed to assess its efficacy against bacteria. They used to cleave *Klebsiella* CPS.

### 2.2.2.4. Holins (Transmembrane Proteins):

Holins are small transmembrane proteins that phage genomes encode. They are an essential part of bacteriophages. They are essential in the lysis of bacterial host cells during the late phase of the phage replication cycle. They oversee managing the timing of host cell lysis during the lytic cycle. Throughout the phage infection

process, holins accumulate in the bacterial cell membrane until they achieve a necessary level. When a particular amount of holins approaches a certain level, pores or lesions occur in the cytoplasmic membrane of bacterial cells. These lesions generate an abrupt rise in membrane permeability, resulting in the production of endolysins, enzymes that destroy the bacterial cell wall. Holins vary greatly in structure, size, and manner of action. They can be classified into two distinct groups based on their membrane permeabilization methods. The canonical holins and pinholins. Canonical holins generate huge cytoplasmic membrane pores, whereas pinholins cause minor lesions without generating big pores. Holin has excellent time-based regulation throughout the lytic cycle. They enable the phages to collaborate on the synthesis of adequate progeny particles within the host cell prior to beginning lysis. This monitored distribution of progeny phages increases the likelihood of transmission and replication success.

Overall, holins serve an important role in guaranteeing the effective dispersal of phage progeny by assuring the prompt and precise lysis of the host bacterial cell. Surprisingly, lysins lack a signal peptide that transports them to the periplasmic region to reach the PG, so phages create tiny hydrophobic proteins called holins that export lysins through the bacterial inner membrane. Nonetheless, there are some lysins that carry a secretion signal and employ the standard host sec system for accessing the PG. Some lysins transported by the host sec system have N-terminal signal sequences that act as type II signal anchors or uncleaved signal peptides. Because of the protein's capacity to leave from the membrane and be released into the periplasmic space, this signal sequence is known as SAR (signal arrest release) sequence.

### 2.2.3. Experiences in Phage Therapy:

There have been countless cases of phage therapeutic usage during the last 100 years, albeit many are circumstantial and lack records reflecting efficacy or other clinical information. There are two types of implementations in general. The first is humanitarian use, which occurs when an infection is fatal or when a major infection

cannot be managed using already approved therapies and procedures. (Luong et al., 2020). Such therapies are typically unique, with experimental evidence that a variety of phages can, at least in vitro, attack the strain of bacteria producing the illness. These therapies are typically based on the notion that phage destruction of bacteria in a research setting may be mimicked in the body of the patient. Initial assessment of pathogenic strains for phage sensitivity is of the utmost importance because phage infection descriptions of antibiotic-resistant bacteria can differ tremendously, and only hardly can it be with assurance anticipated that a few phages or combination of phages will infect the bacterial isolate without previous evaluation. (Schooley et al., 2017). The other approach is to conduct randomized clinical studies, which can provide information on security, effectiveness, and dose, along with additional clinical factors. Few clinical studies for phage therapy have been done, even though their accomplishments and errors are immensely enlightening. Between 2017 and 2019, three fundamental case reports of customized phage therapy were published under the US Food and Drug Administration's (FDA) emergency Investigational New Drug (eIND) procedure. These instances were crucial in reviving curiosity about phage therapy for severe bacterial illnesses in the United States. Conventional antibiotic therapy was ineffective in every case, and the use of phages targeted at pathogenic organisms along with antibiotics has been linked to effective microbiological and therapeutic outcomes. Essential lessons were drawn from every scenario, which aided in structuring negotiations about prospective scientific areas for phage treatments and the organized understanding foundation required to transition phage treatments from subjective observations to a logically anchored area. The first case was an individual close to death from a widespread *Acinetobacter baumannii* infection after 5 months of ineffective typical antimicrobial therapy. This scenario revealed that numerous phages uniquely effective against a multi-drug-resistant bacterium might be found and purified for intravenous administration on a clinically meaningful schedule. The example also proved that by repeating intravenous infusion, substantial dosages of phages might be administered to several dispersed

areas of illness. Aside from the clinical outcome, the selection of bacteriophage-resistant *A. baumannii* demonstrated that the phage had an obvious antibacterial effect in vivo. Subsequently, the researchers proved that a second-generation phage cocktail effective towards the A. baumannii identified by the first phase of phage therapy could be created. This opens the possibility of further rounds of phage therapy if the selection of phage-resistant mutations limits the therapeutic effect.

The second pivotal case employed two significant new perspectives through which phages could be deployed in highly significant ways in combination with antibiotics). In this instance, a guy with an inserted Dacron aortic arch prosthetic could not react favorably to repeated regimens of intravenous antibiotics treatment over a course of three-year duration. After an extended duration of antibiotic repression without sterilization, the patient was suffered with a gaping sinus adjacent to the region of the transplant that was constantly discharging *Pseudomonas aeruginosa* following continued intravenous ceftazidime administration. The patient was given a lytic phage (OMKO1) that was attached to the outer membrane protein M of *P. aeruginosa's* mexAB- and mexXY-multidrug efflux systems). Attacking these efflux pumps enhanced this organism's sensitivity to ceftazidime over twofold and to ciprofloxacin over tenfold. Aside from its effect on sensitivity to antibiotics, OMKO1 has been demonstrated to be extremely detrimental to the biofilm produced on the transplanted devices. This phage was injected into the septic opening. Antibiotics were stopped after another month of treatment, and the transplanted area was kept sterile.

A young woman with cystic fibrosis underwent dual orthotopic transplantation of her lungs for a prolonged *Mycobacterium* infection of the lungs that eventually stopped responding to antimycobacterial medications in the third case. Following maximum combination antimicrobial treatment, her *M. abscessus* infection reappeared 5 weeks after lung transplantation and spread to her injury, lungs, epidermis, and liver. By evaluating a database of phages that were previously chosen for efficacy against *M. smegmatis,* a phage cocktail targeting her *M. abscessus* infection was developed. In this scenario, employing bacteriophage

recombineering of electroporated DNA (BRED), it was required to remove the repressor gene from two of the critical phages in the program to transform them from temperate to lytic phages. This patient's extensive aberrant mycobacterial disease was cured after she was treated intravenously with an amalgam of three lytic phages. This was the first-time modified phages were used, and it extended the scope of phage treatments to mycobacterial infections.

Since these three occurrences, there has been a surge in personalized phage treatments in the United States and Europe. Although not all these cases proved successful, they did lay the framework for the research studies that began in earnest during the last 18 months. (Khalid et al., 2021)

### 2.2.4. Phage Therapy Implications:

#### 2.2.4.1. Beneficial Attributes of Therapeutic Bacteriophages:

There are numerous general qualities to consider when selecting phages for therapeutic application.

- First, the phages must efficiently destroy a particular bacterial pathogen in vitro condition, with no appreciable residual bacteria.
- The phages must be viable and stable across a wide concentration range so that long-term storage at the refrigerator's temperature does not result in a significant loss of infection capacity.
- The phages must be easy to grow in number, prepared in high-titer formulations, and purify easily to use in therapeutic preparations.
- The phage formulations must be uncontaminated and devoid of endotoxins and other potentially dangerous impurities.
- The phage genetic material should not contain any harmful genes that have been reported or assumed to be potentially hazardous.
- The phages should be unable to function as universal transducing phages. Some of these variables can be determined using bioinformatics, but others must be confirmed experimentally.

- It is also beneficial to comprehend the incidence and root causes of resistance to each phage, as well as co-resistance patterns. For example, if a medical professional utilizes two phages in a cocktail, it is best not to use a single cellular receptor, as receptor degradation results in resistance against both phages.

#### 2.2.4.1. Formulation, Dosage, and Application of Phages:

Most bacteriophages can be replicated to high titers in either solid or liquid media, highly concentrated, and filtered to a high degree of purity. A conventional research facility preparation may generate $>10^{12}$ phage particles, enough for hundreds or perhaps thousands of treatments depending on dosages and longevity. For in vivo studies, it is critical that the preparations be sterile (often by commercial accreditation) and have endotoxin levels below FDA-approved thresholds. Additional processes may be necessary to minimize endotoxin levels in phages generated on Gram-negative hosts, while phages generated on non-LPS-containing hosts are normally free of endotoxin. There is barely any clinical evidence defining appropriate phage doses or pharmacokinetic aspects of treatment. Furthermore, phage pharmacokinetics differs from antibiotic pharmacokinetics because pathogen eradication relates to phage multiplication, therefore Phage levels in a patient could fluctuate greatly according to when the phage infection occurs and where the pathogenic bacteria have been detected. Phage safety is generally quite good; problems from reasonably large dosages are less troubling than with potent small-molecule antibiotics. (Dedrick et al., 2019).

#### 2.2.4.2. Do Immune Responses Affect the Therapeutic Efficacy of Phages?

Immune responses that impede phage activity may occur when supplied via other ways, particularly intravenously, and are less of a risk when administered topically for a short period of time. Immune responses may be mitigated, at least for a while, in instances where the patient is immune compromised, such as after the transplantation of an organ, Delivering the same phage cocktail to an older immunocompromised individual, on the other hand, resulted in therapy failure due to a strong IgG-mediated antibody reaction that was significantly suppressive to at

least two of the cocktail's phages. Immune responses to phages have been observed in both human and animal model systems, however, they are not always neutralizing. (Hodyra Stefaniak et al., 2015).

#### 2.2.4.3. Potential Phage Therapy Obstacles: Strain Modulation and Resistance:

Phages' capacity to successfully eradicate bacteria under laboratory conditions makes them appealing antimicrobials for clinical application, but two major difficulties frequently occur. For instance, there is significant variation among clinical specimens of similar species for various bacterial illnesses, and this variation extends to differences in bacteriophage attacking patterns. As a result, a phage that is beneficial for one individual may be ineffective for another if it does not infect and eliminate both strains. This variation is significant for some pathogens (such as *M. abscessus and S. aureus*) but less so for others (such as *Mycobacterium tuberculosis*) (Moller et al., 2021). There are two potential answers to this problem: screening for and detecting phages with the broadest host ranges capable of infecting as many diverse clinical isolates as feasible and constructing phages mixtures containing enough phages to ensure high chances. A second barrier is bacteriophage resistance. Phage resistance is common in many various biological systems, like antibiotic resistance, albeit there is considerable diversity. There are two approaches to this: use a cocktail of multiple phages for the target strain or sequentially inject individual phages.

#### 2.2.4.4. Phage Resistance:

When phages are widely compromised, resistance is to be expected, and resistance management strategies are critical. However, the prevalence and severity of resistance vary widely amongst bacteria. These discrepancies can be explained to some extent by whether phage receptor reduction or mutation is tolerated without causing viability loss. While receptor mutation is frequently involved in resistance, there are a variety of additional mechanisms, such as restriction, CRISPR-Cas,

prophage-encoded mechanisms, and abortive infection systems. (Bernheim et al., 2020).

### 2.2.5. Phage Antibiotic synergy (PAS):

Clinical carelessness after excessive usage is certainly one of the causes of the occurrence of antibiotic resistance in microorganisms. Antibiotic resistance is an enormous loss for the world's healthcare, and a lack of antibiotic therapeutics has produced a dire requirement for alternate methods of treatment. Bacteriophage (phage) therapy is being studied as a viable treatment option for the growing number of antibiotic-resistant bacteria. Sublethal quantities of some antibiotics that promote the release of progeny phages from bacterial hosts are referred to as phage-antibiotic synergy (PAS). The use of phages in conjunction with antibiotics is a potential technique for lowering antibiotic doses and preventing antibiotic resistance throughout therapy. Comeau AM et al invented the term "phage-antibiotic synergy" (PAS) in 2007. The investigators discovered that activation with sublethal concentrations of β-Lactam and quinolone antibiotics contributed to considerably greater plaque size and number, suggesting that an enhanced adsorption rate, smaller latent period, and greater burst size occurred during the development of plaque. (Kim et al., 2018). Furthermore, (Uchiyama et al., 2018) investigated 21 different antibiotics for their combined effect with the *Pseudomonas aeruginosa* phage, the majority of which were efficient in suppressing bacterial cell wall formation or protein synthesis. (Ryan et al. 2012) used T4 phage individually or in conjunction with cefotaxime and T4 to treat *Escherichia coli*. The study found that in the absence of the antibiotic, the T4 phage had a latent duration of 24 minutes, which was decreased to 18 minutes when cefotaxime was added. Furthermore, the initial T4 phage concentration increased from $5x10^6$ to $5x10^7$ plaque forming unit (PFU)/ml, indicating improved propagation as well as a surge in phage adsorption efficiency. In conclusion, these data reveal that the incorporation of antibiotics causes modifications to plaque size, latency period, and burst size during phage development, and can thus be utilized to detect the synergistic effect of phages and antibiotics.

### 2.2.5.1. Phage-antibiotic synergy in treating Biofilms:

Bacterial biofilms are populations of bacteria that are connected to an abiotic or biological surface, such as pipes, catheters, stents, tubes, and prosthetic hip joints. The cell population is encased in an extracellular matrix composed of secreted lipids, extracellular DNA, amino acids, and polysaccharides known together as "**extracellular polymeric substances"** (EPS). These compounds promote surface adherence while also shielding against antibacterial. agents. Conversely, biofilms are a harmful habitat for tenacious bacteria, offering considerable resistance to antimicrobial drugs without requiring genetic changes and drug-resistance genes (Huber et al., 2003). As a result, biofilms are much harder to eliminate than planktonic bacteria treated alone with antibiotics. Potentially, biofilms are being evaluated as a non-antibiotic treatment. Particularly, various research has investigated the combined action of phages and antibiotics for bacterial eradication in biofilms. One of the previous research projects investigated the efficiency of phage Sb-1 alone or in conjunction with several kinds of antibiotics for the eradication of *S. aureus* biofilms. Treatment with phage Sb-1 alone greatly reduced persistent bacteria but did not eliminate the biofilm, according to the results reported. However, combining phage Sb-1 with rifampicin/daptomycin dramatically reduced EPS and cleared *S. aureus* biofilm. Furthermore, simultaneous phage Sb-1 and antibiotic therapy is an efficient method for biofilm reduction. Toxic genes expressed by phage genetic material are expressed as specialized enzymes such as holin, endolysins, and polysaccharide depolymerases, which participate in the degradation of bacterial cell walls after bacterial infection. A combination of phage-encoded hydrolytic enzymes and particular antibiotics greatly disrupts the structure of bacterial biofilms and releases tenacious microorganisms contained in the biofilm into the nutritional surroundings. A 6-hour administration with KPO1K2 and ciprofloxacin, for example, effectively removed a biofilm generated by *Klebsiella pneumoniae* and resulted in a 4.5-fold decrease in the total number of bacteria interred in the biofilm (Gordillo Altamirano et al., 2019).

### 2.2.5.2. Phage-antibiotic Synergy in Clinical Trials:

Several research has been conducted to investigate the implications of combining phages with antibiotics in vitro and in vivo, but there have also been some successful clinical trials. (Bao et al.,2020) described a case of an individual who acquired a chronic urinary tract infection (UTI) caused by extensively drug resistant *Klebsiella pneumoniae* (ERKp) that resisted all available antibiotics examined except polymyxin B and tigecycline. The UTI was not treated despite critical care therapies, including tigecycline medication. Following comprehensive evaluation and informed agreement, the patient was recruited in a phage treatment clinical trial. In vitro, phage cocktail III (KP164, KP152, KP6377, KP154, and HD 001, KP155) and SMZ-TMP could totally limit ERKp growth for more than 24 hours. As a result, following five days of uninterrupted treatment with the above treatment strategy, which included consumption of trimethoprim-sulfamethoxazole (SMZ-TMP) two times daily and bladder washing with phage cocktails III, the ERKp could not be obtained from the patient's urine sample, and the signs and symptoms of urinary tract infection vanished fully. Furthermore, there was no evidence of reappearance after six months of discharge. Several antibiotics were unsuccessful in curing a kidney transplant patient who developed a urinary tract infection with an extended-spectrum-lactamase (ESBL)-positive *K. pneumoniae* strain in the first month after the transplant. Even though the ESBL *K. pneumoniae*, in this scenario, was responsive to meropenem, the infection occurred and eventually progressed into epididymitis following continuous meropenem treatment. A Georgian phage showed remarkable lytic activity against these ESBL-K. pneumoniae strains. The urethral symptoms reported by the patient entirely disappeared after one day of medication with meropenem paired with this phage by oral and bladder irrigation, respectively, and samples from the urine remained negative for 14 months after therapy. (Kuiper et al, 2019).

### 2.2.5.3. PAS-Related Genetic Mechanisms:

Phage infection begins with phages adhering to receptors on bacteria's outermost components, including lipopolysaccharides (LPS), proteins, teichoic acids, and flagella. Although the evolution of phage-resistant bacteria is almost certainly unavoidable, multiple studies indicate that phage selective stress may expedite bacterial genetic changes, encouraging them to withstand phage infection at the expense of their efficiency. Reduced infectiousness, poor food intake, susceptibility to antibiotics, and colonization problems are examples of fitness compromises. This discovery establishes the groundwork for the use of phages. Along with the immediate killing impact caused by phages on host bacteria, phage-produced selective stress is effective in controlling bacterial growth. Surface elements of bacteria, like receptor molecules that reside on phages, play an important role in the characteristics of diseases. Among the outermost surface components are lipopolysaccharides (LPS), outer membrane proteins, teichoic acid, capsules, pili, siderophores receptors, and efflux pumps. Their constituents are frequently regarded as virulence markers, antibiotic resistance-related variables, and normal growth indicators, as they can regulate host adhesion and deterioration, as well as antibiotic efflux (Leon et al., 2015). Additionally, phage infection causes evolutionary selective pressure on the ompU gene, which expresses the outer membrane porin in *Vibrio cholerae* and serves as a phage infection sensor in the bacteria. During this selective stress, *V. cholera*e ompU or toxR genes alter, resulting in phage resistance. *V. cholerae*, too, exhibits a natural compromise between phage resistance and bacterial pathogenicity. Particularly, these alterations reduce pathogenicity by at least a hundred times since the mutant strains lack the capacity to induce cholera and hence lose an innate capacity to transmit illness. These findings suggest that adaptability to phage infection entails evolutionary competitiveness compromises and give molecular mechanisms to comprehend the influence of phage infection on *V. cholerae* transmission along with the seeding of the surrounding hosts (Li et al., 2021).

### 2.2.6. Encapsulation:

Gastrointestinal flora control is a powerful tool for preventing and treating disorders caused by microbiome dysregulation. Aside from traditional probiotic products, phage delivery has lately shown considerable promise in changing gut microbiota composition and influencing gene regulation of gastrointestinal tract bacteria. On the other hand, the phage protein structure is susceptible to environmental stimuli during preservation and delivery process, resulting in a loss of infection competence and microflora management capabilities. Encapsulation is a great approach for increasing phage stability and accurately adjusting the delivery dose. Encapsulation carriers made of various functional materials, notably enzyme-responsive and pH-responsive polymer molecules, were employed to defend phages from extreme environment and liberate them in the colon. At the same time, various types of carriers exhibited distinct structural and functional properties, which altered their degree of protection and delivery effectiveness. This study systematically summarizes current advances in phage encapsulation as well as dissemination research, with a focus on the function features of carrier systems related to their protective capacity and colon-targeted distribution.

Billions of microorganisms, including viruses, bacteria, and fungi, live in the large intestine or colon, forming an ever-evolving gut microbiota. The gastrointestinal microflora has been suggested as the "eighth organ" of humans, playing an important role in a variety of physiological functions such as nutritional metabolism, protection against infection by pathogens, and upkeep of the gastrointestinal mucus layer (Valdes et al., 2018). Intestinal flora dysbiosis has been linked to both the development and progression of various disorders, including inflammatory bowel disorder, cancer of colorectal, major depression, and alcoholic liver disease. As a result, altering gut flora has lately become a study focus for illness prevention or treatment. Antibiotic treatment, probiotic management, transplantation of fecal microbiota, and phage administration are some ways that have been explored to manage gut microbiota and boost human health. Though antibiotics are effective in treating bacterial infections, they kill both infectious

agents as well as beneficial bacteria in the gut, resulting in an additional disruption of the microbiota in the gastrointestinal tract (Llopis et al., 2016). Furthermore, antibiotic-resistant microorganisms offer a significant obstacle to treatment with antibiotics. Other "classic methods" for modulating gut microflora, such as probiotics administration and fecal microbiota transplantation, are fraught with problems due to inadequate gut colonization and the potential transmission of toxic components from the donor. As a result, new ways for regulating gut flora in a more efficient and manageable manner are of the utmost importance (Ott et al., 2017). With the growing understanding of intestinal microbiology, colon-specific phage administration has been suggested as a potential way to modify intestinal flora in vivo. Multiple phages or phage combinations are currently being employed to manage the gut flora and preserve the equilibrium of the intestinal tract. remarkably, a recent clinical trial discovered that merging probiotics with a phage cocktail substantially raised the proportion of gut-beneficial bacteria (e.g., *Lactobacillus*) and reduced likely pro-inflammatory species (e.g., *Desulfovibrio*), successfully reducing GIT inflammation of contributors than using probiotic alone (*Bifidobacterium*). Phage viability is frequently compromised by extreme conditions (e.g., elevated temperatures, pH imbalances, and digestive degradative enzymes), significantly limiting their advantageous outcomes (Grubb et al., 2020). Phage encapsulation is a popular method for improving phage resilience and retaining their considerable vitality during long-term preservation and intestinal passage. *Salmonella* phage Felix O1 trapped in S100/sodium alginate hydrogel, for example, retained full activity after 2 hours of exposure to simulated gastric juice, whereas free phages were entirely inactivated. Thus far, various beneficial substances like fibers, fluid emulsion, and hydrogels have been used to create phage encapsulation carriers (Vinner et al., 2018). Yet, there is an absence of an in-depth investigation that focuses solely on the intended property, inherent constraints, and most recent advancements in relevant encapsulating polymers and carrier methods utilized for phage distribution and gut flora management.

Furthermore, the key parameters influencing phage effectiveness during preservation and oral administration are unknown.

### 2.2.6.1. The phage-mediated modulation of gut microbiota:

Bacteriophage-mediated modulation of intestinal flora occurs in the colon because of relationships among bacteria and bacteriophages (i.e., the phage life cycle). As an inherent predator of bacteria, the phage may kill or lysogenize within its host cell via the lytic replication cycle or the lysogenic process, and the resulting phage is known as virulent phage or temperate phage. Excess infections or pathogenic bacteria produce unwanted compounds that include bacterial toxins, which are transported into the bloodstream systemically and cause accompanying problems (Ofir et al., 2018). Once in the colon, pathogenic phages can precisely destroy host pathogens and reduce undesirable metabolites via detection and invasion with little harm to general microorganisms such as beneficial bacteria. Furthermore, symbiotic bacteria or probiotics will occupy and colonize the pathogenic bacteria's domain, regaining the equilibrium of the gut while encouraging physical wellness. For instance, colon administration of a phage mixture comprised of three virulent phages attacking adherent-invasive E. coli dramatically reduced pathogenic *E. coli* colonization in mice gut and reduced colitis signs and symptoms. A recent investigation found that the total number of *E. faecalis* strains in the gastrointestinal tract of patients with alcohol-related hepatitis was extremely higher (about 2800-fold higher than controls group) and that the exotoxin (cytolysin) produced by *E. faecalis* was the primary inducer of hepatocyte loss and hepatic damage. They also colonized mice with *E. faecalis* and examined the curative effects of a lytic phage mixture targeting cytolysin-positive *E. faecalis* (Duan et al., 2018). Their findings showed that phage delivery dramatically reduced the overall number of *E. faecalis* in mice feces and cytolysin levels in mouse liver, resulting in a lesser extent of damage to the liver and inflammation after prolonged ethanol feeding. This study suggested that phage administration could be used as an innovative treatment method for severe liver damage caused by intestinal pathogens (Yang et al., 2023).

#### 2.2.6.2. The primary parameters impacting phage viability:

During the preservation and colon delivery process, the environmental parameters (e.g., the ambient temperature, pH) and intestinal variables (e.g., digestive juice, bile salts) have the greatest influence on phage survivability. A comprehensive and detailed understanding of these influence elements is beneficial in proposing an optimal encapsulation method.

##### 2.2.6.2.1. Temperature:

Outer capsid proteins and interior genetic components make up a phage. As a result, most phages are susceptible to heat and will be deactivated at elevated temperatures due to the disintegration of capsid structural proteins. Previous research demonstrated that viable phages of Salmonella phage LPST153 were reduced by 6 logs after heating at 70 °C for 1 hour, and no phage remained when the temperature was elevated to 80 °C (Islam et al., 2020). In another investigation, researchers discovered that after 60 minutes of incubation at 50 °C, a phage cocktail consisting of *Salmonella enterica* phage, *E. coli* phage, *Shigella fexneri* phage, and was totally deactivated (Rahimzadeh et al., 2021). As a result, it is suggested that the process and phage storage temperatures should be at room temperature or lower. (e.g., 4 °C, 20 °C). However, the formation of ice crystals in phage solution may result in phage deactivation, necessitating the addition of a cryoprotectant like glycerol. (Marton et al., 2021).

##### 2.2.6.2.2. pH:

The pH of the surrounding atmosphere is another significant variable regulating phage viability. Under extreme pH circumstances, particularly low pH, the protein arrangement of a phage may denature, which leads to phage deactivation. It has been observed that *E. coli* O157:H7 phage HY01 was highly stable in the pH range of 4-10 but was completely inactivated at pH 4 or pH > 11 (Malik et al., 2017). In a similar manner our prior research on the pH stability of Salmonella phage LPST94 revealed that following 1 hour of incubation at 37 °C, it kept stable in the pH range of 5-11, although its viability decreased significantly at pH 5 or pH > 11. In further

investigation, *Aeromonas hydrophila* phage ZPAH7 showed a pH stability range of 4 to 13 but entirely stopped activity at pH 4.0. According to the literature, most phages appear to be comparatively stable in neutral or near-neutral environments (Islam et al., 2020).

**2.2.6.2.3. Gastrointestinal Route:**

Oral administration is the easiest and most accessible method for phage delivery since it is non-invasive, causes no physical discomfort, is easy to administer, and is generally accepted. As a result, in most therapeutic or preclinical research, phage distribution is primarily accomplished via the oral route (Sarker et al., 2017). The gastrointestinal system is made up of the mouth cavity, esophagus, stomach, small intestine, and large intestine (colon). Although partially liberated phages can transit via the digestive tract, they appear to have a poor rate of recovery from the gut. <1% of fecal phages were collected from healthy individuals in Bangladesh during an oral T4 phage investigation, indicating a substantial decline in alive phages through the gastrointestinal passage. (Sarker et al., 2012).

**2.6.2.2.4. Gastric Acid:**

When oral phages enter the body, they are combined with saliva (lysozyme and amylase) via the process of mastication and undergo a pH change in the mouth cavity (6.6-7.4). According to one study, 71% of phages could be retrieved from human spit after 7 days of incubation at 37 °C, indicating strong phage stability. Given the short retention length (5-65 s) of phage in the oral cavity, it appears that the action of saliva on phages is unimportant (Bachrach et al., 2003). After gulping down, phages pass through the esophagus and into the stomach in a matter of seconds. Many hypersensitive phages have been found to sustain their viability exclusively at neutral pH ranges, even though a few of them may survive in slightly acidic environments at pH 3 (e.g., λ phage) (Dabrowska & K, 2019). Hydrochloric acid, pepsin, and mucin are the main components of gastric juice. The presence of HCl creates an extremely acidic environment in the stomach, with a pH ranging from 1 to 3, resulting in the acid-mediated breakdown of capsid protein and nucleic

acid, along with a reduction in the viable phage population. This acid exposure is an important component in causing a significant decrease in phage concentration during the administration procedure. Furthermore, digesting proteins in gastric fluid (e.g., pepsin) can break down phage capsid protein and enhance acid permeability, resulting in phage structural destruction. Under typical physiological settings, the gastrointestinal travel period is 1-2.5 hours, which is sufficient to severely damage the phage composition and even result in the entire deactivation of given phages. Because acidity-neutralizing substances or antagonists have been used to preserve phage viability in the stomach, excessive usage of antacids may result in substantial negative effects (Nehra et al., 2018).

**2.6.2.2.5. Bile salts and Trypsin:**

Phages reach the small intestine via gastric emptying after gastric digestion. The small intestine has a relatively moderate environment (pH 6.5-7.4) with a transit period of 2-4 hours, and it contains complicated elements including bile salts and digesting enzymes such as trypsin (Yao et al., 2014). The effects of bile salts and trypsin on phage survivability have been studied in vitro/ex vivo. (Ma et al., 2008) discovered that after 3 hours of incubation in 1% and 2% simulated bile solutions, the viable count of Salmonella phage Felix O1 reduced by 1.28 and 1.68 log units, respectively, indicating a substantial decrease in phage survivability. In compliance with trypsin, the research found that T2 and phages were resistant to it in vitro, whereas coliphage P1 was quickly deactivated by it. Thus, phage trypsin resistance may vary depending on strain, which may be connected to phage degradation of enzyme binding sites.

**2.6.2.2.6. Phage-specific antibody:**

Another putative influencing factor for phage survivability in the intestine is phage-specific antibodies. Exogenous phages will undoubtedly be identified and consumed by mononuclear phagocytes such as dendritic cells. Antigen-presenting cells (APCs) then present phage-derived antigens (e.g., capsid protein) to naive T cells. Then, these naive T cells are stimulated and developed into T helper cells,

which can activate naive B cells into plasma cells, which produce huge numbers of antibodies (e.g., IgA, IgG, sIgA) (Popescu et al., 2021)/ Through specific identification, these antibodies will attach to and neutralize phages in the intestinal epithelium. Researchers found in a murine model that oral phage at a high dose (2x $10^{10}$ PFU/mouse daily) generated IgG and sIgA despite being required for an extended exposure duration (35 days for IgG, 78 days for sIgA), with a significant rise in sIgA related with phage absence in feces. As a result, certain antibodies may have a detrimental impact on phage survivability in the gut, resulting in a decrease in viable phages (Majewska et al., 2015). Due to the severe circumstances in the digestive tract, particularly gastric acid, few free phages may reach the large intestine (colon). Importantly, phages' positive function will only be understood if they reach the colon in a live state. As a result, it is critical to preserve their ability to survive during intestinal travel. Several encapsulation substances and carrier applications have been developed to protect phages while transporting them to the colon, allowing phages to multiply in host cells via the lytic cycle or incorporate into the host genetic material via the lysogenic cycle, removing infectious bacteria or transforming bacteria genes to encourage intestinal equilibrium.

**2.2.6.3. Encapsulation material:**

The choice of encapsulating polymers is critical to phage distribution effectiveness. Given the goal and method of phage distribution, the materials employed to construct delivery vectors should be non-hazardous, able to decompose, and harmless to the human being. Meanwhile, the encapsulating materials should be resistant to a variety of severe conditions. Furthermore, as previously stated, the retention duration of consumed chemicals in the intestinal tract is restricted. Although these encapsulated phages survive harsh conditions and make it to the colon in good health, they will be evacuated with faeces leading to barely any phage discharge into the colon (Duran-Lobato et al., 2020). When phages are moved in the colon, they must be fully discharged. Based on colonic environmental conditions, enzyme-responsive substances (e.g., pectin, chitosan) that can be deteriorated by specific digestive enzymes synthesized by intestinal microbes to

produce phages and pH-responsive components (e.g., Eudragit S100, sodium alginate) that may disintegrate or expand at colonic pH to emit internal phages are chosen as ideal building blocks for obtaining a colon-targeted distribution.

**2.2.6.3.1. Enzymes Responsive Material:**

Numerous microorganisms inhabit the large intestine (colon), which can create a variety of hydrolases (e.g., -glucosidase, pectinase) to ferment and destroy some compounds (e.g., chitosan and pectin) that can't be broken down in the stomach and small intestine (Wang et al., 2022). As a result, these enzyme-responsive polymers offer a new way of transferring phages and achieving specific distribution in the colon.

**2.2.6.3.2. Chitosan**:

Chitosan is a naturally produced cationic linear polysaccharide derived from chitin via alkaline deacetylation. Chitosan's skeletal structure is made up of glucosamine subunits and N-acetyl glucosamine units connected by β-(1, 4) glycosidic linkages. Chitosan's molecular chain contains significant groups of amino acids that can be hydrogenated and carry enormous positive electrical charges in an acidic state (Bakshi et al., 2020). Then, through electrostatic contact, cationic chitosan can combine with negatively charged molecules (e.g., sodium tripolyphosphate, sodium alginate) to produce an encapsulating matrix. Chitosan as the outermost layer (shell) in the stomach can prevent or prolong the diffusion of gastric juice into the carrier, which is then destroyed by a specialized polysaccharide hydrolase to liberate encapsulated phages. (Rahimzadeh et al., 2021) used an ion gelation approach to create chitosan nanoparticles to encapsulate a mixture of phages composed of three phage strains (*Salmonella enterica* phage, *Shigella fexneri* phage, and *E. coli* phage). In vitro, release studies revealed that free phages were completely inactivated in stomach circumstances, but encapsulated phages retained their vitality and released smoothly in the intestinal environment. Furthermore, chitosan can be utilized as an outer covering carrier to raise the overall thickness of the inner

carrier (for example, alginate hydrogel), hence improving the physical durability of the carrier matrix under challenging circumstances.

#### 2.2.6.3.3. Pectin:

Pectin is a polymeric substance found in the cell walls of a variety of terrestrial vegetation species, including vegetables and fruits. Pectin's core structure is a linear chain of α-(1, 4) linked galacturonic acids, which can be slightly methoxylated to vary its functional characteristics. Pectin is classified as low methoxy pectin (DM 50%) or high methoxy pectin (DM > 50%) based on the extent of methoxylation (DM) (Maric et al., 2018). Pectin cannot be degraded by pepsin in the stomach, but it can be broken in the colon by pectinase, which is produced by intestinal bacteria and produces short-chain fatty acids while liberating encapsulated compounds. (Dini et al., 2012) used $Ca^{+2}$ crosslinking to encapsulate *E. coli* phage CA933P in low-methoxylated homogenized pectin hydrogel. Non-encapsulated phages were entirely inactivated within 10 minutes at pH 2.5 (with 0.5 mg/mL pepsin), whereas the viable quantity of encapsulated phages fell by just 0.4 log units even after 3 hours of treatment. They also discovered a 48% discharge of phages from hydrogel formulation into the simulated intestinal fluid (SIF) following 4 hours of incubation at 37 °C.

### 2.2.6.4. pH-responsive materials:

The acidity variations between the stomach, small intestine, and colon allow for the use of pH-responsive material to transport phages to the target site. These materials, acting as "shielding," not only keep phages alive in difficult environments (e.g., acidic pH, pepsin, bile salts), additionally, they can carry and discharge them in the colon via pH-triggered dissolution/expansion (Bazban-shotorbani et al., 2017).

#### 2.2.6.4.1. Sodium Alginate:

The most frequent pH-responsive substance utilized for encapsulation of phages is sodium alginate. Sodium alginate is a naturally existing anionic linear polysaccharide composed of β-D-mannuronic acid and α-L-guluronic acid units

connected by a β-(1, 4) glycosidic bond (Dekamin et al., 2018). Through $Na^{+}$ exchange with divalent cations (e.g., $Ca^{+2}$), the α-L-guluronic acid units can create an "egg box" configuration to enclose phages, resulting in molecular chains of alginate interwoven creating a thick gel matrix. Mannuronic acid has a pKa of 3.38, while guluronic acid has a pKa of 3.66. The carboxyl groups on the molecular structure of sodium alginate become protonated (-COO changes to -COOH) in gastric juice (pH 3.0) to generate insoluble alginic acid, which protects internal phages from the degradation of gastric acid and digesting enzymes. The alginate hydrogel swells and releases encapsulated phages in intestinal fluid (pH > 4.0). As a result, alginate gel expands and discharges in a pH-dependent manner. However, the ability to survive encapsulated phage in only alginate gel remains to be considerably compromised due to the introduction of harmful elements through the porous exterior of alginate gel (Tang et al., 2013). As a result, sodium alginate has been mixed with other materials to enhance phage protection. For example, when scientists tested the protective effect of an alginate-caseinate composite hydrogel on Salmonella phage Felix O1, they discovered that after 2 hours of simulated gastric fluid (SGF) treatment (pH = 2.0), the free phages were entirely deactivated, while the total amount of viable encapsulated phages dropped by merely 1.2 log units (Ergin et al., 2021).

#### 2.2.6.4.2. Eudragit S100:

Eudragit S100 is a synthesized pH-responsive material formed from methyl methacrylate and methacrylic acid in a 1:2 ratio, capable of retaining its structural strength in acidic environments (pH 7) and dissolving in alkaline ones (pH > 7). The active carboxylic groups in Eudragit S100 provide shielding from encapsulated compounds. These carboxylic groups are changed into carboxylate in a relatively acidic condition (a pH range of 5-7) and create a water-insoluble layer that can tolerate stomach acid and impede the diffusion of water vapor. Eudragit S100 has been extensively used as an encapsulating medium for phage targeting in the colon (Thakral et al., 2013). (Vinner et al., 2017) used Eudragit S100 in conjunction with sodium alginate to create pH-responsive micron-sized particles that entrapped the

C. difficile phage CDKM9. In their study, phages encapsulated in pure alginate particles were completely inactivated after being exposed to a simulated stomach juice however, , the incorporation of Eudragit S100 greatly increased the acidity retention of phages with just a 1.5 log reduction in phage quantity. As a result, an amalgamation of pH-responsive materials and other materials (particularly enzyme-responsive materials) is recommended.

**2.2.6.5. Other Materials:**

Along with the pH-responsive and enzyme-responsive materials previously discussed, other polymeric substances such as poly (ethylene oxide), poly(vinylpyrrolidone), poly (vinyl alcohol), cellulose diacetate, and whey protein are used to encapsulate phages. Furthermore, some tiny compounds, such as cholesterol, phosphatidylcholine, trehalose, lactose, and leucine, play a significant role in the formation of phage carriers (emulsions, liposomes, particles). These matrix substances also performed well in terms of phage viability under extreme circumstances.

**2.2.6.5.1. Emulsions:**

Emulsions are an appropriate medium with outstanding effectiveness in encapsulation that provides an ideal setting for phage. Conventional emulsions are categorized into two categories based on phase composition: oil-in-water (O/W) emulsion and water-in-oil (W/O) emulsion. Furthermore, numerous emulsions can be created by dispersing the first emulsion into another continuous state. In other words, an O/W or W/O emulsion can be further uniformed and disseminated in the oil or water phase to generate an oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) multiple emulsion. Due to the hydrophilic nature of phages, W/O and W/O/W emulsions are widely utilized to entrap them. (Rios et al., 2018) developed a W/O/W numerous emulsions to encapsulate *P. aeruginosa* phage JP004, and the encapsulation effectiveness was high. Following encapsulation, the watery core of the emulsion can create a favorable environment for the inner phages, promoting phage survival during delivery and preservation.

#### 2.2.5.6.2. Liposomes:

Liposomes are circular molecules with a cytomembrane-like framework that contain phospholipids (for example, cephalin, lecithin, and soybean phospholipids), cholesterol, and other components. An aqueous phase in the liposome core allows delicate phages to be integrated and entrapped. Furthermore, the liposome's phospholipid bilayer membrane can provide mechanical defense for inner phages. Liposomes have various advantages as a successful encapsulating technology, including ease of manufacture, biological adaptability, the ability to degrade, and affordability, which can be accomplished through simple procedures such as solvent injection procedures, thin film hydration process, and reversed evaporation (Shah et al., 2020). Liposomes have an intense preference for epithelial cell membranes due to their cytomembrane-like shape, which improves their adherence to intestinal cells. Furthermore, the positive electrical charge of cationic liposomes may combine with negatively charged parts of mucus (e.g., sialic, sulfonic acid residues) to improve mucosal adherence. As a result, one of the major benefits of liposomes for colon administration is their lengthy intestine-holding time, which improves the effectiveness of treatment. (Lai et al., 2020). Furthermore, due to their exceptional biocompatibility, liposomes could shield phages from immunological clearance by preventing phagocytes from identifying and trapping phages. The potential of liposome transporters to permeate bacterial biofilms is another key characteristic. In other situations, persistent pathogen invasion in the gut is associated with the formation of bacterial biofilms, which create significant barriers to treatment with phages. Extracellular polymeric molecules on the biofilm layer protect internal bacteria against phages, which cause chronic and recurring intestinal illness. Liposomes have been shown to have excellent anti-biofilm action, and their mechanical and chemical characteristics (e.g., diameter, surface charge) have an important influence on their functionality. The potential of liposome transporters to permeate bacterial biofilms is another key characteristic. In other situations, persistent pathogen invasion in the gut is associated with the formation of bacterial biofilms, which create significant barriers to treatment with phages

(Tytgat et al., 2019). Extracellular polymeric molecules on the biofilm layer protect internal bacteria against phages, which cause chronic and recurring intestinal illness. Liposomes have been shown to have excellent anti-biofilm action, and their mechanical and chemical characteristics (e.g., diameter, surface charge) have an important influence on their functionality.

**2.2.5.6.3. Hydrogels:**

Hydrogel is a three-dimensional in nature hydrophilic polymeric matrix that is cross-linked by either chemical or physical design as a transition material between the liquid and solid states. This conventional network arrangement is composed of two sections, namely a liquid portion (water) and a solid portion (polymers), which provides hydrogel with numerous notable qualities, including its rigidity and ability to absorb water. Porous networks in a watery setting can absorb enormous amounts of water by hydrolysis and capillary action, expand, and slowly liberate encapsulated chemicals. Extrusion dripping, electrostatic complexation, emulsion-templating, and antisolvent precipitation are all traditional ways of preparing hydrogels (Batista et al., 2019). Because of their substantial porosity and a huge certain portion of the surface, hydrogels have remarkable encapsulation effectiveness for delicate compounds. (Colom et al., 2017) successfully encased three *Salmonella* phages (, UAB_Phi78, UAB_Phi20, and UAB_Phi87) into alginate/$CaCO_3$ hydrogel microspheres made by extrusion-dripping technique, and all phage encapsulation rates were approximately 100%. Furthermore, hydrogel can shield inner phages from acidic and enzymatic breakdown while prolonging their retention duration during gastrointestinal travel, making it an appropriate medium for phage administration.

Many gastrointestinal and venous diseases have been linked to dysbiosis of the gut microbiota. Phage delivery has demonstrated huge potential in gastrointestinal regulation in situ by adjusting the makeup of gut microbiota or controlling bacterial gene interference in numerous disorders in the past few years as a unique technique due to their targeted competence to specific host bacteria. However, poor

environmental and gastrointestinal circumstances will cause phage deactivation and lowered effectiveness, offering a significant obstacle to phage administration. Luckily, an encapsulating method has been used to increase phage survivability and integrity during its transportation and storage in the gut. Several encapsulation transporters (e.g., fibers, hydrogel, particle) have been tested for their capacity to retain phage integrity during preservation, maintain phage viability, and release phage in the colon during gastrointestinal transit.

**2.2.6.6. Disadvantages of phage therapy:**

**2.2.6.6.1. Narrow host spectrum:**

Certainly, the development of effective broad-spectrum antibiotics effectively eliminated phage treatment as a viable alternative. One of the key challenges was the limited host spectrum of most phages (confined to the strain level in many cases), which necessitated a phage armament along with an understanding of the infecting variant for successful deployment. Furthermore, phages are complicated biological structures that must be replicated on an appropriate host. (Hill et al., 2018).

**2.2.6.6.2. Bacterial Resistance to Phages:**

Bacterial resistance to phage, just like antibiotic resistance develops in bacteria when they are exposed to those antibiotics, phage resistance can also easily develop (Taati Moghadam et al., 2020). In some in vitro experiments resistance can develop within 24 hours of phage exposure. Use of newly isolated phages for control of *Pseudomonas aeruginosa* PAO1 and ATCC 10145 biofilms (Örmälä & Jalasvuori, 2013). Phage resistance can develop by various methods like adaptive immunity systems restriction-modification systems, abortive infection systems, receptor alteration/mutations, and one of the most popular the CRISPR system, etc. although continuous exposure to a group of phages may allow bacteria to develop resistance and even if that resistance becomes permanent in the environment research suggests that there will always be novel infective phages available in the environment.

**2.2.6.6.3. Adverse Immune Response:**

Although bacteriophages are not harmful to human or animal cells our immune system may still recognize them as a foreign body and produce antibodies against them decreasing their PFU to lower than dose levels phages must also not be given in too high a dose to prevent severe allergic immune response (Jończyk-Matysiak et al., 2017).

**2.2.6.6.4. Unexplored Environmental Consequences:**

After phage therapy, the phages from the human body can be released into the environment which may have unintended consequences that have not been majorly explored yet (Meaden & Koskella, 2013).

**2.2.6.6.5. Endotoxin Release on Cell Death of Bacteria:**

Lytic phages cause the lysis of bacteria at the conclusion of their antibacterial action, freeing different bacterial components like endotoxin (LPS) from gram-negative bacteria. This may cause adverse reactions in a host such as an inflammatory cascade response which may lead to sepsis, shock, organ failure, or even death. But this problem is also present in rapidly acting antibacterial antibiotics and recombination of phage can be done to produce temperate phages with similar antibacterial activity in vivo as proven by Paul VD, who develo1ped recombinant lysis-deficient Staphylococcus aureus phage P954 with deactivated endolysin gene with in vivo antibacterial mortality capacity of 80% LD80 (Paul et al., 2011).

**2.2.6.6.6. Characterization and Selection of Suitable Phage From Scratch Takes Time:** Similar to antibiotic characterization which involves a long process of in vitro, ex vivo, in vivo etc. phage characterization is also a long process that may end up giving us an ineffective phage at the end, therefore it is crucial to isolate large number of phages and then start characterization process which is a very time consuming and effort taking process (Kutateladze & Adamia, 2010). It is suggested to go straight to genetic sequencing after isolation to prevent waste of

time if the phage turns out to contain virulent gene or lysogeny characteristics if we are to look for therapeutic phages (Wittebole et al., 2014).

#### 2.2.6.6.6. Inability to Infect Intracellular Pathogenic Bacteria:

Most phages are very efficient at adsorption, and it is assumed to take only 5 mins to adsorb onto the surface receptor of host bacteria but when their host enters eukaryotic cells the phages lose their access to their receptors and are unable to infect these cells. These bacteria are called intracellular pathogens. Important species of intracellular bacteria belong to the Salmonella, Listeria, Brucella, Rickettsia, and Legionella genera (Doss et al., 2017; Sulakvelidze et al., 2001). Although conflicting research has arisen which points out the ability of some phages to infect salmonella in poultry so further research is required (Wernicki et al., 2017).

# CHAPTER 3: MATERIALS AND METHODOLOGY

## 3. Material and Methodology:

### 3.1. Synergistic effect of phages with antibiotics:

#### 3.1.1. Bacteriophages:

I acquired well-isolated and well-characterized bacteriophages designated KPA and KPM against *Klebsiella pneumonaie* from the Applied and Environmental Geomicrobiology lab of the Department of Microbiology's bacteriophages research group. KPA and KPM bacteriophages are members of the *siphovirodae* and *podivirodae* families, respectively.

#### 3.1.2. Isolation and Characterization of Host Bacterium and Its Antibiotics Resistance Determination

The pathogenic strain of *K. pneumoniae* was obtained from the urine specimen of a 30-year-old catheterized female patient undergoing spinal surgery for a spinal tumor. This strain was Gram stained following the protocol described previously, and this strain was grown on eosin methylene blue agar (EMB) purchased from Sigma-Aldrich for observing colony morphology and comparing it with colony morphology of *K. pneumoniae* described in previous literature. This strain was tested for biofilm production using the microtiter plate assay and biofilm strength was calculated by using the formula previously described by (Stepanović et al. 2007). This strain was tested against thirteen antibiotics according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Table 3.1). These antibiotics are often used for the treatment of illnesses caused by bacteria in the order Enterobacterales. The antimicrobial resistance profile of the host isolate was determined by a disc diffusion assay and the antibiotic discs used in this study were manufactured by Oxide UK.

| Antibiotics | Concentration µg/disk | Susceptible concentration in µg | Resistant concentration in µg |
|---|---|---|---|
| Amoxicillin | 10 µg | ≤8/4 | ≥32 |
| Piperacillin | 100 µg | ≤4/8 | ≥16/8 |
| Ceftazidime | 30 µg | ≤4 | ≥16 |
| Cefepime | 30 µg | ≤2 | ≥16 |
| Cefazolin | 30 µg | ≤16 | ≥32 |
| Cefixime | 5 µg | ≤1 | ≥4 |
| Gentamicin | 10 µg | ≤4 | ≥16 |
| Doxycycline | 30 µg | ≤4 | ≥16 |
| Chloramphenicol | 30 µg | ≤8 | ≥32 |
| Sulfamethoxazole trimethoprim | 25 µg | ≤2/38 | ≥4/76 |
| Meropenem | 10 µg | ≤1 | ≥4 |
| Fosfomycin | 200 µg | ≤64 | ≥256 |
| Nitrofurantoin | 300 µg | ≤32 | ≥128 |

**Table 3.1.** shows the CLSI-defined susceptible and resistant antibiotic concentrations and the antibiotic-loaded concentrations of the discs used in this study.

### 3.1.3. Bacteriophages Against *K. pneumoniae*:

Phages named KPA and KPM used in this study were isolated and characterized by our bacteriophages research group of Applied, Environmental and Geomicrobiology Laboratory of Quaid-i-Azam University, Islamabad, Pakistan from hospital wastewater samples against the *K. pneumoniae* strain isolated from urine sample of elderly female patient with urinary tract infection. The strain used in this study was susceptible to both phages with the efficiency of plating of KPA to our *K. pneumoniae* strain was 93% while the efficiency of plating of KPM to our *K. Pneumoniae* strain was approximately 100% that was determined by dividing the number of plaques of tested phage against tested strain to the number of plaques of the tested phage against its own host strain. The titer of these bacteriophages was increased by adding 100µl of bacteriophages filtrate in host bacterial culture in its log phase ($OD_{600}$ 0.37) in 50 ml nutrient broths, after 24 hours of bacteriophages infection to host bacterium, both phages were purified by treating broth with 10% chloroform of the total volume of phages inoculated bacterial broths for releasing the entrapped bacteriophages from infected host cells, after 1 hour of incubation, chloroform was separated by using separating funnel and the aqueous portion was spun at 10,000 rpm for 5 minutes, the supernatant was recovered and filtered through Millipore syringe filters with 0.22 µm syringe filters, 100µl from both phages' filtrates was serially diluted and PFU was calculated out by using double layer agar assay of the dilutions.

### 3.1.4. MIC Determination:

Firstly, we determined the MIC of *Klebsiella Pneumoniae* to 3 different antibiotics (Gentamicin, Cefepime, and Meropenem) among 13 tested antibiotics. Stock solutions with a concentration of 512µg/ml were prepared. MICs were calculated using a 96-well microtiter plate method described by (Rahman et al., 2004). Briefly, overnight-grown bacterial suspension was diluted in double-strength nutrient broth to $OD_{600}$ 0.3. Into each well 100µl of bacterial suspension and the same volume of

antibiotic dilutions were added up to the 11th well to obtain the final concentration of 0.25µg/ml. The microtiter plate was incubated at 37°C for 24hrs. Controls for plate sterility and bacterial growth without antibiotics were also added. The lowest concentration of antibiotic-inhibited bacterial growth, which was visible with the naked eye was considered the MIC and was confirmed by taking OD at 600nm in microtiter plate reader. The MIC results were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibiotic breakpoints and concentration are presented in Table 3.2. Four sub-inhibitory concentrations of three selected antibiotics were chosen for the phage-antibiotics synergy study (Table 3.2).

| **Antibiotics Resistant ranges** | **Antibiotics MIC** | **Selected concentrations for PAS(µg/ml)** | | | |
|---|---|---|---|---|---|
| | | **½** | **1/4** | **1/8** | **1/16** |
| Gentamicin ≥16 µg/ml | 8 µg | 4 µg | 2 µg | 1µg | 0.5µg |
| Cefepime ≥16 µg/ml | 16 µg | 8 µg | 4 µg | 2 µg | 1 µg |
| *Meropenem ≥4 µg/ml | 2 µg | 1 µg | 0.5 µg | 1.25 µg | 0.125µg |

**Table 3.2.** shows the minimum inhibitory concentration of our three selected drugs and their sub-inhibitory concentration used in this phage-antibiotic synergy study. We discovered that strain is cefepime resistant, moderate to gentamicin, and susceptible* to meropenem.

### 3.1.5. Stability of KPA and KPM with Sub-Inhibitory Concentrations of Antibiotics:

100 µL of bacteriophages (KPA and KPM) filtrates were inoculated with 100 µL of the selected antibiotic dilutions and incubated for 60 minutes at room temperature. After incubation these were diluted and subjected to bilayer agar assay and the number of plaques of KPA and KPM phages in the antibiotic dilutions were counted and compared to the PFU of the controls (phage filtrates without antibiotics).

### 3.1.6. Effect of Different MOIs of KPA and KPM Alone and in Combination on Turbidity Reduction and Colony Forming Unit Reduction of Host Bacterium

To evaluate the effect of different MOIs of KPA and KPM on host bacterium reduction, host bacterium diluted in double strength nutrient broth to obtain final $OD_{600}$ 0.5 (CFU $1.5\times10^8$/ml) was inoculated with KPA and KPM phages with the MOIs of 20, 10, 1, 0.1 and 0.01 in 1:1 in microtiter polystyrene flat bottom plate and incubated for 24 hours, 100µl of host bacterium was added with 100µl of diluent (normal saline 0.9% NaCl) used for the phage dilution was used as control and diluent alone and nutrient broth alone was used as negative control. Reduction in $OD_{600}$ of phage treated bacteria at different MOI was observed after 24 hours in microtiter plate reader and compared with the OD of control (bacteria without bacteriophages). After 24 hours samples were drawn from each well, 10, 100 and 1000 folds diluted and spread on eosin methylene blue agar to find out the reduction in colony-forming units.

Cocktail of KPA and KPM was made based on the results obtained from the above experiment by mixing both at MOIs that didn't eradicate host bacterium completely to know the synergistic effect of both phages. Sub-inhibitory MOI of KPA and KPM were combined in 1:1 and mixed with the host bacterium with $OD_{600}$ 0.5 in the ratio of 1:1 in microtiter plate and after 24 hours, $OD_{600}$ was checked and

compared with the controls (host bacterium, host bacterium + KPA, and host bacterium + KPM). After 24 hours the sample was drawn from each well, diluted, and spread on eosine methylene blue agar to find out the reduction in CFU of the host bacterium.

### 3.1.7. Synergistic Effect of KPA and KPM and their Cocktail with antibiotics on Host bacterium:

100µl of sub-inhibitory concentrations of antibiotics were mixed with 100µl of host bacteria added with bacteriophages in double-strength nutrient broth at desired MOI. In this experiment, the OD of the host bacterium at 600nm was 0.5 while the sub-inhibitory concentration of bacteriophages was used. KPA and KPM mixed in 1:1 was tested with sub-inhibitory concentrations of antibiotics on host turbidity reduction and CFU reduction. OD at 600nm was checked after 24 hours and compared with the OD of the control. After 24 hours samples were drawn from each well, diluted, and spread on eosine methylene blue agar to find out colony-forming units of *K. pneumoniae*.

### 3.1.8. Synergistic Effect of KPA and KPM and their Cocktail with Antibiotics on Biofilm Formation Inhibition:

Host bacterium diluted in double-strength nutrient broth to obtain OD 0.5 at 600nm was mixed with bacteriophages and bacteriophages cocktail with 100µl of different concentrations of antibiotics and incubated for 24 hours at 37°C in the static incubator. After 24 hours, all the wells were washed with tap water, dried, and stained with 0.1% crystal violet, and incubated for 15 minutes. After incubation, all the wells were washed, air dried, and crystal violet was dissolved in 96% ethanol and biofilm was quantified by checking absorbance at 570nm and compared with the control.

### 3.1.9. Biofilm Reduction by KPA, KPM and their cocktail

10ml of 24 hours old culture of host bacterium was poured in sterilized glass petri dishes in biosafety hood, sterilized glass slides were immersed in culture and plates

were incubated at 37°C in static incubator for 24 hours, after incubation 10ml of the phage filtrate of KPA, KPM and mixed was added and thoroughly mixed, and incubation of 24 hours in a static incubator at 37°C was given. On control slides 10ml of normal saline was added and incubated in the same conditions. After incubation, all the slides were washed, dried, stained with 1% crystal violet, washed, dried, and checked for biofilm reduction pattern under light microscopy.

### 3.2. Microencapsulation of phages in different polymeric substances:

#### 3.2.1. Bacteriophage Isolation:

I have received well-isolated and well-characterized bacteriophages designated KPA and KPM against *Klebsiella Pneumonaie* from our bacteriophage research group in the Applied and Environmental Geomicrobiology lab of the Department of Microbiology. KPA and KPM are the lytic phages and belong to *siphoviridae* and *podivirodae* families, respectively.

#### 3.2.2. Stability of pure phage lysate at different gastronomic pH:

Before encapsulating phages in different polymeric substances, KPA and KPM phage was tested for pH stability in 0.9% NaCl solution regulated to various pH levels (pH 2 to 7.4). 1998µl of different pH solutions and 2µl of phage were added and incubated for 1 hour. To ensure sterility, we run a negative control with 2µl of phage lysate in 1998µl of normal saline. We further diluted each pH solution containing phages and performed DLA to determine their stability.

#### 3.2.3. Microencapsulation of Phages in different Polymeric substances:

Phages are highly unstable in different gastronomic pH and different polymeric substances are used to microencapsulate phages to increase the stability, shelf life of phages, enabling their control release and improving their delivery to target sites. Polymeric substances used are highly biocompatible, non-toxic, obtained from natural sources, economically affordable and have ease of gelation.

Different polymeric substances used for encapsulation are as follows:

- 2% of Sodium Alginate
- 2% Sodium Alginate: 1%Agarose
- 2% Sodium Alginate: 1% Gelatin
- 2% Sodium Alginate: 1% Polyethylene Glycol (PEG)

**3.2.3.1. Microencapsulation of phages within alginate beads:**

Phages were encapsulated into alginate beads using a relatively traditional method by (Krasaekoopt et al., 2004). To make the capsules, a mixture of 5ml of KPA phage with ($9.7x10^{13}$ PFU/ml) and 45ml of distilled water containing 2% (w/v) sodium alginate (Sigma-Aldrich, Munich, Germany) solution was dispensed into 1% CaCl2 solution using a syringe. They were gently shaken at room temperature for about 30 minutes to fully cross-link the calcium alginate beads walls. Before moving on to the next experiment, the beads were filtered and washed with distilled water. The same experiment was performed with KPM phage containing ($2.0x10^{13}$ PFU/ml).

**3.2.3.2. Synthesis of Agarose-alginate capsules:**

To improve stability (avoid deterioration) and regulate the release of encapsulated phages hybrid encapsulation of phages with sodium alginate, and agarose was performed. To create the Ag-Al hydrogels, the subsequent steps were taken. 2% of sodium alginate solution and 1% of agarose solution can be prepared by dissolving the 1 g of sodium alginate and 0.5g of agarose in 45 ml of distilled water under constant stirring at room temperature, the solution was heated in the microwave and stirred occasionally until completely dissolved. To entrap the phages in Ag-Al beads, we added 5ml of pure phage of KPA containing ($9.7x10^{13}$ PFU/ml) and KPM with ($2.0x10^{13}$ PFU/ml), respectively in Ag-Al solution, they were promptly put into syringes with 21 G hypodermic needles. The needle's tip was held 30mm over the surface of the CaCl2 solution. At room temperature, the Ag-Al solution was added gradually drop by drop into a 1% CaCl2 solution while gently agitating. Before proceeding to the next experiment, the beads were filtered and washed with distilled water. To see the development and swelling behavior of Ag-Al

microcapsules harboring phages with the naked eye, different colors were introduced to the initial solutions.

**3.2.3.3. Synthesis of Al-Gelatin capsules:**

2% of sodium alginate solution and 1% of gelatin solution can be prepared by dissolving the 1 g of sodium alginate and 0.5g of gelatin in 45 ml of distilled water under constant stirring at room temperature, the solution was heated in the microwave and stirred occasionally until completely dissolved. To entrap the phages in Al-Gelatin beads, we added 5ml of pure phage of KPA containing ($9.7x10^{13}$ PFU/ml) and KPM with ($2.0x10^{13}$ PFU/ml), respectively in Al-gelatin solution and immediately loaded them into syringes with 21 G hypodermic needles attached. The needle's tip was held 30mm above the surface of the CaCl2 solution. At room temperature, the Al-Gelatin solution was added dropwise into a 1% CaCl2 solution while gently stirring. Before moving on to the next experiment, the beads were filtered and washed with distilled water. To monitor the development and swelling behavior of Al-Gelatin microcapsules retaining phages with the naked eye, various colors were introduced to the preliminary solutions.

**3.2.3.4. Synthesis of Al-PEG capsules:**

2% of sodium alginate solution and 1% of PEG solution can be prepared by dissolving the 1 g of sodium alginate and 0.5g of PEG in 45 ml of distilled water under constant stirring at room temperature, the solution was heated in the microwave, and stirred occasionally until completely dissolved. To entrap the phages in Al-PEG beads, we added 5ml of pure phage of KPA containing ($9.7x10^{13}$ PFU/ml) and KPM with ($2.0x10^{13}$ PFU/ml), respectively in Al-PEG solution and immediately loaded them into syringes with 21 G hypodermic needles attached. The end of the needle was kept 30mm above the surface of the $CaCl_2$ solution. The Al-PEG solution was added dropwise into 1% $CaCl_2$ solution under gentle stirring at room temperature. The beads produced were filtered out and rinsed with distilled water before being placed for the next experiment. Different pigments were added

to the initial solutions to observe the formation and swelling behavior of Al-PEG microcapsules carrying phages with the naked eye.

### 3.2.4. Phage Loading Efficiency:

The microcapsules loaded with phages generated in previous procedures were treated with a breaking solution (50mM sodium citrate, 0.2M sodium bicarbonate, and 50mM Tris-HCl, pH 7.5) to get the phage loadings., by dissolving 2 capsules in 1ml of breaking solution and incubate overnight at room temperature. The phages liberated from the capsules were determined by phage titer by performing a double-layer agar assay. Phage loading efficiency was determined by comparing the phages released from the capsules with free phages in the supernatant to the total number of entrapped phages. Phage Loading Efficiency can be calculated by the formula given below:

$$= 100 - \frac{(\text{Free phages in } CaCl_2 \text{ Soln.})}{(\text{Total phages entrapped})} \times 100$$

### 3.2.5. Accessing the preservation and stability of capsules upon storage:

To test the preservation and stability of encapsulated bacteriophages, capsules were maintained in phosphate buffered saline (PBS) at 4°C and samples were taken every 8 days and after 8 weeks. Under the storage settings, no phage was released during the duration of this study.

### 3.2.6. Dried Capsules Characterization:

All the capsules (Alginate, Alginate +Agarose, Alginate +Gelatin, Alginate + PEG) will be air dried. For morphological characterization, optical microscopy of capsules was performed. Capsules were placed on a glass slide on a mechanical stage and examined with a 4X magnification lens. The weight of each capsule was determined by placing one capsule of each polymer on a digital weighing balance and recording the reading of each capsule in grams.

### 3.2.7. Effect of drying on the viability of phages in capsules:

To observe the effect of drying on the viability of phages in capsules, we performed a plaque assay and compared the phage titer of dried capsules with control.

### 3.2.8. Stability of capsules in different gastronomic pH solutions:

Phage release in different pH solutions was determined by incubating 2 dried capsules in 1ml of each pH solution like 2 (stomach), 5.7 (caecum), 6 (duodenum), 6.7 (rectum), and 7.4 (small intestine) and incubated for 2 hours. To determine the free phages in the supernatant we centrifuge it and perform the DLA. Microcapsule-carrying phages were incubated in the breaking solution and to determine the stability and entrapment of phages in capsules a Double layer agar assay was performed.

### 3.2.9. Stability of free and microencapsulated phages in simulated gastronomic conditions:

To simulate the behavior of both free and encapsulated phages in the GI system, they were incubated in “simulated gastric fluid” (SGF) pepsin and in “simulated intestinal fluid” (SIF) pancreatin according to approach used by (Richards et al., 2021).

#### 3.2.9.1. Phage release in Simulated Gastric Fluid (SGF):

First, the stability of free and microenca

psulated phages was investigated in SGF which was prepared by dissolving 32mg of pepsin in 10ml of 0.2%(w/v) NaCl solution at pH 2.5 adjusted with 1M HCl. 2 capsules per 1ml of SGF solution were added at 37°C for 2hr. Free phage samples were obtained, diluted, and promptly tested for phage survival. Phage survival of microcapsule-carrying phages was determined by performing a double-layer agar assay.

#### 3.2.9.2. Phage release in Simulated Intestinal Fluid (SIF):

Phage release in the simulated intestinal fluid was determined by incubating the 2 capsules in 1ml of Simulated Intestinal Fluid (SIF) which was prepared by

dissolving 10mg/ml of pancreatin in 50mM $KH_2PO_4$ at pH 6.8 by gently shaking and incubating for up to 2hrs. Double-layer agar assay determined the stability of microcapsule-carrying phages and free phages in the supernatant.

# CHAPETR 4: RESULTS

## 4: Results:

### 4.1. Synergistic effect of Phages with antibiotics:

#### 4.1.1. Isolation and Characterization of Host Bacterium and Its Antibiotics Resistance Determination

The host bacterium isolated from the urine specimen of a 32-year-old catheterized patient who had undergone spinal surgery to remove a spinal tumor was Gram-stained and appeared Gram-negative and produced mucoid, large, convex, pink-colored colonies with a dark center on eosin methylene blue agar (Fig. 4.1), and these are the distinctive feature of *K. pneumoniae*.

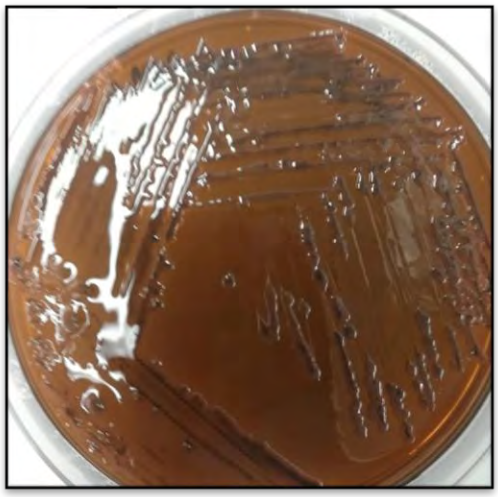

**Fig 4.1**: depicts the colony morphology of *K. pneumoniae* on eosine methylene blue agar.

The isolated *K. pneumoniae* strain was tested for biofilm forming potential by microtiter plate assay and the OD at 540 nm of the negative control was 0.0960±001 and the OD of *K. pneumoniae* strain was 0.9430±0.32, which is ten times higher than the OD of the negative control and indicates that this *K. pneumoniae* strain is a strong biofilm producer.

The antibiotics resistance pattern of this strain was assessed by disc diffusion assay following the CLSI guidelines, this strain appeared as resistant to 11 tested antibiotics (Table 3) and sensitive to two antibiotics.

| Antibiotics | Concentration µg/disc | Zone of inhibition | S/R/I |
|---|---|---|---|
| Amoxicillin | 10 µg | 0mm | Resistant |
| Piperacillin | 100 µg | 0mm | Resistant |
| Ceftazidime | 30 µg | 0mm | Resistant |
| Cefepime | 30 µg | 13mm | Resistant |
| Cefazolin | 30 µg | 0mm | Resistant |
| Cefixime | 5 µg | 0mm | Resistant |
| Gentamicin | 10 µg | 16mm | Intermediate |
| Doxycycline | 30 µg | 9mm | Resistant |
| Chloremphenicol | 30 µg | 29mm | Sensitive |
| Sulfamethoxazole trimethoprim | 25 µg | 0mm | Resistant |
| Meropenem | 10 µg | 34mm | Sensitive |
| Fosfomycin | 200 µg | 0mm | Resistant |
| Nitrofurantoin | 300 µg | 11mm | Resistant |

**Table 4.1:** shows the resistance pattern of *K. pneumoniae* used in this study.

### 4.1.2. Bacteriophages Against *K. pneumoniae*:

The bacteriophages KPA and KPM used in this study were obtained from the Bacteriophage Research Group at the Laboratory of Applied and Geomicrobiology, Quaid-i-Azam University, Islamabad, Pakistan. The sensitivity of the *K. pneumoniae* strain to these phages was tested (Fig 4.2a and 4.2b) and the plating efficiency of both phages on the *K. pneumoniae* strain was calculated by dividing the average number of phage plaques against the tested strain by the average number of phage plaques divided against its original host. The KPA efficiency was 0.93 while the KPM plating efficiency was 0.86. An efficiency greater than 0.5 indicates that the phage is virulent.

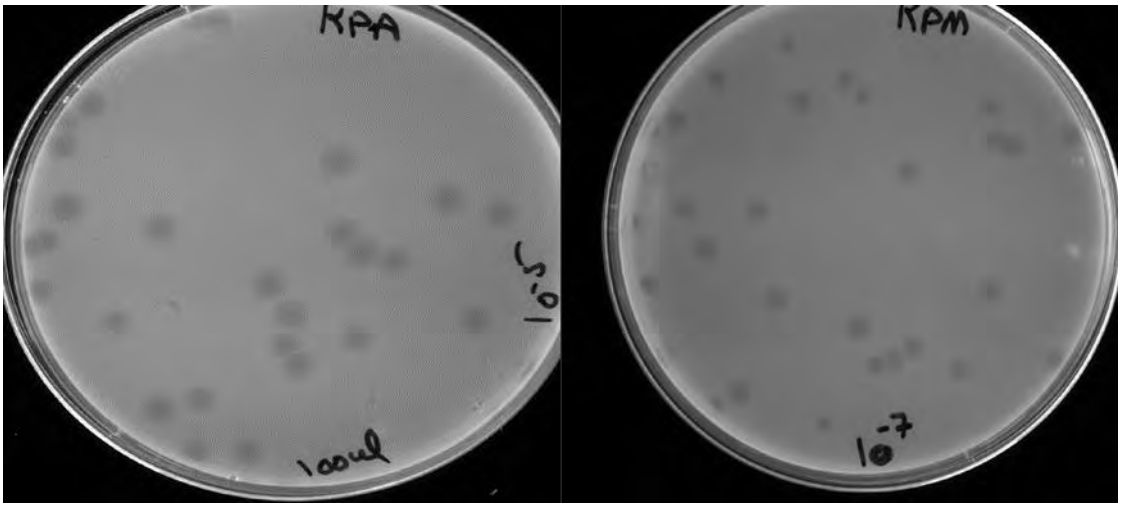


**Fig 4.2a and 4.2b:** depicts the plaques of KPA and KPM against *K. Pneumoniae* respectively.

### 4.1.3. MIC Determination:

Three antibiotics, cefepime, gentamicin and meropenem, were chosen to evaluate their synergistic effects with KPA and KPM in the eradication of *K. pneumoniae*. The MIC for cefepime was 16 µg, for gentamicin 8 µg and for meropenem 2µ g against *K. pneumoniae*. *K. pneumoniae* was susceptible to meropenem according to CLSI guidelines, resistant to cefepime, and gentamicin had an intermediate effect. The four sub-inhibitory concentrations (Table 3.2) of these antibiotics were tested with bacteriophage for their synergistic effect.

### 4.1.4. Stability of KPA and KPM with Sub-Inhibitory Concentrations of Antibiotics:

The stability of KPA and KPM was tested in four sub-inhibitory concentrations of cefepime (8µg, 4µg, 2.6µg, and 2µg) gentamicin (4µg, 2µg, 1.3µg, and 1µg) and meropenem (1µg, 0.5µg, 0.3µg, and 0.25µg). After one-hour incubation of KPA and KPM in sub inhibitory concentrations of selected antibiotics, the PFU was determined by a bilayer agar assay and compared to the PFU of the control without antibiotics. Sub inhibitory concentrations of selected antibiotics did not adversely affect phage stability. No increase in KPM was observed in all the sub-inhibitory concentrations of antibiotics but the increase in plaque size of KPA was observed in sub-inhibitory concentrations of Cefepime and Meropenem and plaque size was increased to 5mm from 3mm (control) (Fig 4.3).

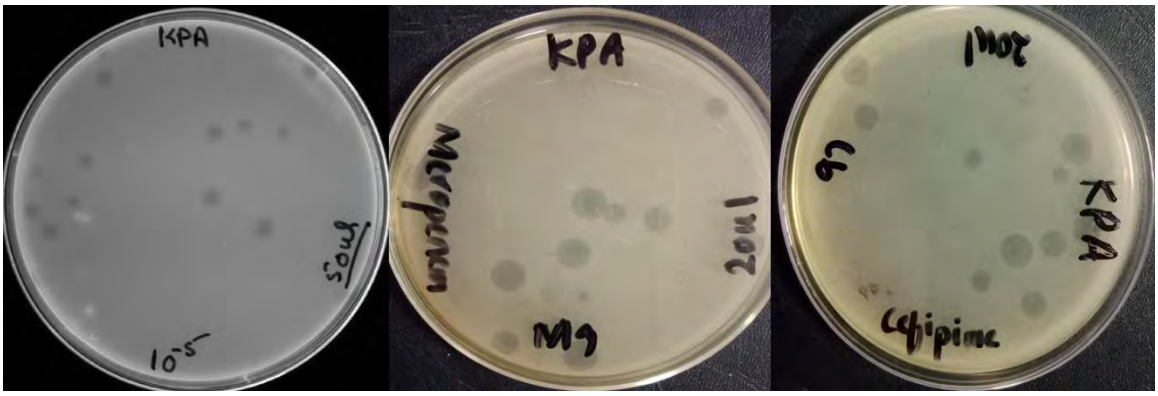


**Fig 4.3.** demonstrate the increase in plaque size of KPA with sub-inhibitory concentrations of meropenem and cefepime.

### 4.1.5. Effect of Different MOIs of KPA and KPM Alone and in Combination on Turbidity Reduction and Colony Forming Unit Reduction of Host Bacterium

The effect of different MOIs of KPA and KPM on host bacterium turbidity and CFU reduction was tested. KPA appeared to be the most efficient, killing all host bacteria at MOI 20, 10 and 1, while KPM killed all host bacteria at MOI 20 and 10 but not at MOI 1 and lower MOIs (Fig. 4.4a and 4.4c). The combination of KPA at

MOI 0.1 and KPM at MOI 1 resulted in a significant reduction in host bacterium CFU compared to the use of KPA alone (Fig. 4.4b and 4.4d).

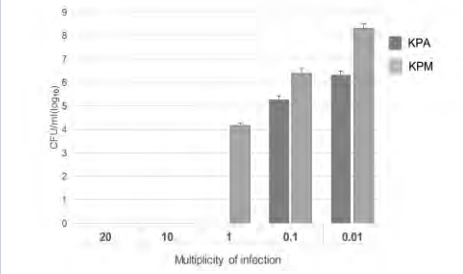


Fig.4.4a

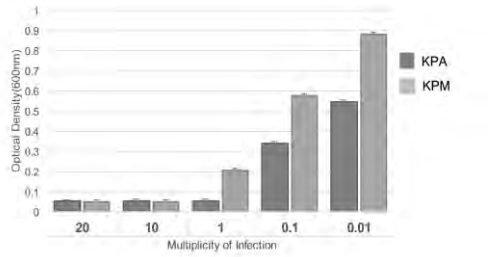


Fig. 4.4b

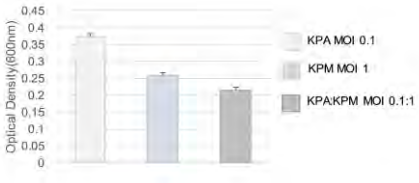


Fig. 4.4c

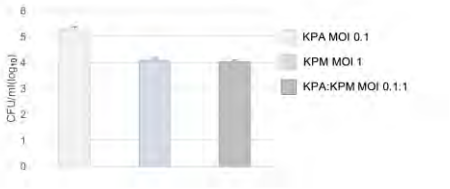


Fig. 4.4d

**Fig 4.4a and 4.4b** shows that KPA kill all the host bacteria at MOI 20, 10, and1, while KPM demonstrate the efficient killing of host bacteria only at MOI 20 and 10. **Fig 4.4c and 4.4d** shows that combination of KPA and KPM at MOI (0.1:1) result in significant reduction of host bacterium CFU, as compared to KPA and KPM used alone.

### 4.1.6. Synergistic Effect of KPA and KPM and their Cocktail with antibiotics on Host bacterium:

The synergistic effect of KPA, KPM and their cocktail with four sub-inhibitory concentrations of cefepime (8 µg, 4 µg, 2µg and 1µg), gentamicin (4 µg, 2 µg, 1µg and 0.5µg) and meropenem (1 µg, 0.5 µg, 1.25µg and 0.125 µg) on turbidity and CFU reduction was evaluated. Cefepime caused 100% killing of the host bacterium when used at a concentration of 8 µg and 4 µg with KPA at a MOI of 0.1, and at a

concentration of 2 µg with KPA at a MOI of 0.1, there was a three-log reduction in CFU of the host bacterium compared to CFU reduction by cefepime alone in the same concentration (Fig. 4.5a and 4.5b). KPM at MOI 1, when used with 8 µg of cefepime, resulted in a 6-log reduction in CFU compared to cefepime used alone at the same concentration, and at the 4µg concentration of cefepime with KPM at MOI 1, a 5-log reduction in CFU was observed (Fig. 4.5a and 4.5b). Cefepime at a concentration of 8 µg and 4 µg with a cocktail of KPA and KPM resulted in a 100% reduction in CFU and the lower sub-inhibitory concentration of cefepime with KPA and KPM cocktail resulted in a significant reduction in turbidity and CFU compared to reduction caused by cefepime, cefepime with KPA and cefepime with KPM (Fig. 4.5a and 4.5b).

Gentamicin with KPA and KPM at sub-inhibitory concentrations did not cause a significant reduction in CFU and turbidity of the host bacterium but the cocktail of KPA and KPM at all the sub-inhibitory concentrations caused significant reduction in CFU and turbidity of host bacterium (Fig 4.5c and 4.5d).

Meropenem at sub-inhibitory concentration with KPA, KPM and their cocktail caused maximum reduction of host bacterium compared to Cefepime and Gentamicin. Meropenem at the concentration of 1µg, and 0.5µg with KPA, KPM and their cocktail caused 100% reduction of the host bacterium and meropenem at the concentration of 0.25µg with cocktail of KPA and KPM caused 100% host bacterium reduction. Meropenem at the concentration of 0.25µg with KPA cause three log reduction of host bacterium while with KPM cause 2 log reduction of host bacterium and Meropenem at the concentration of 0.125µg with KPA and KPM cocktail caused four log host bacterium reduction (Fig 4.5e and 4.5f).

By this study, Meropenem and cefepime showed synergistic effect with KPA, KPM and their cocktail and Gentamicin combined with bacteriophages. They didn't appear as promising combination to be considered for phage-antibiotic synergy.

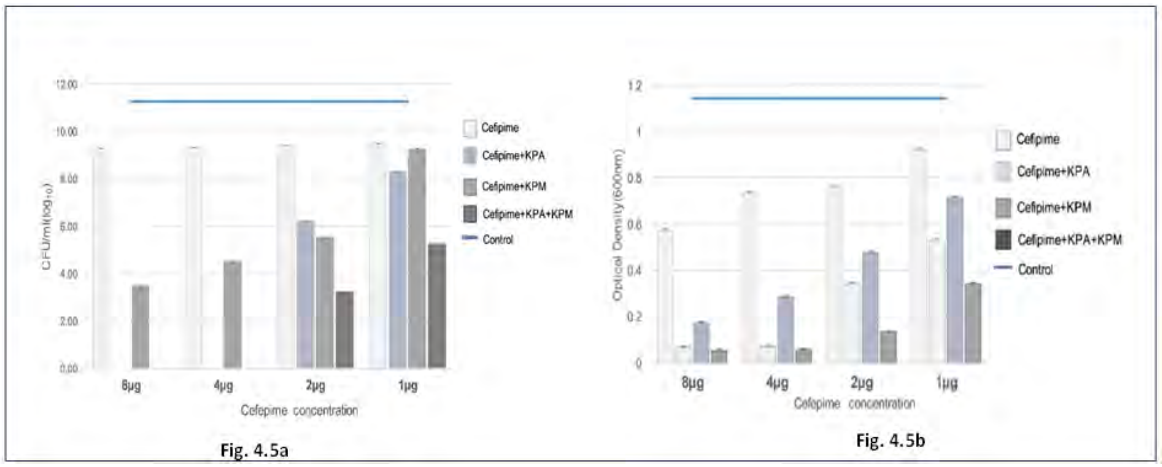


Fig. 4.5a Fig. 4.5b

**Fig 4.5a and 4.5b** shows that cefepime at 8µg and 4µg causes 100% killing of host bacterium with KPA phage, while at 2µg resulted in 3-log reduction of host bacterium, but in case of KPM 8µg of cefepime results in 6-log reduction and 4µg of cefepime results in 5-log reduction of host bacterium as compared to control. Cefepime at concentration of 8µg and 4µg with phage cocktail KPA: KPM results in 100% reduction of host bacterium.

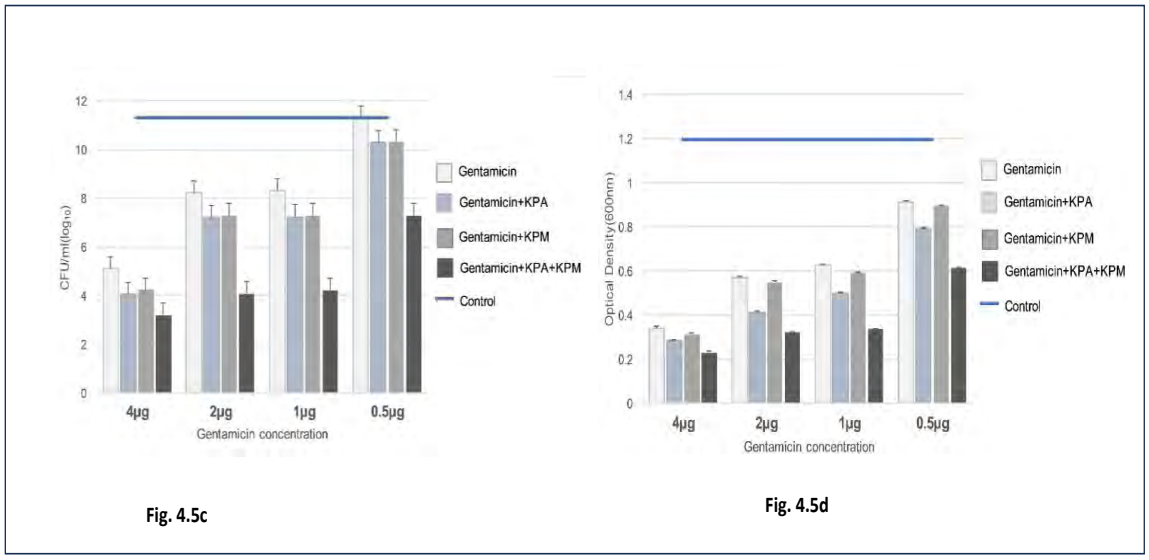


Fig. 4.5c Fig. 4.5d

**Fig 4.5c and 4.5d.** shows that gentamicin at sub-inhibitory concentration with KPA and KPM individually does not result in significant reduction of host bacterium while the sub-inhibitory concentration of gentamicin with phage cocktail KPA: KPM results in significant reduction in turbidity and CFU of host bacterium.

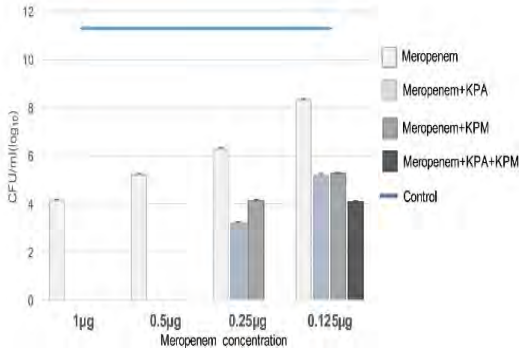


Fig 4.5e

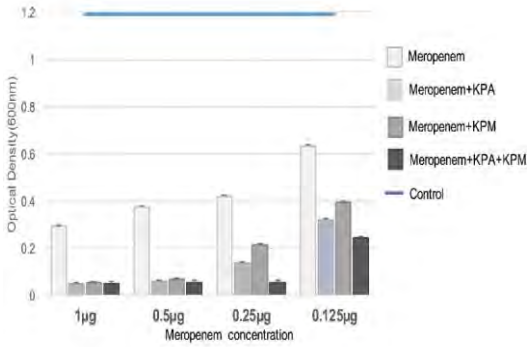


Fig. 4.5f

**Fig 4.5e and 4.5f** demonstrates that meropenem at 1µg and 0.5µg results in 100% killing of the host bacterium when used with KPA, KPM and KPA: KPM phage cocktail, however meropenem at 0.25µg with phage cocktail KPA: KPM results in 100% reduction while with KPA and KPM individually results in 3-log and 2-log reduction respectively. Meropenem at 0.125µg with phage cocktail KPA: KPM results in a 4-log reduction of the host bacterium.

### 4.1.7. Synergistic Effect of KPA and KPM and their Cocktail with Antibiotics on Biofilm Formation Inhibition

Biofilm formation inhibition potential of KPA, KPM, and their cocktail at the MOI of 0.1:1 was evaluated in this experiment. The biofilm formation inhibition potential of KPA was higher than KPM, but their combination worked better than both alone (Fig 4.6a).

Biofilm formation inhibition was observed when KPA and KPM were used with all the subinhibitory concentrations of cefepime but the combination of KPA with cefepime inhibited biofilm formation greater than KPM with cefepime. KPA and KPM cocktail with cefepime at its sub-inhibitory concentration significantly inhibited biofilm formation compared to an antibiotic, KPA, KPM, and their cocktail alone (Fig 4.6b).

The cocktail of bacteriophages with gentamicin worked better than gentamicin alone, gentamicin with KPA, gentamicin with KPM, KPA alone, KPM alone, and their cocktail alone (Fig 4.6c). No biofilm was observed when the cocktail of KPA and KPM was used with 4µg and 2µg of gentamicin.

All sub-inhibitory concentrations of meropenem with KPA, KPM, and their cocktail inhibited biofilm formation. No biofilm was observed when KPA and KPM were used at 1 g and 0.5 µg meropenem and when a cocktail of KPA and KPM at 1 µg, 0.5 µg, and 0.125 µg meropenem was used (Fig. 4.6d).

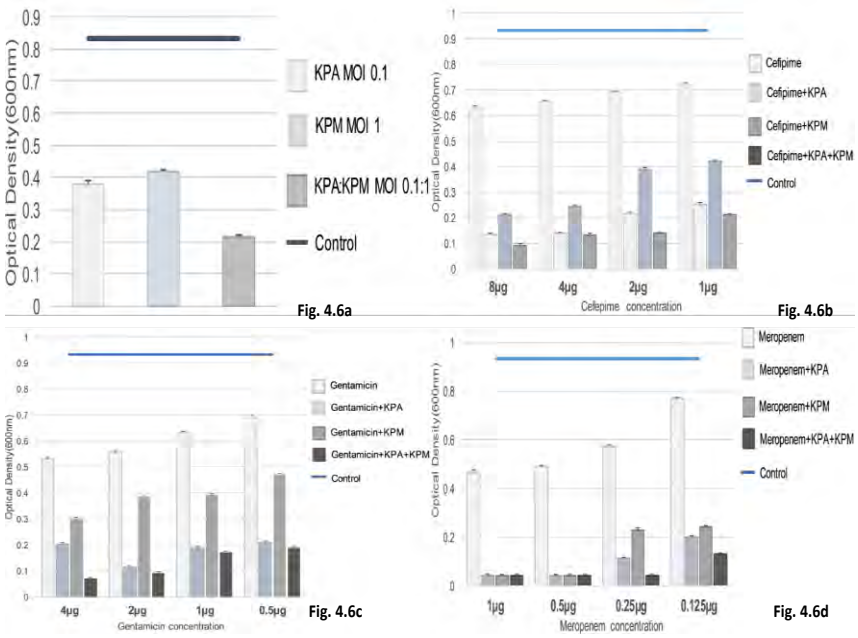


**Fig 4.6a** shows that biofilm formation inhibition potential of KPA was greater than KPM, but their cocktail (KPA: KPM) worked better than both alone.

**Fig 4.6b** shows that combination of KPA with cefepime inhibited biofilm formation greater than KPM with cefepime. However, phage cocktail (KPA: KPM) with cefepime at its sub-inhibitory concentration significantly inhibited biofilm formation compared to an antibiotic, KPA, KPM, and their cocktail alone.

**Fig 4.6c** demonstrates that cocktail of bacteriophages with gentamicin worked better than gentamicin alone, gentamicin with KPA, gentamicin with KPM, KPA alone, KPM alone, and their cocktail alone.

**Fig 4.6d.** shows that no biofilm was observed when KPA and KPM were used at 1 µg and 0.5 µg meropenem and when a cocktail of KPA and KPM at 1 µg, 0.5 µg, and 0.125 µg meropenem was used.

### 4.1.8. Biofilm Reduction by KPA, KPM and their cocktail:

Biofilm was formed on glass slides by immersing in bacterial broth culture in petri dishes, these biofilms were treated with KPA phage, KPM, and their cocktail for 24 hours, these slides were washed thoroughly with tap water, dried, and stained with 0.1% crystal violet and examined by light microscope. The control slide had biofilm of distinctive geometry (4.7a), no proper biofilm was observed on the slide treated with KPA phage (fig 4.7b), KPM treated slide had dense biofilm but lower than the control (fig 4.7c), and no biofilm was observed on slides treated with a cocktail of KPA and KPM (fig 4.7d).

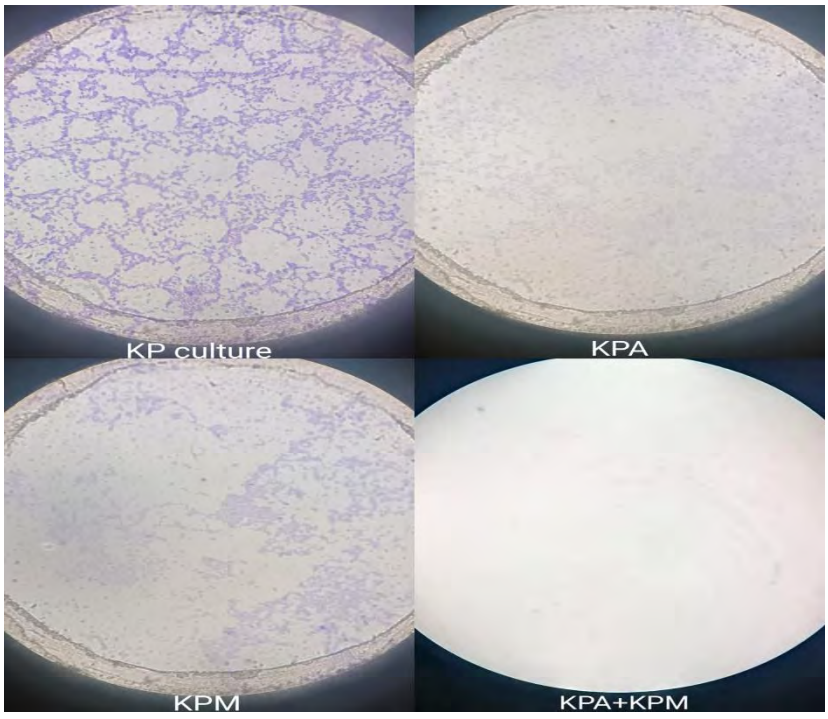


**Fig 4.7a:** shows that the control slide had biofilm of distinctive geometry,

**Fig 4.7b:** shows no proper biofilm was observed on the slide treated with KPA phage,

**Fig 4.7c:** shows that KPM treated slide had dense biofilm but lower than the control,

**Fig 4.7d:** demonstrates that no biofilm was observed on slides treated with a cocktail of KPA and KPM.

### 4.2. Encapsulation results:

#### 4.2.1. Stability of pure phage lysate at different gastronomic pH:

Before moving towards the encapsulation, we determine the stability of pure phages lysate of KPA and KPM at different gastronomic pH levels such as 2 (stomach), 5.7 (caecum), 6 (duodenum), 6.7 (rectum), and 7.4 (small intestine).

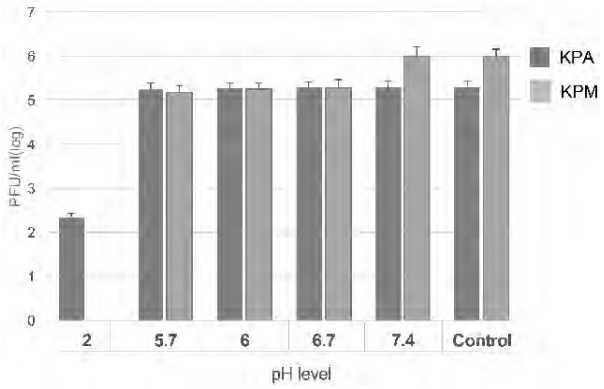


**Fig 4.8:** shows the stability of KPA and KPM at various gastronomic pH.

Results from our experiment shows that KPM phage is completely unstable at pH 2, while KPA phage somehow shows stability but not completely. The reason for the unstability at pH 2 is highly acidic pH that can denatures the phages proteins and render them ineffective. But as the pH levels increseas and become alkaline phages shows great stability especially at the intestinal pH 7.4 because the proteins in phages are less likely to undergo denaturation ar a pH closer to neutral, allowing them to remain functional.

#### 4.2.2. Microencapsulation of Phages in different Polymeric substances:

We have used different polymeric substances for phage encapsulation like sodium alginate. Agarose, Gelatin, and Polyethylene Glycol. All the materials used for encapsulation are biodegradable, biocompatible, non-toxic, having effective gelation mechanisms, specific permeability control that allows the targeted release of phages, cost effective and easy to handle.



**Fig. 4.9a** **Fig. 4.9b** Fig. **4.9c**

**Fig 4.9a:** shows the microcapsules of KPA and KPM phage made with different polymeric substances in $CaCl_2$ solution.

**Fig 4.9b and 4.9c:** shows the morphology of wet stained capsules of different polymeric ssubstances.

### 4.2.3. Phage Encapsulation Efficiency:

The encapsulation efficiency of phages in different polymeric substances were calculated according to formula:

$$= 100 - \frac{\text{(Free phages in } CaCl_2 \text{ Soln.)}}{\text{(Total phages entrapped)}} \times 100.$$

The results can be calcuated by determining the free phages in supernatant and entrapped phages in the capsules after placing them in breaking solution.

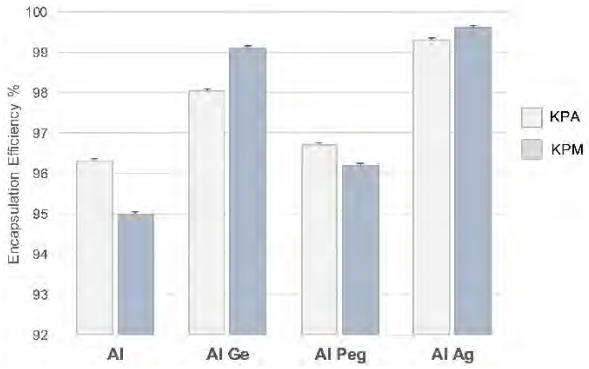


**Fig 4.10:** shows that the highest encapsulation efficiency of 99.3% and 99.6% have been achieved in case of (Alginate + Agarose) in KPA and KPM respectively.

### 4.2.4. Characterization of Dried Capsules:

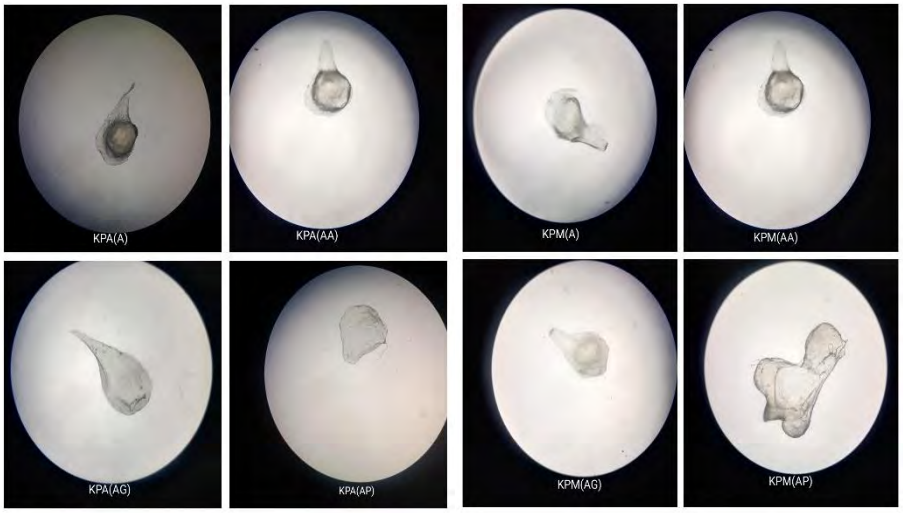


**Fig 4.11 (a) and Fig 4.11(b):** demonstrate the optical microscopy of different polymeric capsules of KPA and KPM with 4X magnification lens.

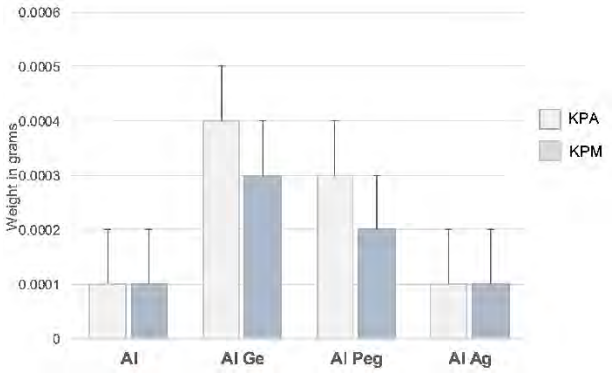


**Fig 4.11(c):** shows the weight of each dried capsule in grams. alginate +gelatin recorded the highest weight of 0.0004g and 0.0003 grams in the case of KPA and KPM respectively.

### 4.2.5. KPA and KPM phage entrapment and release from different polymeric substances:

To determine phage entrapment in different polymeric substances, we determined the free phages present in supernatant and subtracted them from the total phage titer (pfu/ml) used for encapsulation, and to determine phage release from different capsules, we placed each capsule in breaking solution followed by DLA and determined the number of phage release in the form of pfu/ml.

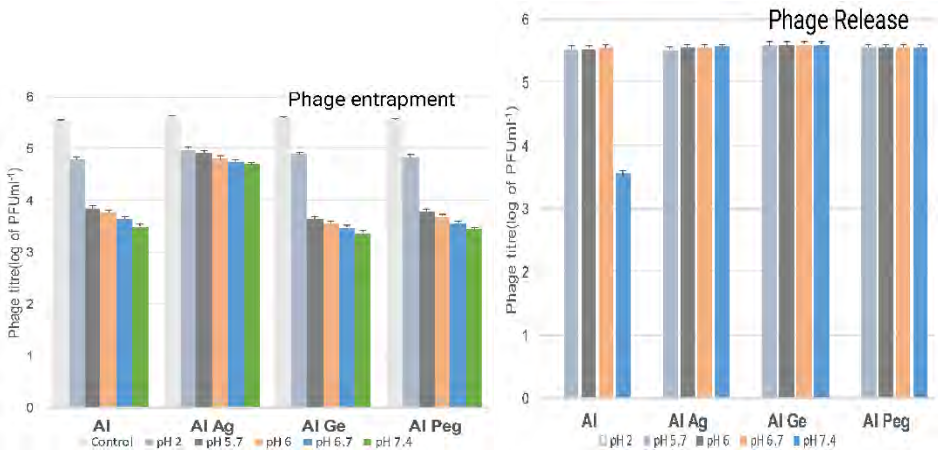


**Fig 4.12(a):** shows the phage entrapment and **Fig 4.12(b):** shows the phage release from the different polymeric substances in case of KPA. Highest entrapment efficiency has been shown by alginate + agarose capsules in case of pH 2 followed by the remaining pH levels, where stability is the main problem because of the

extreme gastric conditions of stomach that can denatures the phage protein structure and inactivate them. In the case of phage release, maximum phage release has been observed in case of (Alginate + Gelatin) because at the pH>4 alginate + gelatin hydrogel undergoes structural changes and begins to swell and releases encapsulated phages in the intestinal fluid. As a result, alginate+ gelatin expands and discharges in a pH -dependent manner.

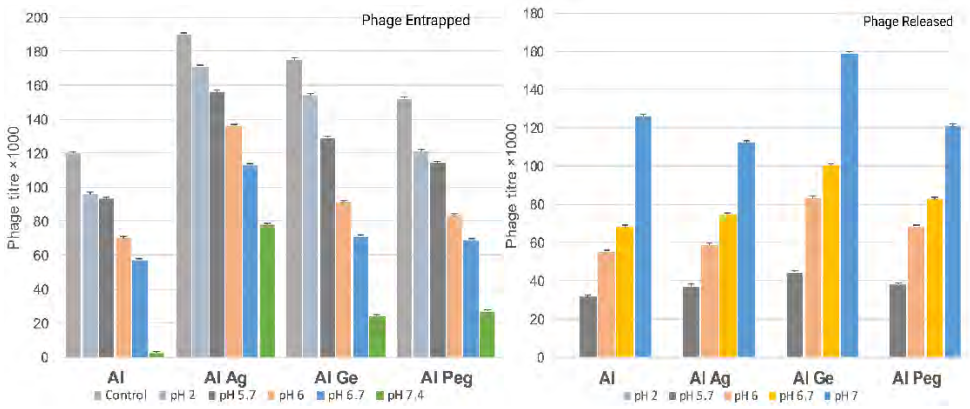


**Fig 4.12(c):** shows the phage entrapment and **Fig 4.12(d):** shows the phage released from the different polymeric substances in case of KPM. However, the highest phage entrapment has been achieved in case of Alginate + Agarose in case of pH 2 followed by the remaining pH levels where stability is the main issue because of the severe gastric conditions of the stomach. In the case of phage release, highest phage release was observed in case of Alginate + Gelatin at pH 7.4 that is the pH of the intestine, at the intestinal pH, the alginate + gelatin capsules expand and release encapsulated phages in the intestinal fluid in a pH dependent manner.

### 4.2.6. KPA and KPM phage entrapment and release in simulated gastric fluid (pepsin) and simulated intestinal fluid (pancreatin):

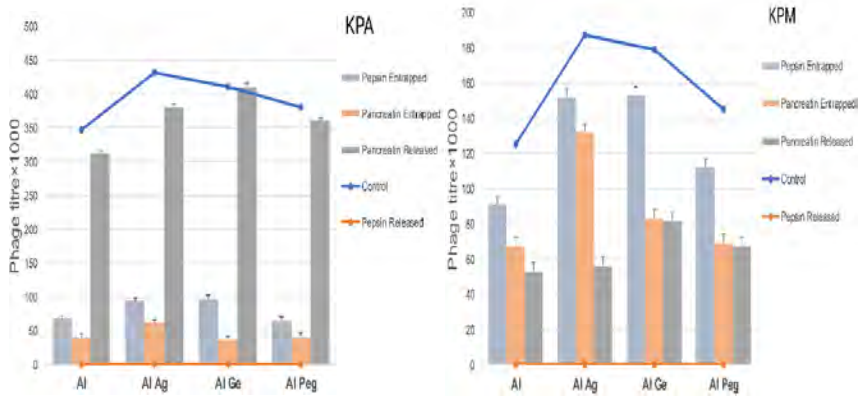


**Fig4.13 (a) and 4.13 (b):** shows the entrapment and release of phages KPA and KPM respectively in pepsin and pancreatin. Phage entrapment in the case of pepsin can be determined by calculating the free phages in supernatant and subtracted from total phage and has achieved maximum entrapment in case of alginate + gelatin in case of KPA and KPM respectively. Whereas in the case of pancreatin maximum phage entrapment has been observed in case of alginate + agarose in case of KPA and KPM respectively. No phage release has been observed in case of pepsin in any of the polymeric substances in case of KPA and KPM, that shows the encapsulated phages are stable in the gastric pH and will reach the intestine without any degradation or loss, whereas in case of pancreatin, maximum phage release has been observed in case of alginate + gelatin in case of KPA and KPM respectively.

# CHAPTER 5:

# DISCUSSION

**5. Discussion:**

Researchers have put forward phage therapy as a substitute for antibiotic therapy to combat the increasing incidence of drug-resistant bacteria. Phages, also known as bacteria-eating viruses, are prevalent in nature and could infect and eradicate microorganisms. Particularly, phage's capacity to combat multidrug-resistant bacteria offers multiple benefits over antibiotics, including a high degree of specificity, minimal dosage, cheap production expenses, antibiofilm effectiveness, and excellent safety. Rather than substituting antibiotics with phages, scientists believe that combining both types of antibacterial agents may be more successful than using either alone. Furthermore, a collaborative strategy could provide benefits such as improved bacterial removal, enhanced invasion into biofilms, and a reduced propensity for bacteria to evolve phage and/or antibiotic resistance (Abdelkader, Gerstmans et al. 2019). Phage-antibiotic synergy is defined as an increase in phage production after exposure to sublethal concentrations of antibacterial drugs, and it is seen as a plausible therapeutic approach (Kamal and Dennis 2015). The combination of phages and antibiotics has been widely employed to improve the elimination of drug-resistant bacteria as well as minimize antibiotic resistance. Already isolated lytic phages (KPA and KPM) by the Bacteriophages Research Group of Applied and Geomicrobiology Research Group of Quaid-I-Azam University, Islamabad were tested with cefepime, gentamicin, and meropenem to evaluate their synergistic effect with these antibiotics at their sub-inhibitory concentrations against multi-drug resistant biofilm forming uropathogenic *K. pneumoniae* isolated from the urine sample of catheterized patient undergone spine surgery for tumor removal. The cocktail of both phages was prepared based on the sub-effective MOIs of KPA and KPM and its efficacy in host bacteria eradication and biofilm was tested and the efficacy of the cocktail was higher than both phages alone. The previous study conducted reported that TM3 single phage was not effective compared to the poly-phage cocktail, the increased efficiency of the phage cocktail was due to synergetic effect (Naghizadeh, Torshizi et al. 2019).

In our study the stability of KPA and KPM was evaluated in sub-inhibitory concentration of selected antibiotics, KPA and KPM was incubated in subinhibitory concentration of selected antibiotics and then DLA was performed to find out stable

PFU and was compared with control, both phages were stable in all the tested concentrations of antibiotics but increased in plaque size of KPA was observed in all the concentrations of meropenem and cefepime. Few reports suggested that a few antibiotics stimulate bacteria to produce more phages under certain conditions, which increases plaque size. (Comeau, Tétart et al. 2007) observed increased release of phages by uropathogenic *E. coli* in the presence of sub-lethal concentrations of aztreonam and cefixime. Subinhibitory antibiotic concentrations cause an increase in burst size, which leads to an increase in plaque diameter as this increase in the size of the plaque with subinhibitory conc. of antibiotics were also explained by (Chan, Turner et al. 2018). In our opinion, the sub-inhibitory concentration of some antibiotics reduces the phage latent time by causing early cell lysis of the host bacterium.

Meropenem and cefepime at their sub-inhibitory concentrations with KPA and KPM and their cocktail efficiently reduced host bacterial cell count. However, a 100% reduction in host bacterial number was observed when KPA and their cocktails were used with 8µg and 4µg of cefepime and when KPA, KPM and their cocktail 1µg, and 0.5µg of meropenem, and their cocktail when used with 0.125µg of meropenem, it caused 100% reduction of host bacterial cells. Gentamicin in combination with KPA and KPM doesn't eradicate host bacterial cells efficiently, but their cocktail worked well. Similarly, one of the experiments performed by (Arumugam, Manohar et al. 2022) on Phage-antibiotic synergy demonstrate that when Carbenicillin (32 µg/mL) at 1/4 of the MIC in conjunction with Citrophage at $10^6$ PFU/mL resulted in a six-log reduction compared to antibiotics alone and a two-log reduction compared to the phage alone. Combination therapy was used to boost the effectiveness of antimicrobials.

Biofilm formation can greatly hamper bacterial infection therapy because these structures successfully shield bacterial cells against a variety of chemical as well as physical stimuli, including antimicrobial substances Moreover, the effect of different dilutions of phages and phage cocktails on turbidity reduction and biofilm inhibition was also evaluated. KPA, KPM and their cocktail inhibited biofilm

formation of host bacterium in the presence of all the subinhibitory concentration of meropenem and cefepime and phage cocktail inhibited biofilm formation of host bacterium in the presence of 4µg and 2µg/ml of gentamicin. Biofilm-associated prosthetic material infections are increasing worldwide and are difficult to treat with the most available antibiotics. A study conducted on the eradication of the biofilm of *K. pneumoniae* by the addition of phages with antibiotics and the increased effectiveness of the antibiotics in the presence of phages was observed. A study conducted by (Shlezinger, Khalifa et al. 2017) reported that vancomycin resistant *Enterococcus faecalis* biofilm was significantly eradicated by the synergistic effect of phages with vancomycin.

The effect of phages and phage cocktail on already established biofilm was also examined, KPA resulted in the greatest reduction of biofilm, as validated by light microscopy compared to KPM while their cocktail completely eradicated biofilm from glass slides. Similarly, different phages such as vB_Eco4-M7 and ECML-117 and phage cocktails specific against STEC-biofilm along with antibiotics rifamycin (400-500xMIC) were used to study the efficacy of STEC cell eradication and biofilm density reduction, the results shows that they appear to be potential anti-STEC drugs when used in conjunction with STEC-specific bacteriophages (Akturk, Oliveira et al. 2019). The eradication of biofilm is by depolymerases of phages that help phage to degrade the biofilm matrix.

It is generally recognized that viruses are typically irreparably destroyed by low pH, chemical solvents, dehydration, and temperature (Stanford et al., 2010). The present investigation found that when exposed to a simulated acidic stomach environment and, to a lesser extent, simulated intestinal conditions, the viability of free phage KPA and KPM was abruptly compromised. As a result, maintaining phage stability is a critical factor in the development of efficient microencapsulation innovations. Furthermore, microencapsulation techniques should use physically moderate conditions, and the materials used should be conducive to the phage and not impair its biological functionality. Moreover, if the aim is to transfer viable phage to the gut, the encapsulating polymers must shield the phage from acidic conditions and

digestive enzymes found in stomach fluid and disintegrate or expand readily in a mildly alkaline intestinal condition. The present encapsulation procedure was carried out in a mild aqueous environment, and our findings reveal that the encapsulating and coating procedures had no negative impacts on phage survival. Results of (Krasaekoopt et al., 2004) on the encapsulation of probiotic-producing bacteria in chitosan-alginate microcapsules, demonstrated a high phage loading efficiency of 93%, similarly our phage KPA and KPM also displayed a remarkable phage loading efficiency of 99.3% and 99.6% in case of alginate + agarose respectively, indicating successful encapsulation. When the phage-containing sodium alginate mixture was put into a calcium chloride gelation solution, the droplets of solution instantly produced gel microcapsules, encapsulating the phage in a three-dimensional framework of ionically interlinked alginate. We have used different polymeric substances with sodium alginate for hybrid encapsulation like agarose, polyethylene glycol, and gelatin. Previously, spermine-alginate and poly(dl-lactide-co-glycolide) were utilized for rotavirus microencapsulation, but only about 14% and 30% of the initial viral load were entrapped within microcapsules, respectively (Sturesson et al., 2000). As a result, it seems that Ca-alginate matrices are compatible with encapsulated phages KPA and KPM. When phage is delivered orally, gastric juice survivability is required. The nonencapsulated phage KPA and KPM were very sensitive to low pH settings. This is consistent with a prior publication that found none of the three *Vibrio vulnificus* phage strains survive in SGF within 2 minutes at pH 2.5 to 2.7 (Mhone et al., 2022). Conversely, λ phage tends to be a bit more acid tolerant and was viable in an SM buffer of pH 3.0 after storage at room temperature for 24 hours (Ma et al., 2008). Our phage KPM was not stable at pH 2 while KPA demonstrated somewhat stability at pH 2. In our study, the maximum phage entrapment was reported in the case of alginate + agarose at pH 2, followed by the remaining pH values, whereas maximum KPA and KPM phage releases has been observed in case of alginate + gelatin because at the intestinal pH of 7.4 alginate + gelatin capsules swell and release the encapsulated phages in pH dependent manner. There was a significant strain variability in reaction to acid, although changes in assay buffer and incubation

parameters could potentially impact phage acid tolerance disparities. The alginate gel matrix shrinks at low pH and dissolves at higher pH, protecting phage from harmful gastric environments while being released in a viable state at targeted places (small intestine) (Vinner et al., 2017). The phage's protection strategy by encapsulation is probably accomplished by minimizing the phage's direct interaction with an acidic solution. The viability of bare-free phage declines instantly after being subjected to an extremely low pH SGF. Encapsulation in a polymeric matrix, presumably, shields the phage by slowing proton migration into the bead network (Dini et al., 2012). Gastric emptying times are a key issue to consider while phage KPA and KPM is traveling through the stomach. The travel time through the stomach is highly varied and is influenced by the formulation and size of particles when a solid meal is administered. In our study, maximum phage entrapment in case of pepsin has been observed in alginate + agarose while no release has been reported in case of pepsin that indicates the successful encapsulation of phages because the free phages are highly sensitive to extreme gastric condition of stomach, however the highest phage entrapment in case of pancreatin has been observed in case of alginate + agarose and maximum release has been reported in case of alginate + gelatin.

## Conclusion:

In conclusion, the escalating threat posed by multidrug-resistant *Klebsiella pneumoniae* demands innovative approaches to combat its virulence and antibiotic resistance. This study investigates the synergistic potential of bacteriophages KPA and KPM, alone and in combination, with select antibiotics against multidrug-resistant *K. pneumoniae*, revealing promising reductions in bacterial counts and biofilm inhibition. Moreover, the research pioneers the encapsulation of phages using polymers like alginate, agarose, gelatin, and polyethylene glycol showcasing their resilience against harsh gastric conditions and controlled release within the intestine. These findings underscore the transformative potential of phage therapy to address antibiotic resistance and enhance targeted treatment within the gastrointestinal tract, offering a significant advancement in the battle against challenging bacterial infections.

**Future Perspectives:**

- Exploration of bacteriolytic proteins of both phages should be done to exploit them as therapeutic agents against multi-drug resistant *Klebsiella pneumonaie* instead of using whole phages.
- In vivo testing of both phages should be done.
- Whole Genome sequencing of both phages should be done to ensure safety of phage therapy.
- Designing custom phages through bioengineering to target specific pathogens and improve their properties.
- Development of comprehensive phage libraries for rapid identification of effective phages against various bacterial strains.
- Ongoing research into understanding phage-bacteria interactions and mechanisms to enhance therapeutic outcomes.

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