Elucidating the lytic potential of the isolated phages, PBM-1 & PBM-2 against the Avian Pathogenic *Escherichia coli* (APEC-O1)

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Master of Science in Industrial Biotechnology

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I’d like to dedicate this thesis to the
two strongest pillars of my life:

My beloved Parents

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Saleha Masood

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List of Abbreviations

APEC	Avian pathogenic *Escherichia coli*
MDR	Multi-drug resistant
AMR	Antimicrobial resistant
PBM	Poultry bedding material
IPEC	Intestinal Pathogenic *Escherichia coli*
ExPEC	Extraintestinal Pathogenic *Escherichia coli*
MPEC	Mammary Pathogenic *Escherichia coli*
UPEC	Uropathogenic Pathogenic *Escherichia coli*
SePEC	Septicaemia associated Pathogenic *Escherichia coli*
NMEC	Neo natal Meningitis *Escherichia coli*
E. coli	*Escherichia coli*
PAI	Pathogenicity island
TEM	Transmission Electron Microscopy
MOI	Multiplicity of Infection
CDC	Center for Disease Control and Prevention
N-agar	Nutrient agar
N-broth	Nutrient broth
NCBI	National Center for Biotechnology Information
O.D	Optical Density
MCP	Major Capsid Protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats

PFU	Plaque Forming Unit
CFU	Colony Forming Unit
VLP	Virus like particle
VAPGH	Virion-associated Peptidoglycan Hydrolases
LPS	Lipopolysaccharide
Kb	Kilobase
NAE	No Antibiotics Ever
SD	Standard Deviation
ds	Double stranded
DNA	Deoxyribonucleic Acid

Abstract

Colibacillosis is one of the most catastrophic disease of poultry worldwide, responsible for increased mortality that leads to major economic losses. As antibiotic resistant strains are prevailing, alternative strategies are being suggested to control the infection, one of them is "phage therapy. In post-antibiotic era, phage therapy is a potential alternative treatment as bacteriophages are specific to host cells causing no harm to eukaryotic cells and commensal microflora. In this study; 2 lytic phages, PBM-1 and PBM-2 were isolated from poultry bedding material taken from different sheds of same poultry farm. The isolated phages showed clear plaques on lawn of Avian Pathogenic *E. coli* O1 (APEC-O1) with no attributes of lysogeny. Transmission electron microscopy revealed that both phages belong to *Myoviridae* with an icosahedral head and a contractile tail. Host range was examined against 40 clinical pathogenic strains and both phages showed infectivity against 10 APEC strains. PBM-1 and PBM-2 showed a high adsorption rate over 90% and 95% to host bacterium over a short period of time. Stability studies showed that both phages were stable over a wide range of temperature and pH. Bacterial cell lysis assay revealed that both phages can reduce the bacterial growth up-to 6 hours. Latent period of PBM-1 and PBM-2 is 20 and 25 mins having a burst size of 102 and 97 virions per bacterium. Our results indicate that both studied phages are potential candidates for use in phage therapy. Further genomic and proteomic analysis will help to unravel the therapeutic potential of these phages.

1. Introduction

In Pakistan, poultry industry contributes to 1.3% GDP of the country (Hussain et al., 2015) and is the second largest industry of Pakistan (Memon, 2012). According to economic survey of 2019-2020, Pakistan is 11th largest producer of poultry in the world producing 1,163 million of broiler chicken. Poultry meat contributes to 28% of total country meat production(Jan et al., 2018).Colibacillosis is one of the most catastrophic disease of poultry worldwide, responsible for increased mortality that leads to major economic losses (Dho-Moulin & Fairbrother, 1999). This disease causes significant economic losses in broiler industry every year. Swollen head syndrome (SHS) one form of colibacillosis, can reduce the egg production by 2-3% and has a mortality rate of 3-4% (Kabir, 2010). Pathogenic bacteria affecting the poultry are risk for both economy as well as humans due to reduced production and transmission of pathogens to humans via contaminated poultry products (Mitchell et al., 2015).

Avian pathogenic *Escherichia coli* (APEC) is responsible for causing systemic and infectious disease called "colibacillosis" in poultry flocks. It affects many organs of target organism causing airsacculitis, pericarditis, perihepatitis, peritonitis and if left unchecked septicaemia, leading to death. Mortality rate of colibacillosis in chickens is 1-10% while in broilers mortality rate is even higher (Omer et al., 2010). This disease is prevalent in avians of all ages (9.52-36.73%) (Matin et al., 2017) but is more common in the broiler chickens of ages 4-6 weeks (36.73%) (Rahman et al., 2004). Studies have shown that poultry meat can be the source of transmission of ExPEC to humans (Mellata, 2013).

As, APEC is subgroup of extra-intestinal pathogenic Escherichia coli (ExPEC); variety of serotypes (O1, 2, 4, 6, 7, 8, 16, 18, 75) identified in APEC and strains of similar O types has been

reported to cause diseases such as urinary infections in humans (J. R. Johnson & Stell, 2000) and meningitis in newborns (Bonacorsi & Bingen, 2005). *In vivo* inoculation of APEC strains in mammalian models showed that these pathogens are able to cause disease in mammals and inoculation of human derived ExPEC cause disease in poultry illustrating the non-host specificity of some ExPEC strains (Jakobsen et al., 2012; Tivendale et al., 2010). Phylogenetic and genome analysis of APEC shows that it is closely related to human extra-intestinal pathogenic Escherichia coli (ExPEC), have similar virulence genes so, have a high zoonotic potential (Tivendale et al., 2010).

APEC resides in dry environment and is transmitted to chickens from garbage or fecal contamination. Newborn chicks can get this infection by transmission of pathogen through ovaries(Panth, 2019). This pathogen is easily transmitted from chickens to humans where it colonizes the human gut causing infection(Linton et al., 1977). Many virulence traits have been identified that allow these pathogens to take over, colonize and cause infections in the body outside gastrointestinal tract. Humans get this infection by inhaling the contaminated dust or by eating poorly cooked poultry meat.

There are two major issues that make it hard to control the avian colibacillosis: first one is that there is no reliable method to locate the causative strains of *E. coli* and secondly, the lack of effective vaccines against this pathogenic strain. Despite the finding of safe and potential vaccine antigens, the currently available vaccines are not totally potent(Moriel et al., 2010). This is due to the diverse characteristics of APEC inhibiting the recognition of common properties that can be used as a marker for diagnostic methods and vaccination purposes.

Control measures taken to prevent colibacillosis include vaccination of birds. *E. coli* is susceptible to disinfectants and temperatures greater than 80^0C so, proper cleaning and heat treatment of poultry houses can reduce the risk of infection. Chlorinating the drinking water of birds and proper ventilation of poultry houses reduce the exposure to pathogenic *E. coli* thereby reducing the risk of infection.

Antibiotics such as ampicillin, cefotaxime, chloramphenicol gentamycin, streptomycin, kanamycin, tetracycline, nalidixic acid, sulphamethoxazole-trimethoprim etc. are used to treat colibacillosis but due to imprudent and misuse of these antibiotics has caused an increase number of antibiotic resistant organisms. So, these drugs fail to control the infection with having an effectiveness of only 50% which is a significant public health concern (Xu et al., 2019). As antibiotic resistant strains are prevailing, alternative strategies are being suggested to control the infection, one of them is "phage therapy".

Phages were firstly discovered by Felix d' Herelle and Twort in 1915 and 1917 independently. Bacteriophages are viruses that infect bacterial strains involved in pathogenesis. Phages specifically target their pathogenic host bacterium causing no harm to host or commensal bacteria. This makes them a favourable candidate to be used as an effective therapeutic agent. Bacteriophages are the most abundant life form on earth. They can be isolated from all natural environments such as sewage water, fresh and marine water bodies, soil, food products etc. (Schaechter, 2010)

Phages were initially applied to cure bacterial infections including urinary tract infections, pneumonia and surgical site infections in 1940's but soon they were neglected due to the discovery

of antibiotics. In the present era due to increased antibiotic crisis, phage therapy revitalized its importance for use in clinical purposes (Dewangan et al., 2017).

Although, not all phages are favourable for therapeutic purposes. Many factors such as phage adsorption, stability of phages at specific body temperature and pH, absence of virulence genes in phage genome are considered.

In this study, two phages were isolated from the poultry bedding material against APEC01 and their lytic potential, pH and thermal stability, their adsorption to host (APECO1), latent period and burst size was evaluated by double layer agar assay. Host specificity of these phages against other bacterial strains was checked by spot assay. Transmission electron microscopy (TEM) was done in order to evaluate the morphology of these phages. Reduction in bacterial growth was analyzed after adding the phages at various multiplicity of infection (MOIs) by taking the $O.D_{600nm}$ on spectrophotometer.

2. Literature Review:

Escherichia coli is a rod-shaped, gram negative bacterium; majority of their strains are commensal whereas few of them acquire virulent genes and become pathogens. Pathogenic *E. coli* are intestinal (IPEC) and extraintestinal (ExPEC). ExPEC is further classified into subgroups including uropathogenic *E. coli* (UPEC), new born meningitis causing *E. coli* (NMEC), septicaemia associated *E. coli* (SePEC) and avian pathogenic *E. coli* (APEC) as shown in Fig.1

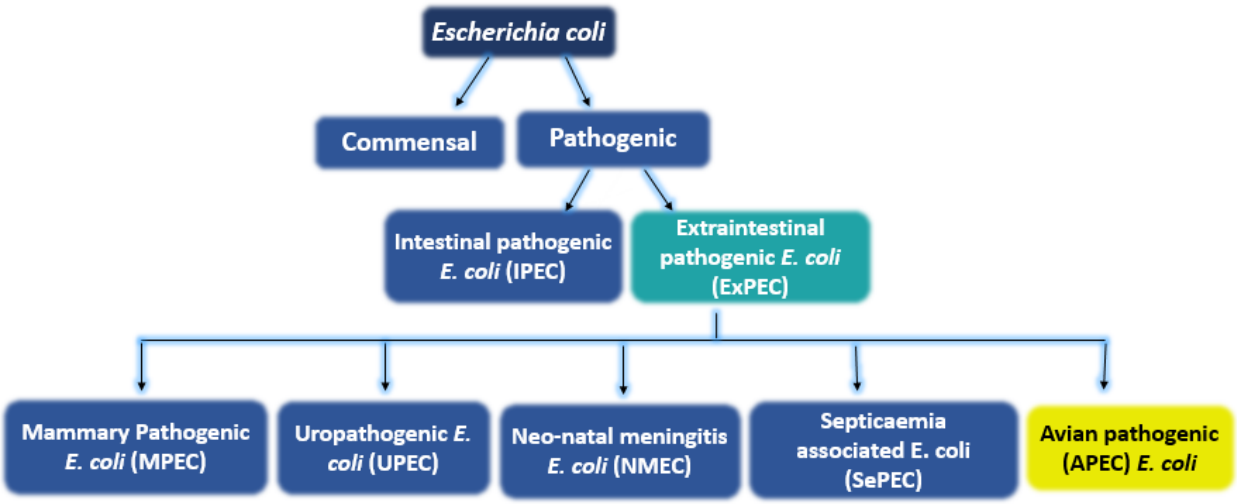


Fig.1 Flow diagram representing pathogenic groups of *Escherichia coli.*

2.1 Pathogenicity of APECO1:

ExPEC differs from other subsets in having certain virulence determinants such as iron acquisition systems, colonizing factors, toxins, serum resistant traits as well as invasions. APEC is classified among extra-intestinal *E. coli* (ExPEC) that shows broad serological diversity. Serogroups that are predominant are O1, O2 and O78 (Dho-Moulin & Fairbrother, 1999; Ewers et al., 2004) APEC causes various diseases excluding intestinal tract in poultry collectively called as colibacillosis and

have diverse symptoms. The first pathogenicity island (PAI) discovered in APEC carry a gene that encode vacuolating cytotoxin and was about 22kb in size (Kariyawasam et al., 2006).

Numerous studies reveal that a single isolate rarely possess all of the virulence genes which shows that APEC strains comprises a heterogeneous group. Different isolates have different virulence factors associated with them, each of them is able to induce avian colibacillosis(Schouler et al., 2012).

Colibacillosis affects all age groups among the poultry and is caused by poor environmental conditions and stress due to concurrent infections. Depending on the organ affected, signs are diverse, non-specific and sudden death may occur. Other symptoms may include; poor growth, lethargy, diarrhea, weakness, reduced appetite etc. A number of conditions can arise including; colisepticaemia, yolk sac infection, egg peritonitis and many others. Clinical presentations may vary accordingly. The severity of disease is determined by age of chicken, co-existing infections, duration of infection and management conditions (Kazibwe et al., 2020).

2.2 Zoonotic Potential of APECO1:

During the recent years, attention has been drawn towards the zoonotic diseases. Center for Disease Control and Prevention (CDC) has released reports on zoonotic potential of ExPEC to caution the public. Initial reports showed that human ExPEC and APEC both have similar phylogenetic background and share the same virulence genes, this explains the zoonotic potential of APECO1 somehow (Manges & Johnson, 2012). Sequencing of APECO1:K1:H7 strain revealed that it is highly similar to human UPEC and NMEC (T. J. Johnson et al., 2007). Zoonotic potential of APECO1 is predominately related to presence of large plasmids. Studies have shown that virulence

genes of APEC and UPEC are mostly present on their large transmissible plasmids (T. J. Johnson et al., 2012).

Virulence genes associated with APEC plasmid such as *sit* operons, aerobactin, salmochelin are also found to be present on UPEC plasmids (Ewers et al., 2007). Moreover, APEC and NMEC share the COIV plasmids in common. APEC plasmid pAPEC-O2-ColV when acquired by commensal *E.coli* isolate contribute to pathogenicity and cause the urinary tract infections (Skyberg et al., 2006) and meningitis in murine models (T. J. Johnson et al., 2010) which shows its ability to cause disease in mammals.

2.3 Antibiotic Resistance:

Antibiotic resistance has spread all over the world due to misuse and overuse of antibiotics. A study conducted by Bashahun and Odoch showed that 96.7% of farmers use antibiotics for disease control while 33.3% use these to enhance the feed intake and promote growth rate. Significant bacterial diseases such as colibacillosis previously treated by antibiotics have become deadly due to associations with multidrug-resistant strains. In a study conducted in Uganda, 87% of *E. coli* strains isolated from broiler chickens were resistant to at least one commonly used antimicrobial agents (Majalija et al., 2010). Another study reported that over 80% of APEC strains exhibited multidrug resistance (Kazibwe et al., 2020).

Although, phenotypic resistance in APEC exists with varying level of resistance level for each drug. In a study conducted in Uganda, resistance levels found out to be 35.7% against Chloramphenicol, 10.7% against Gentamicin, 8.9% against Nitrofurantoin whereas all of strains isolated were found susceptible against Cefixime. Susceptibility to cefixime may be due to the fact that it is recently introduced antibiotic.

Increase in antibiotic resistance and emerging multi drug resistant strains have compelled the mankind to look for alternative ways to treat the infectious diseases. Some of the ways include anti-microbial peptides, pro-biotics, phytocompounds, bacteriocins and CRISPR-Cas, synthetic sRNA, quorum quenching, etc. Bacteriophage therapy serves as a promising tool against these pathogens.

2.4 Bacteriophages:

Bacteriophages are specific viruses that replicate within their host bacterial cells. Phages have a simple structure comprising 60% of protein coat and 40% of nucleic acid i.e. either DNA or RNA (Paez-Espino et al., 2016). Studies have revealed that there are more than 10^{30} bacteriophages on earth, exceeding their host bacteria 10-folds with a weight comprising 10^{9} tons (Wommack & Colwell, 2000; Yu et al., 2017).

Phages are very diverse at genetic level. Despite of their smallest genome, they don't show sequence homology with distinct phages but at protein level, they show some similarities such as major capsid protein (MCP) show conservation in all tailed phages even in archaeal viruses extending to adenoviruses. Studies have shown that the abundance of phages varies with different biomes. Lowest population of 10^{3} VLPS/g was observed in deserts, medial in agricultural land and highest abundance of 10^{9} VLPS/g in forests. Viral abundance is directly proportional to the presence of its host bacterium and inversely proportional to pH, less phage count is observed as pH increases (Dion et al., 2020)

2.5 Classification of Bacteriophages:

According to ICTV (International Committee on Taxonomy of Viruses) 2015 taxonomy release, BAVS (Bacterial and Archeal Viruses Subcommittee) has classified the phages based on their morphology and genomic sequences into 873 species, 204 genera, 14 subfamilies (Adriaenssens et al., 2018; Adriaenssens & Brister, 2017). About 96% of known phages belong to Myoviridae, Podoviridae, Siphovirdae.

Presence of one type of nucleic acid and capsid made up of protein is the fundamental characteristic of bacteriophages. Phages are categorized into 3 groups depending on the type of nucleic acid they possess: single stranded DNA phages, double stranded DNA phages or RNA phages. Majority of bacteriophages have genome comprising of double stranded DNA. In terms of capsid symmetry, phages are divided into 2 groups i-e; isometric (polyhedral) or helical (spiral) (Wernicki et al., 2017).

Different morphotypes of virus are described by Ackermann in a book chapter (Ackermann, 2009). The figure below gives a detailed overview of morphotypes of viruses.

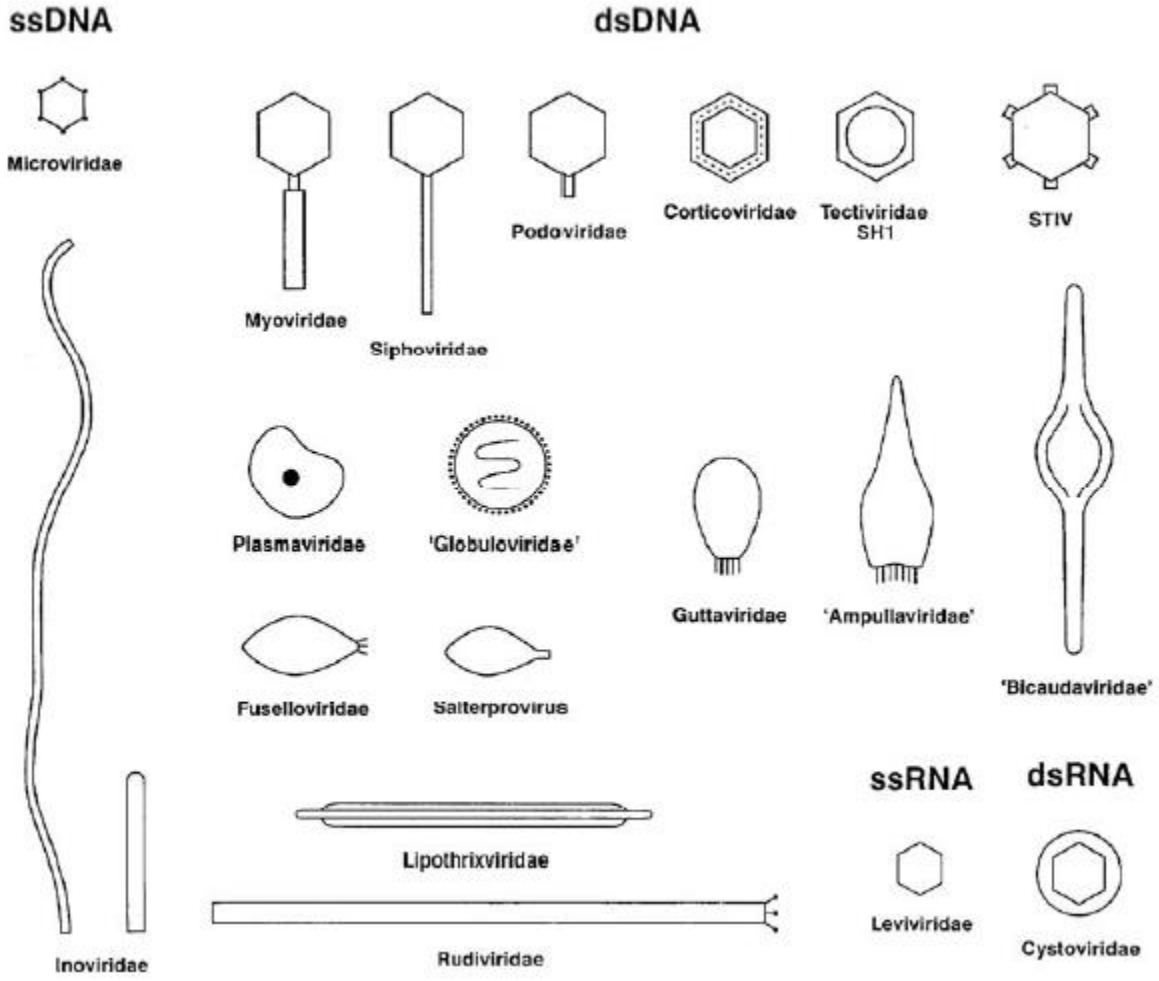


Fig 2.5 Morphotypes of viruses. Adopted from Ackermann, 2009

2.6 Lifecycle of bacteriophages:

Bacteriophages can be divided into two categories i.e. lytic or lysogenic based on their life cycles; dynamics of which play a key role in their implication as a therapeutic agent. Lysogenic phages are those that integrate their genomes into host bacterial cells. During binary fission when bacterial cells replicate, the daughter cells inherit the viral genetic material integrated into their genomes. Lysogenic phages are also called temperate phages; later in time, due to environmental or physiological stress, lysogenic phage can expunge from bacterial genome and enter into lytic cycle.

Lytic bacteriophages are those that infect the bacteria and quickly cause lysis of the cell resulting in destruction of bacterial cells. During the lytic lifecycle: (1) phage gets attach to specific receptor (attachment site) present on the surface of bacterial cell; (2) causes the lysis of bacterial cell wall with the help of enzymes called endolysins and injects its genetic material into the bacterial cell; (3) phage takes control of host cell machinery and starts replicating through bacterial cell transcription and translation; (4) new viral particles formed escape the cell through bacterial lysis. These new phage particles will now infect other susceptible cells, repeating the same process again and again. It highlights the whole mechanism of phage therapy i-e utilizing the viruses as "self-augmenting" drugs that kill the susceptible pathogenic bacterial cells and are more efficient as compared to antibiotics that are unable to self-amplify (Kortright et al., 2019). These lytic phages seems to be the best candidate for use in therapeutic purposes.

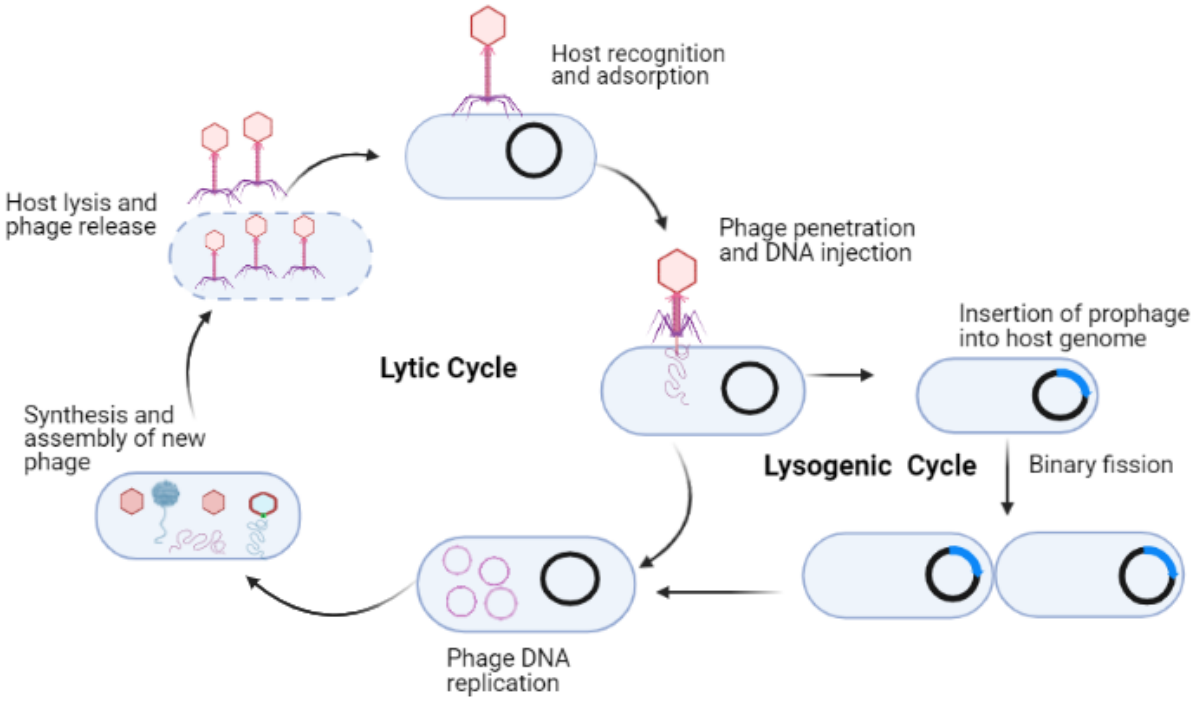


Fig 2.6 Life cycle of bacteriophages

2.7 Bacteriophage as a therapeutic agent:

Phages have a complex and co-evolving relationship with bacteria. They strictly are parasites of bacteria. A specific phage will only attach itself to a specific bacteria or sometimes few strains of that specific bacteria. Binding receptors present on bacterial cell wall are sugar or protein moieties that the phage recognizes and gets attach to the cell. These binding receptors are responsible for the specificity. On completion of each lytic cycle, every bacterial cell is lysed to release 10 to 200 virions. Number of virions released determine the burst size of phage.

For therapeutic use, it is obligatory to isolate active lytic phages against the pathogenic bacteria responsible for causing infection to patient, amplify these phages and administer them so that they

come in contact with their host. In case of osteo-articular infection, the wound is washed with the phage solution before its closure at the end of surgery. Phage administered will continue to amplify as long as their host will be available; once their host bacteria is eliminated, phages will also not survive and will degrade (Brives & Pourraz, 2020).

Temperate phages are not suitable for phage therapy as they integrate their genome in bacterial DNA; due to this lysogenic conversion, bacteria may acquire new genes which may be pathogenic such as toxins encoded by phage which increase the virulence of bacteria (Fortier & Sekulovic, 2013) or even antibiotic resistant determinants (Haaber et al., 2016). However, in case of some bacteria such as *Clostridium difficile*, temperate phages are used as no strictly lytic phages have been isolated to date (Hargreaves & Clokie, 2014). Likewise in case of emergency, due to time constraint, temperate phage can be used as a therapeutic agent when lytic phages are not available. Unfortunately, all lytic phages are not even exempted from concerns. Lytic phage's genome may consist of more than 50% hypothetical genes with no known function at all. They even contain supplementary proteins that alter the bacterial physiology in ways that are not fully comprehended (Philipson et al., 2018). During lytic infection cycle, when viral genome is injected into host cell, some genes are expressed without the production of daughter viruses, the bacterial DNA act as a reservoir for foreign genes of unknown function. This reasoning ought to warrant the research in phage genetics to find out the way towards ensuring safety of phage therapy.

The mode of action of phages is highly specific which allows its consideration as a therapeutic agent. A number of critical antimicrobial resistant (AMR) cases has been treated by phage therapy. They can be used as an alternative to antibiotics or can be used along with them in combinatorial therapy. Studies have reported the synergistic potential of phage and antibiotics (Chaudhry et al., 2017).

2.8 Strategies that can be employed for phage therapy:

Different strategies that can be employed for phage therapy include the application of phage cocktails, phage in combination with antibiotics, application of phage enzybiotics, engineered phages and the use of phages along with CRISPR-Cas system (Wei et al., 2020).

Instead of using a single phage, a combination of phages also termed as phage cocktail, can be employed against bacterial infection. Single phage has a specific host range that limits the efficacy of phage therapy. Application of phage cocktail overcomes the problem of narrow host range in monophage therapy and is therefore, more effective. In former Soviet Union countries, phage cocktail is employed to treat *Staphylococcus aureus* infection. Metagenomic analysis showed the absence of prophage sequences, antibiotic resistance genes or virulent genes which employs the clinical safety of phage cocktail against *S. aureus* infection (McCallin et al., 2018). Other than broader host range, phage cocktails also resolves the issue of phage resistant strains (Nsaif Jasim et al., 2018). A cocktail comprising of five phages has been shown effective in killing *Acinetobacter baumannii* (AB5075) in wound of mouse dorsal-infected model (Regeimbal et al., 2016). A cocktail of 7 phages have been reported effective against *Clostridium difficile in vitro* and *in vivo* hamster models. Optimized cocktail was given orally to the hamsters after 36 hours of infection which delayed the onset of disease by 33 hours as compared to control group (Nale et al., 2016).

Phage genome encode a number of enzymes that have the ability to breach the bacterial cell wall such as endolysins, polysaccharide depolymerases and virion-associated peptidoglycan hydrolases (VAPGH). Instead of using the whole phage, phage enzymes known as enzybiotics can be used as an alternative to antibiotic treatment and have some advantages over phage therapy. These

enzymes are stable and specific i-e they don't cause any harm to eukaryotic cells or any disruption in commensal microflora. Secondly, they have a broader host range as compared to a single phage and they less likely give rise to resistant strains.

VAPGH help in adsorption of phage genetic material into host by degrading the bacterial cell wall made of peptidoglycan. Antimicrobial activity of VAPGH was first reported when disruption of bacterial cell wall was observed prior to production of phage progeny (Rodríguez-Rubio et al., 2013). When the infection cycle of lytic phage ends, phage progeny is released by lysing the host cell wall through lysins. Jacob et al. in 1958, discovered a class of proteins encoded by phage genome that is capable of lysing the bacteria and named it as endolysins (Jacob & Fuerst, 1958). They belong to class of peptidoglycan hydrolases that have the ability to disrupt the bacterial peptidoglycan. A lysin encoded by phage MR11 against *S. aureus* is reported to completely lyse many strains of methicillin resistant *S. aureus* (MRSA) as well as vancomycin resistant *S. aureus* (VRSA). Moreover, no resistance against endolysin was observed (Rashel et al., 2007; Son et al., 2010)

Another class of enzymes encoded by phage is polysaccharide depolymerases that have the ability to hydrolyze the capsule, lipopolysaccharide (LPS) and biofilm i-e polysaccharide components of bacteria. Bacterial biofilm and capsule resist the action of antibiotics, phagocytes and disinfectants. Depolymerases work by destroying the protective barriers of bacterial pathogen made up of polysaccharides (Maciejewska et al., 2018) so that, bacteria again became susceptible to antimicrobial agents. Dpo48, a polysaccharide depolymerase isolated from phage IME200 reported to degrade the capsule of bacterial pathogen (Liu et al., 2019).

Phage and phage enzymes used in combination with antibiotics have a better therapeutic potential than each individual treatment and also reduce the emergence of resistant strains. Antibiotic resistant bacteria susceptible to phages, acquire resistance against phages due to selection pressure exerted but at the same time, these phage resistant strains are susceptible to antibiotics. This concept is known as "trade off" in biology according to which if an organism acquires genes then it has to lose some of its other genes.

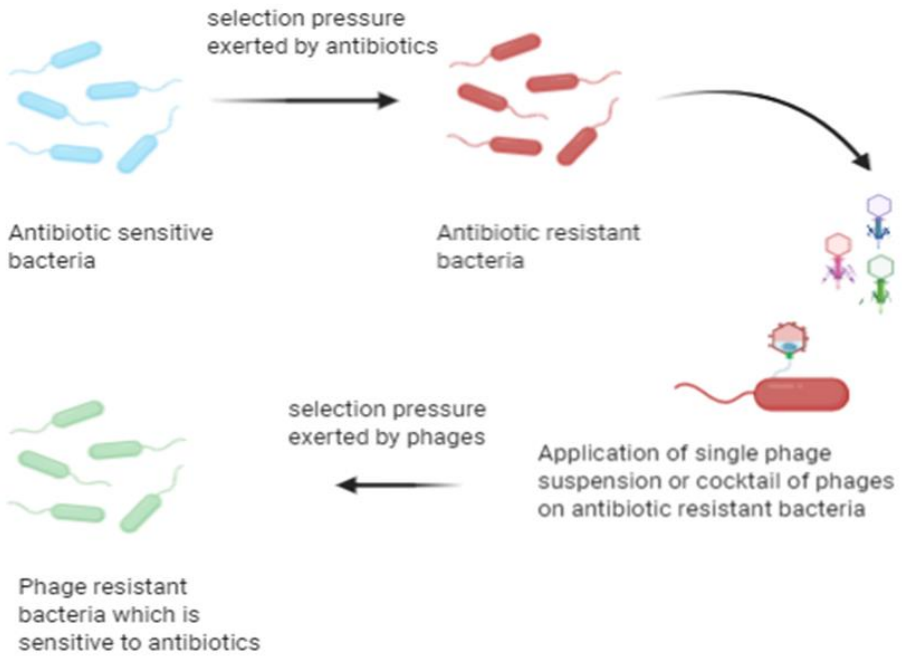


Fig 2.8 Concept of trade off: an organism can't increase its traits without decrease in another

Synergistic effect of antibiotics and phages was first proposed in 2007, when the phenomena of propagation of bacteriophages was observed triggered by sub lethal concentrations of specific antibiotics (Comeau et al., 2007; Ryan et al., 2012). Synergistic effect of polysaccharide depolymerase, a phage enzyme and ciprofloxacin, an antibiotic has shown better results in removal

of *Klebsiella pneumoniae* biofilm than the treatment of ciprofloxacin alone (Verma et al., 2010). It was concluded that synergy of antibiotics with phage and phage enzymes has better efficacy in eradication of bacterial biofilms. Shlezinger et al. reported that synergistic effect of *Enterococcus faecalis* phage EFLK1 and vancomycin on antibiotic resistant *E. faecalis* strains was more effective in eradication of planktonic cells as wells as biofilm (Shlezinger et al., 2019). Nisin combined with phage has shown the eradication of planktonic and biofilm cells of *S. aureus* (Duc et al., 2020). In rat models, *P. aeruginosa* infection was effectively inhibited by the synergistic effect of ciprofloxacin and phage (Oechslin et al., 2017). It was reported that phage cocktail and sulfamethoxazole-trimethoprim when used synergistically, inhibit the resistance against phages and treat the urinary tract infections caused by *Klebsiella pneumoniae* (Bao et al., 2020). A patient infecting from multi-drug resistant *A. baumannii* and *K. pneumonia* was cured after treatment with phage cocktail in combination with meropenem and colistin (Nir-Paz et al., 2019). However, some antibiotics have an adverse effect on phages by blocking their replication (Kronheim et al., 2018). It should be noted that dose of antibiotic and the order of addition have a great impact in efficacy of combination therapy.

Advances in genetic engineering and its application has promoted research in life sciences. Genetic engineering of phages addresses the problem of narrow host range of phages as well as phage resistance in bacterial strains. With the help of genetic engineering, phage therapeutic efficacy can be increased by broadening its lysis spectrum or delivering exogenous proteins. One way to broaden the lysis spectrum is to engineer the genes encoding the receptor binding proteins (RBPs) into tail fibers/spikes of phages (Dams et al., 2019). A study reported the chimeric phage formation by homologous recombination of orf18, an anti-receptor gene present in phage DT1 of *Streptococcus thermophilus* and phage MD4 belonging to Siphoviridae. Chimera phage have

obtained the host range of MD4 (Duplessis & Moineau, 2001). Another chimeric T2 phage of *E. coli* was constructed by engineering the genes of phage IP008 coding for long tail fibers through homologous recombination into phage T2 in order to broaden its lysis spectrum (Mahichi et al., 2009). Ando et al. constructed a platform using yeast, in 2015 that can modulate the host range of phage by modular swapping the tail components of phage. Synthetic phages, thus obtained, have the ability to target the new bacterial population (Ando et al., 2015). Dunne et al. established a library of synthetic phages with the help of bioinformatics and RBP structure analysis and displayed a polyvalent phage having a broader host range (Dunne et al., 2019).

Yehl et al. synthesized phages by identifying the host determining regions of phage T3 and through site-directed mutagenesis of these regions to change the host range and repress the resistance (Yehl et al., 2019). All that successful reports encouraged scientists to exploit ordinary phages and synthesize novel phages that can target specific pathogens. An *E. coli* T7 phage was engineered that carried the enzyme dispersin B, an enzyme that has the ability to degrade extracellular polysaccharides of bacterial biofilm, more efficiently than wild type/unengineered phage (Lu & Collins, 2007). A successful case of modified phage cocktail was reported, a 15-year old patient suffering from cystic fibrosis and a diffused infection of *M. abscessus* was treated by modified phages (Dedrick et al., 2019).

Phages can be combined with CRISPR-Cas system to increase their therapeutic potential which is also a form of phage engineering. Adaptive immunity in bacteria and archaea is provided by Clustered regularly interspaced short palindromic repeats (CRISPR-Cas) system against foreign invaders such as viruses (Barrangou et al., 2007). This system works by integrating the short genomic segments of invader's DNA into CRISPR array which then produce short RNAs known

as CRISPR RNAs or crRNAs, these resultant RNAs guide the protein complex known as Cas to target and degrade foreign genetic elements (Koonin et al., 2017).

Emergence of bacterial resistance can be inhibited by using site specific interference of CRISPR-Cas system in combination with high infection rate of phages to deliver the CRISPR-Cas system into bacterial DNA with the help of phages in order to eliminate the pathogenic bacteria (Greene, 2018). Target pathogenic bacteria which contain a CRISPR-Cas system, only a mini-CRISPR array is needed to integrate into genome of phage for the production of crRNAs which match the antibiotic resistant bacterial genome. In other words, phage exploits the endogenous CRISPR-Cas system to attack its host (Li & Peng, 2019). Recently, CRISPR-Cas3 system delivered through phage infection in *Clostridium difficile* was repurposed as antimicrobial, targeting pathogenic bacteria after encoding of CRISPR array (Kurt et al., 2021). For target bacteria not containing an endogenous CRISPR-Cas system, an exogenous nuclease of CRISPR can be introduced. Many studies report, the efficient and specific bacterial targeting by the use of exogenous CRISPR-Cas9 system with phagemids (Citorik et al., 2014). Another study reports the delivery of CRISPR-Cas13 through phage capsids to attack the antimicrobial resistant genes. Similarly, an exogenous CRISPR-Cas system was delivered in phage ϕNM1 of *S. aureus* to target pathogenic bacteria (Bikard et al., 2014).

2.9 *In vivo* testing against colibacillosis:

Huff et al. (Huff, Huff, Rath, Balog, & Donoghue, 2002) exhibited the preventive efficacy of 2 bacteriophages against colibacillosis. 7 d old chickens were administered with SPR02 with PFU/ml of 2.6×10^8 and DAF6 with PFU/ml of 2.35×10^9 followed by bacterial challenge given to these birds at same day (7 d), a day later (8 d) and two days later (10 d). *E. coli* with 5.6×10^4 CFU/ml

was injected into thoracic air sac of birds at 7, 8 and 10 d of their age. High rate of mortality (63%) was observed in chickens who were not sprayed with phages and challenged with pathogenic *E. coli* on the same day (7 d). Significant reduction in mortality was observed for chickens who were sprayed with phages prior to bacterial challenge. 7% mortality rate was observed in chickens who were challenged with pathogenic bacteria at the same day (7 d of their age) after spraying with bacteriophages. Mortality rate increases to 27% in chickens who were challenged with *E. coli* a day later and again the mortality rate drops to 3% when the chickens were challenged with pathogenic *E. coli* two days later after spraying SPR02 and DAF6. The results suggested that application of bacteriophages was quite effective against colibacillosis.

Most important result obtained from this study was unplanned. One group of broiler chickens at 1 d of their age was comparatively less active, has a low hatch weight and don't look good quality chickens. Moreover, significantly high mortality rate (20%) in control group was observed that was not treated with phages. Chicks that were treated with phages showed significantly low mortality rate (3%). They suspect that this group might suffered from colibacillosis already and bacteriophage application in form of aerosol spray had a therapeutic effect in treating the chickens.

Again, Huff et al. (Huff, Huff, Rath, Balog, Xie, et al., 2002) injected phage SPR02 in chicken models to evaluate the efficacy of phage. In the first group, 3 d old chicken models were injected in their air sacs with 10^3 CFU/ml of pathogenic *E. coli* combined with 10^3 or 10^6 PFU/ml of bacteriophage or 10^4 CFU of pathogenic *E. coli* combined with 10^4 or 10^8 PFU/ml of bacteriophage. In second and third group, 1 week old chickens were challenged with pathogenic *E. coli* and treated with bacteriophages immersed in drinking water. Significant reduction in mortality was observed in the first group where both bacteria and phages were added simultaneously. This was due to the fact that phage infect and start replicating in host before the

establishment of bacterial infection. No effect in mortality was observed in second and third group suggesting phage SPR02 had no protective effect when given to chicken models in drinking water.

In the next study, Huff et al. (Huff et al., 2003) determined the efficacy of both phages SPR02 and DAF6 either through aerosol spray or intramuscular injection to treat colibacillosis in broiler chickens. In the first study, 7 day old chickens were challenged with 10^4 CFU/ml of pathogenic bacteria followed by bacteriophage SPR02 and DAF6 spray. Significant reduction in mortality i-e 50% to 20% was observed when bacteriophages were sprayed immediately at same day of challenge. However, no therapeutic effect of phages was observed when bacteriophages were sprayed after 24 or 48 hours delay. In second study, one group consist of untreated control group and the other group was given an intramuscular injection of bacteriophages in thigh after challenged with 10^4 CFU/ml of pathogenic bacteria. Intramuscular injection of phage SPR02 and DAF6 was given immediately to one group, 24 and 48 hours later to other groups. Significant reduction from 53% to 17% in mortality rate was observed that was injected immediately, 46% to 10% when injected after 24 hours delay and 44% to 20% after injecting with 48 hours delay. These results imply that route of administration played an important role in determining the therapeutic efficacy of bacteriophages for eradicating the disease.

Huff et al. (Huff et al., 2004) analyzed the efficacy of combination therapy i-e bacteriophage SPR02 and DAF6 in combination with antibiotic enrofloxacin. Broiler chickens were challenged with 6×10^4 CFU/ml of *E. coli* by injecting in left thoracic air sac. In the first group, no phage was injected for treatment purposes, this group is regarded as control group. In the second group, only bacteriophages were injected through intramuscular route in infected chickens for treatment purposes. In third group, only enrofloxacin (50ppm in drinking water) was given to infected chickens. In the last group, 50ppm enrofloxacin was also injected in drinking water of chickens

for 7 days consecutively in addition to intramuscular injections of bacteriophages. High mortality rate i-e 68% is observed in untreated group. While in group treated with phage by intramuscular injection 15% mortality rate is observed which is comparatively higher than 3% mortality rate observed in group treated with antibiotic enrofloxacin. However, 0% mortality rate was observed for group treated with combination therapy (antibiotic with phages). These results imply that synergistic effect of bacteriophages and antibiotics have a better therapeutic potential than each individual treatment.

Oliveira et al. (Oliveira et al., 2010) evaluated the *in vivo* performance of phage cocktail in natural infected and experimentally infected chickens. 2 Myoviridae phages; phi 78E and phi 61E, 1 Siphoviridae phage phi258E were mixed to form a phage cocktail having 5.0×10^7 PFU/ml and were administered in naturally infected chickens. Chickens were challenged with 1×10^8 CFU/ml of APEC H839E strain. Phage phi 78E was administered both orally and by aerosol spray at different titers, 10^7 and 10^9 PFU/ml, in experimentally infected chickens. Group treated with 10^9 PFU/ml showed significant reduction ($p < 0.05$) in mortality (~ 45%), morbidity (~ 60%) and pathology (~ 2.5) scores as compared to the untreated/control group mortality(~ 75%), morbidity (~ 100%) and pathology (~4) scores. Lesions found in the carcasses of phage treated group were less severe as compared to control group. Based on these results, this study concluded that phage therapy can be used as an alternative to antibiotics to treat infections caused by APEC.

El-Gohary et al. (El-Gohary et al., 2014) established colibacillosis in experimental chicken models by indirect exposure to pathogenic *E. coli* and determined the potential of bacteriophages in protecting the chicken models from colibacillosis. In that study, surface of litter was sprayed with suspension having 2.8×10^8 CFU/ml of pathogenic *E. coli.* In phage treated groups, 8×10^8 PFU/ml suspension of bacteriophage SPR02 was sprayed on litter. Mortality of the treated group

is 5% as compared to the control group having a mortality rate of 25%. Results of this study suggested that spraying the bacteriophage in environment is a practical and effective method to hinder the onset of bacterial diseases like colibacillosis.

3. Materials

3.1: Media used for culturing of bacteria and their phages

Below mentioned are the media used for isolation, enrichment and plating of bacteria and bacteriophages. Recipes of all media used are given below. They were dissolved in distilled water and autoclaved before use.

Table 3.1.1: Luria Agar (L-agar)

Sr. No	Ingredients	Quantity in g/L
1	Yeast extract	5
2	Tryptone	10
3	Sodium chloride	10
4	Agar	14

Table 3.1.2: Luria Broth (L-broth)

Sr. No	Ingredients	Quantity in g/L
1	Tryptone	10
2	Yeast extract	5
3	Sodium chloride	10

Table 3.1.3: Semi-solid Agar

Sr. No	Ingredients	Quantity in g/L
1	Tryptone	10
2	Yeast extract	5
3	Sodium chloride	10
4	Agar	5

3.2: Solution and Buffer used for phage isolation and propagation

Following solution and buffer were used for isolation and propagation of phages

Table 3.2.1: Phage Buffer (TM buffer)

Sr. No.	Ingredients	Quantity in ml/L
1	5M NaCl	3
2	1M Tris-Cl (pH=7.4)	4
3	1M $MgSO_4$	1
4	0.1M $CaCl_2$	1

Table 3.2.2: Ringer's solution

Sr.No.	Ingredients	Quantity in g/L
1	NaCl	7.2

2	KCl	0.37
3	$CaCl_2$	0.17

3.3: Equipment used during the study:

Equipment	**Manufacturer**
Refrigerator	Caravell
Shaking incubator	JSR
Tabletop Balance	ShiMADZu
Hot Plate	Velp-Scientifica
Centrifuge Machine	Hermle
pH meter	WTW inoLab
Laminar Flow cabinet	Esco
Microcentrifuge	Sigma
Vortex Mixer	Heidolph
Spectrophotometer	Optima
Incubator	Memmert

3. Methods

3.4 Sample collection:

Chicken bedding material from different sheds of a poultry farm located in Rawalpindi was collected in sterilized falcon tubes. The samples were immediately processed for bacteriophage isolation.

3.5 Isolation of bacteriophages:

To isolate the phages, 20g of poultry bedding material was suspended in 40 ml of $1/4^{th}$ strength Ringer's buffer and kept in shaking incubator overnight to release the bacteriophages from bedding/ fecal samples. Later on, the suspensions were centrifuged at 6000 rpm for 10 min and supernatants were filter sterilized with 0.22µm filters to remove bacterial cells.

3.6 Enrichment of bacteriophages:

Approximately 40 ml of Luria-Bertani (LB) medium was inoculated with 1ml of APEC culture (24 hours old) and 1ml of phage suspension and was kept in shaking incubator at 37^0C for 24 hours. After 24 hours of incubation, flask was withdrawn from the incubator and 500µl of chloroform was added (at final concentration of 1%) to get rid of bacterial cells. Chloroform may inactivate some phages i-e their lytic potential is affected. However, T-phages remain unaffected. After 15 mins of incubation, at room temperature, bacterial debris settled down at the bottom of flask. Suspension was poured into falcons and centrifuged for 10 mins at 6000rpm to get rid of bacterial debris. Supernatant was taken with the help of sterile syringes and filter sterilized, first

with 0.45µm and then with 0.22 µm to further purify the suspension which is now called as phage lysate.

To determine the lytic potential of phages, double layer agar assay and spot assay were performed.

3.7 Evaluation of lytic activity of phage by spot assay:

APECO1 was streaked on nutrient agar plate and incubated overnight at 37^0C. A single colony of APEC was picked with the help of wire loop and cultured in 30ml LB broth in a flask. The flask was kept in shaking incubator at 120rpm and 37^0C temperature overnight. After overnight incubation, 100µl of bacterial culture was mixed with 3-4ml semisolid LB agar (LB agar with 0.5-0.7% agar) in a test tube and was poured onto LB agar plates to make a bacterial lawn. The plates were allowed to incubate for 5-7 min at room temperature. 5ul of phage lysates were pipetted and spotted onto bacterial lawn. Plates were incubated overnight at 37^0c after drying of spots.

3.8 Purification of bacteriophages:

To purify the phages, 1ml of bacterial culture (APECO1) was pipetted in 30 ml of LB broth in 100ml flask. With the help of pipette or wire loop, a well isolated spot from L-agar plate was picked and inserted into the flask containing bacterial culture. Area around the spot was stabbed with sterile tip and piece of agar containing the phage was sucked on. The mixture containing bacteria, LB broth and phage was kept at 37^0C at 120 rpm overnight in shaking incubator.

After overnight incubation, 1% chloroform was added to remove the bacterial debris and the mixture was centrifuged at 6000 rpm for 20 min. Supernatant was taken, poured into new falcon tube and was filter sterilized; first by 0.45 µm and then later on by 0.22 µm filters. The whole procedure was repeated 6 to 7 times to have a pure phage lysate.

3.9 Determination of Plaque forming Unit (Pfu/ml) by double layer agar assay:

For this assay, a single colony of APEC was picked, cultured in the 30ml of LB medium and kept in the shaking incubator at 120rpm and 37^{0}C temperature overnight. Next day, 1ml of APEC culture (24 hours old) was pipetted and cultured into 40ml of LB broth for 3-4 hours to get the exponential phase. $O.D_{600nm}$ was measured using spectrophotometer. When it reached 0.5-0.7, culture is taken off from shaking incubator.

Phages were serially diluted ten-folds (10^{-1} to 10^{-10}) using TM buffer (10mM $MgSO_4$, 10mM Tris-HCl, pH=7.2). In a test tube, 100µl of diluted phage lysate and 100ul of exponentially growing bacteria were added with the help of a pipette and the test tubes were incubated at 37^{0}C for 15-20 minutes so that the phage adhered to its host appropriately. After that, 3-4 ml of semisolid agar (at 50-60)°C was added in the relevant test tube and the whole mixture was poured immediately on N-agar plates. The plates were swirled a bit so that molten agar spread out evenly. The plates were allowed to solidify and were kept in the incubator at 37^{0}C for 24 hours. Next day, plaques observed on the APEC lawn were counted on a countable plate indicating the lysis of bacteria and the pfu/ml of phage lysates were calculated by following formula:

Pfu/ml = No. of plaques/ Dilution factor × Volume of virus added to plate

3.10 Determination of Host Range:

Lytic activity of PBM-1 and PBM-2 was determined against 39 bacterial strains by spot assay. Bacterial test strains were incubated in LB broth at 37^{0}C. After overnight incubation, 100 µl of

tested strains were mixed with 3-4 ml of semi-solid agar and poured onto L-agar plates to make double-layer agar medium. 5 µl of phage suspension PBM-1 and PBM-2 was spotted onto L-agar plates containing the tested strains. Plates were incubated overnight at 37^0C. Interaction between phages and tested bacterial strains was confirmed by presence of clear spots on the site of application of phage onto L-agar plates. Tested bacterial strains against which phages showed no lytic activity were scored as negative. Depending on spot morphology, results observed were differentiated into 3 groups: (+ +) clear spots, (+) turbid spots and (-) for no spots at all.

3.11 Transmission Electron Microscopy (TEM):

Phages PBM-1 and PBM-2 were prepared for transmission electron microscopy (TEM) by depositing the 10 µl of purified phage lysate on cuprum grid. Filter paper was used to remove the suspension after 1-2 min and samples were negatively stained with 2% potassium phosphotungstate. After 1 min, sample was successively washed with water 3 times. After 10 mins of air drying, samples were imaged with TEM (NIBGE). Dimensions of both phages were measured by ImageJ online tool (image processing system developed at National Institute of Health Sciences).

3.12 Planktonic cell lysis kinetics of phage PBM-1 & PBM-2:

To evaluate the cell lysis kinetics of phage PBM-1 & PBM-2, exponential phase bacterial culture was infected with phage at different multiplicity of infection (MOI). 1ml of overnight APEC-O1 culture was inoculated in 20ml of LB broth to obtain exponential phase culture having 10^8 CFU/mL, phage was inoculated at 0.01, 0.1, 1 and 10 MOIs and broth was kept in shaking

incubator at 37^0C and 120rpm. Optical density (OD_{600}) was measured after every 1 hour interval upto 8 hours until the O.D starts to increase.

3.13 Thermal and pH stability assay:

Exponential phase of APECO1 was obtained by pipetting 1ml of overnight bacterial culture in 30ml of LB broth for 3-4 hours until 0.8 optical density is reached when measured through spectrophotometer. To measure the thermal stability of phages, known concentration (phage a = 1.37×10^{10} pfu/ml, phage c= 2.4×10^9 pfu/ml) of 1ml fresh phage lysates were mixed with 1 ml of LB broth (pH=7) and incubated at 4^0C, 25^0C, 37^0C, 45^0C, 60^0C, and 70^0C temperatures for 60 minutes. After 1 hour treatment, the falcon tubes were cooled down, 100 µl of phage lysate was taken and was serially diluted in TM Buffer. Phage titers were calculated by double layer agar assay to enumerate the surviving phages.

To measure the pH stability of these lytic phages, known concentration (phage a = 1.37×10^{10} pfu/ml, phage c= 2.4×10^9 pfu/ml) of 1ml fresh phage lysates were poured in 1ml of LB broth having 3, 5, 7, 9, 11 and 13 pH values and incubated at 37^0C for 60 mins. pH of LB broth was adjusted using 1M HCL and 1M NaOH solution prior to autoclaving. After 1 hour treatment of phages at different pH, 100 µl of phage lysates were serially diluted in TM buffer and their titers were calculated by double layer agar assay.

3.14 Kinetics of phage adsorption to host cells:

Phage adsorption assay was carried out as described by Kim (Kim et al., 2019). Exponentially growing host culture (1.2×10^8 CFU/ml) in LB broth was inoculated with phage lysate at ratio of

0.1 MOI. 100 µl of suspension was taken out at 0, 3, 6, 9, 12, 15 and 20 minutes, diluted in 900 µl ice cold TM buffer (phage buffer) and was centrifuged at 12000rpm, 4^0C for 5 minutes. Supernatant was transferred into the new tube and phage titer was determined by double layer agar assay. Phage titer at 0 min was called as original titer (To). Rate of phage adsorption to APECO1 was calculated by T/To.

3.15 One-step growth curve:

One-step growth curve was analyzed by following the method described by Kim (Kim et al., 2019) and Peng & Yuan (Peng & Yuan, 2018). 10 ml of exponentially growing host bacteria (1.2×10^8 CFU/ml) was inoculated by phage at the ratio of 0.1 MOI and incubated at 37^0C and 120 rpm. Phage was allowed to absorb to host cells for the adsorption time determined above. After, the suspension was centrifuged at 6000rpm for 30 minutes. Supernatant was discarded to remove the un-adsorbed free phages and the phage infected pellet was resuspended in 10 ml of pre-heated LB broth. The mixture was incubated at 37^0C and 120 rpm, 100µl aliquots were taken at 10 min interval for 1.5 hour. Phage titers were determined immediately by double layer agar assay.

3.16 Extraction of phage genome:

Phage genome was extracted using zinc chloride precipitation method as described by Santos (Santos, 1991). 1ml phage lysates of both phages, PBM-1 and PBM-2 were mixed with 12.5µl of $MgCl_2$. One microliter of RNAase A (100mg/ml) and 0.4µl of DNAaseI (1 µg/ml) were added and mixtures were incubated for 2-4 hours at room temperature after brief vortexing. Heat treatment was given to inactivate the DNAaseI by again incubating the mixtures at 65-70^0C for 20 minutes. 20µl solution of 2 M $ZnCl_2$ was added to mixture and incubated at 37^0C for 5 mins. The

mixture was centrifuged at 10,000rpm for 1 min, supernatant was discarded and pellet was resuspended in 500µl of TES buffer (0.1M EDTA; 0.1M Tris HCL, pH 8 and 0.3% SDS) with the help of pipette tip. The whole suspension was incubated at 60^0C for 15 min. 60µl of 3M potassium acetate, pH 5.2 was added, mixed and left on ice for 15-20 minutes. Dense white precipitates formed at this step due to protein precipitation. Suspension was centrifuged at 12000rpm for 1min in refrigerated centrifuged, supernatant was picked carefully to avoid the contamination of proteins and transferred to new eppendorf tube. Chilled isopropanol was added in equal amount and the mixture was left on ice for 10 mins to allow the DNA precipitation. Centrifugation was done to form the DNA pellet, washed 2-3 times with 70% ethanol and dried the pellet at room temperature. The pellet was resuspended in 50µl of deionized water. DNA extracted was confirmed by visualizing the bands on 1% agarose gel.

3.17 Storage of bacteriophages:

Luria Bertani (LB) broth was utilized as medium for storing the bacteriophages. Purified phage lysates of PBM-1 and PBM-2 were stored in LB broth at 4^0C, -20^0C and -80^0C for 12 months. Fifty percent glycerol was added in phage lysate at a final concentration of 20%. Both phage samples PBM-1 and PBM-2 were stored in 2 ml Eppendorf tubes in triplicates. PFU/ml of phages were calculated before and after storage by double layer agar method. In double layer agar method, bacteriophages were diluted using TM buffer and diluted phages were incubated with exponential phase APEC-O1 culture for 15-20 mins at 37^0C. After incubation, the suspension was mixed with 3-4ml of semisolid agar and overlaid on N-agar plates. Plaques observed after overnight incubation were counted to calculate phage titer.

4. Results

4.1 Isolation of PBM-1 and PBM-2 phages:

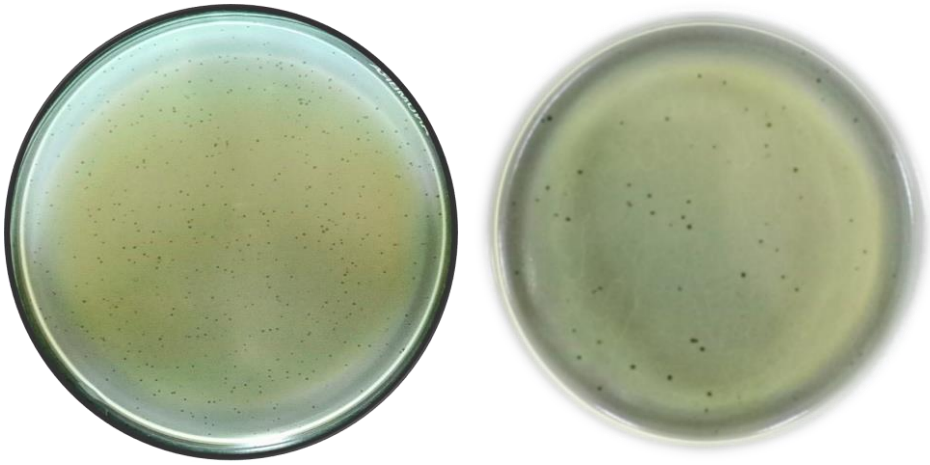
Two APEC phages were isolated from poultry bedding material of different sheds of same farm and were named as PBM-1 and PBM-2 i.e. PBM stands for poultry bedding material. Lytic activity of phages was checked by spot assay. Clear plaques/spots were obtained on site of application of phage on APEC-O1 lawn on L-agar plates in spot assay.

PBM-1 **PBM-2**

Fig 4.1 Spot Assay of PBM-1 and PBM-2: Inhibition zones observed against APEC-O1 lawn on L-agar plates indicates the lytic activity of both phages

4.2 Determination of phage titer and plaque morphology:

Double layer agar assay was performed to determine the phage titer and plaque morphology. Plaques of both phages PBM-1 and PBM-2 obtained against APEC-O1 were transparent, round and smaller than 1mm in size. Titer of PBM-1 is 1.37×10^{10} PFU/ml and of PBM-2 is 2.4×10^{9} PFU/ml.



PBM-1 **PBM-2**

4.3 Highly specific lytic spectrum:

Host specificity of PBM-1 and PBM-2 was determined against 39 bacterial strains. Out of 39, 30 were APEC strains. Both of the phages showed lytic activity against 10 APEC strains (49E, 50E, 74E, 80E, 154E, 159E, 163E, 165E and 167E) out of 30. However, no lytic activity was observed against fecal *E.coli* (n=4), uropathogenic *E. coli* (n=1), neonatal meningitis *E. coli* (n=1), DH5α (n=1), *Klebsiella pneumoniae* (n=1), methicillin-resistant *S. aureus* (MRSA) (n=1), *and Bacillus subtilis* (n=1).

Table 4.3 Host range of PBM-1 and PBM-2 against 39 bacterial strains: Signs ++ represent clear plaques, + represent turbid plaques, - represent no plaques.

Bacterial strain	PBM-1	PBM-2
APEC 48E	-	-
APEC 49E	+	+

APEC 50E	+	+
APEC 51E	-	-
APEC 53E	-	-
APEC 74E	++	++
APEC 75E	-	-
APEC 76E	-	-
APEC 77E	-	-
APEC 80E	++	++
APEC 81E	-	-
APEC 85E	-	-
APEC 151E	-	-
APEC 152E	-	-
APEC 153E	-	-
APEC 154E	+	+
APEC 155E	-	-
APEC 156E	-	-
APEC 157E	-	-
APEC 158E	-	-
APEC 159E	+	+
APEC 160E	-	-
APEC 161E	-	-
APEC 162E	-	-
APEC 163E	+	+
APEC 164E	-	-
APEC 165E	+	+
APEC 166E	-	-
APEC 167E	++	++
FE-96	-	-
FE-98	-	-
FE-99	-	-
FE-100	-	-
Uropathogenic *E. coli*	-	-
Neonatal meningitis *E. coli*	-	-
DH5α	-	-
K.pneumoniae	-	-
Methicillin-resistant *Staphylococcus aureus* (MRSA)	-	-

Bacillus subtilis	-	-

4.4 Determination of phage PBM-1 & PBM-2 morphology via Transmission Electron Microscopy (TEM):

In order to determine the morphology of PBM-1 and PBM-2, transmission electron microscopy was performed. Both phages examined by TEM were classified according to criteria proposed by Ackermann (Ackermann, 2009). Based on morphology, PBM-1 and PBM-2 were classified as member of the *Myoviridae* family of phages having hexagonal capsids. Dimensions measured by the online web tool ImageJ revealed that PBM-1 has a head diameter of 133 ± 6 nm (n=6), contractile tail length is 161 ± 2 nm (n=6) and PBM-2 has a head diameter of 147 ± 6 nm (n=6), contractile tail length is 176 ± 4 nm (n=6).

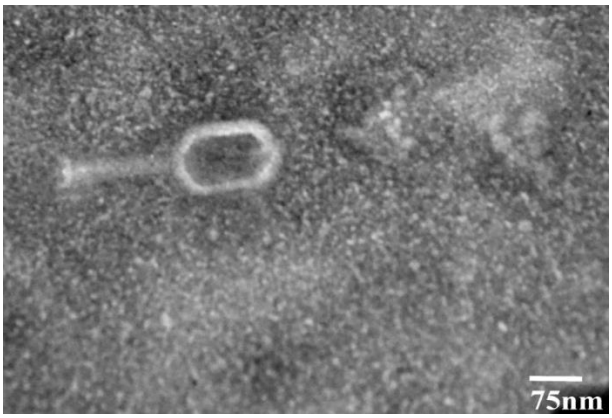


Fig 4.4 (a) Transmission electron micrograph of PBM-1 (Scale bar = 75 nm)

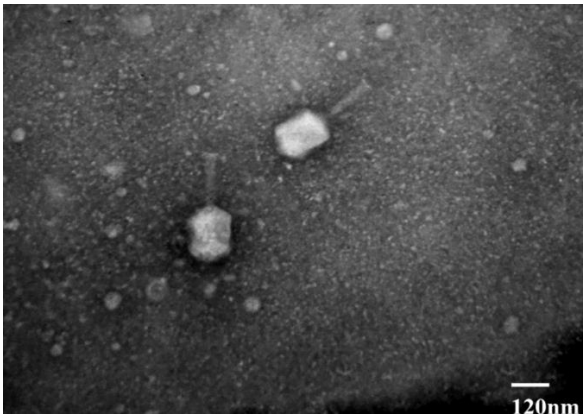


Fig 4.4 (b) Transmission electron micrograph of PBM-2 (Scale bar = 120nm).

4.5 Planktonic cell lysis kinetics of PBM-1 and PBM-2:

Planktonic cell lysis kinetics of PBM-1 and PBM-2 was evaluated and it was observed that APEC-O1 when not inoculated with phages show a continuous increase in optical density $(OD)_{600}$ during the 8 hours of incubation. However, by co-incubating the bacterial strain with phages at different concentrations (0.01, 0.1, 1, 10), both of the phages significantly reduced the bacterial growth and as a result OD_{600} decreases. PBM-1 when applied at 10 MOI reduced the bacterial growth upto 5 hours, and at 1, 0.1 and 0.01 MOI reduced the bacterial growth upto 7 hours; after that the OD_{600} start to increase which might be due to the generation of phage-resistant strains. PBM-2 when applied at 10, 1, 0.1 and 0.01 MOI to APEC-O1 culture reduced the bacterial growth during the first 6 hours; after that the OD_{600} start to increase which might be due to the generation of phage-resistant strains.

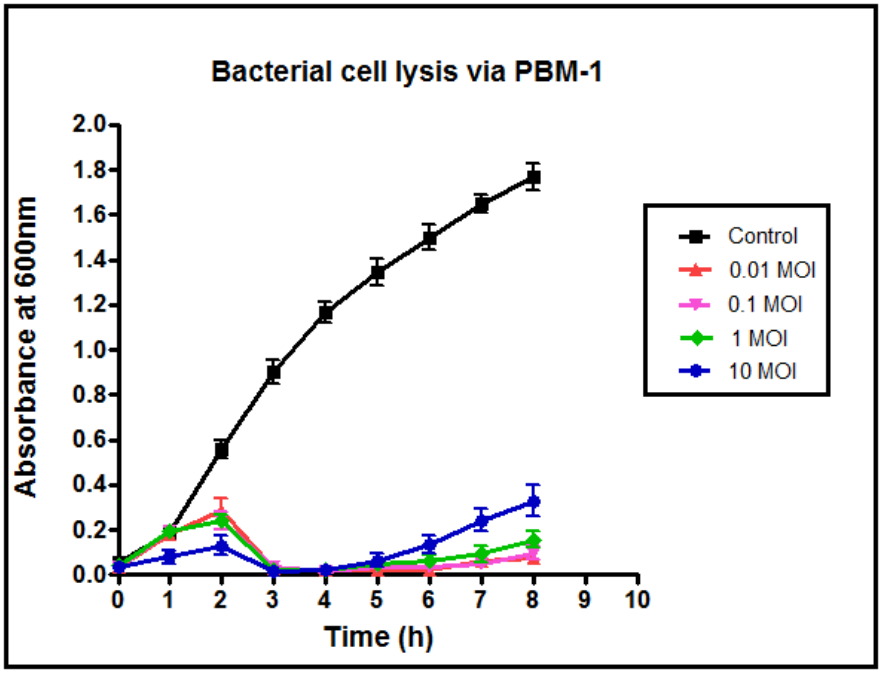


Fig 4.5 (a) Planktonic cell lysis kinetics of phage PBM-1 at an MOI of 0.01, 0.1, 1 and 10. Control represents the APEC-O1 culture not inoculated with the phage. The results presented here are the mean values with SD indicated by error bars from three independent experiments

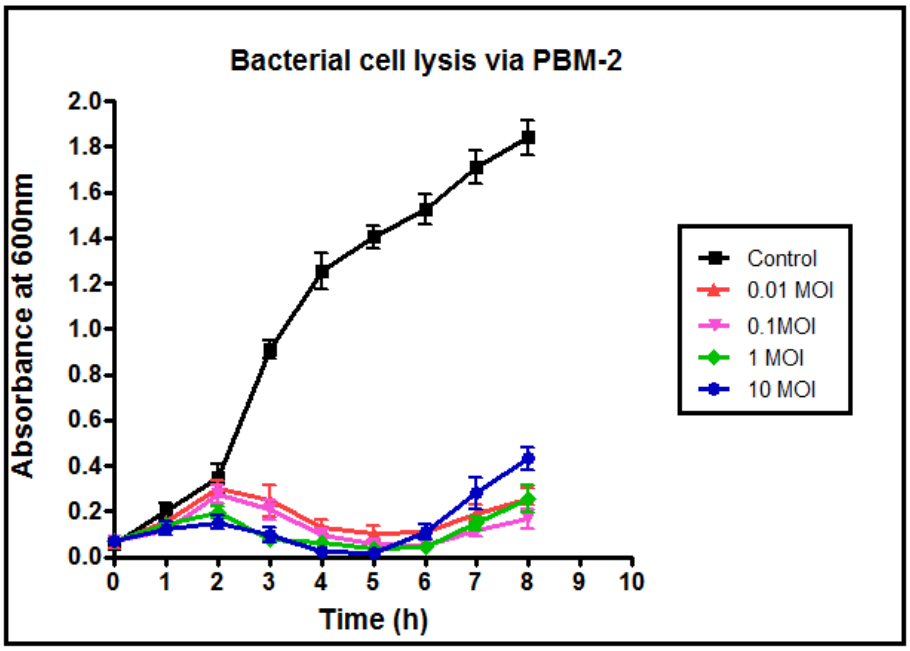


Fig 4.5 (b) Planktonic cell lysis kinetics of phage PBM-2 at an MOI of 0.01, 0.1, 1 and 10. Control represents the APEC-O1 culture not inoculated with the phage. The results presented here are the mean values with SD indicated by error bars from three independent experiments

4.6 Effect of temperature on the survival ability of PBM-1 and PBM-2:

Thermal tolerance of PBM-1 and PBM-2 was analyzed by calculating the phage titer at different temperatures by double layer agar assay. It was observed that both of the phages were thermal tolerant as no significant reduction was observed upto 37^0C ($p > 0.05$). However, after being treating the both phages for one hour at 45^0C and 60^0C, significant reduction was observed ($p < 0.0001$). At 45^0C, 68.5% of PBM-1 and 62.2% of PBM-2 were viable. When temperature arose to 60^0C viability of phages decreased sharply as only 32% of phage PBM-1 and 36.6% of phage PBM-2 were able to survive. At 70^0C, both of the phages lost their infectivity after being treated for one hour.

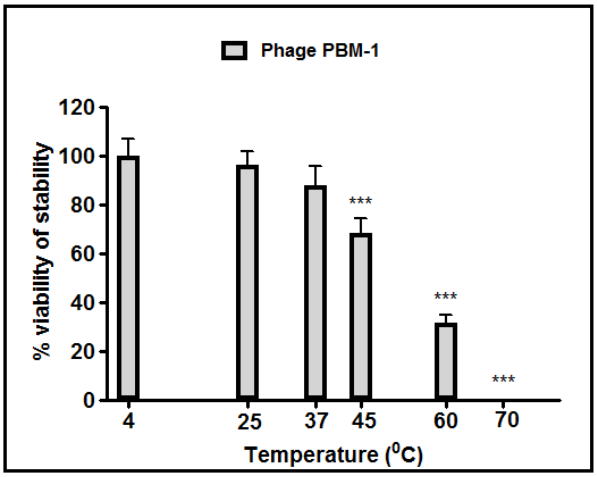


Fig 4.6 (a) Thermal tolerance of PBM-1. Phage was incubated for 1 hour at different temperatures. The results presented here are the mean values with SD indicated by error bars from three independent experiments.

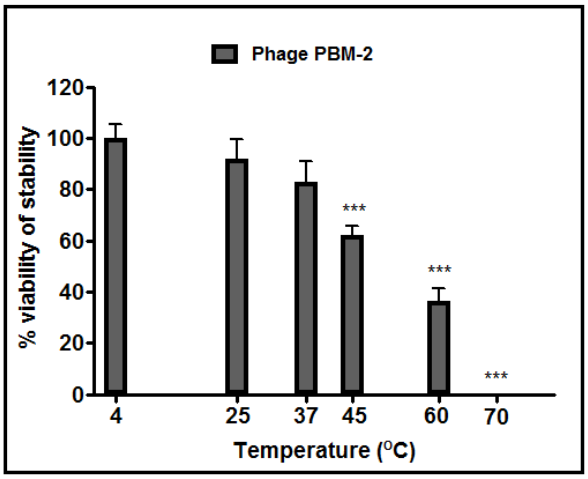


Fig 4.6 (b) Thermal tolerance of PBM-2. Phage was incubated for 1 hour at different temperatures. The results presented here are the mean values with SD indicated by error bars from three independent experiments.

4.7 Effect of pH on the survival ability of PBM-1 and PBM-2:

Viability of PBM-1 and PBM-2 under different pH range was evaluated by calculating the phage titer by double layer agar assay. It was observed that PBM-1 was viable at acidic pH. However, when we move towards the alkaline pH, 60% & 50% of PBM-1 were able to survive at 9 & 11 pH showing a significant reduction ($p < 0.0001$). Viability of PBM-1 decreased sharply i.e. only 10% of phage survived when incubated at pH 13 for one hour ($p < 0.0001$).

PBM-2 showed significant reduction under all pH ranges except 5 and 7. At pH 3, 85.1% phages survived ($p < 0.05$). However, at alkaline pH, phage titer is greatly reduced showing a significant reduction at 9 and 11 pH ($p < 0.0001$). PBM-2 lost its infective ability when incubated at 13 pH for one hour.

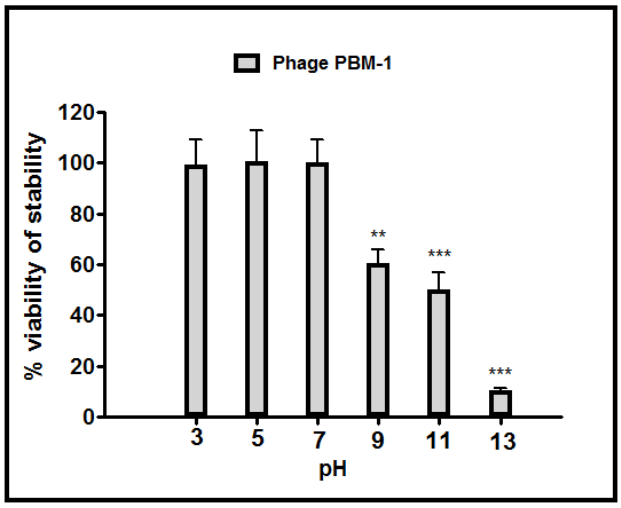


Fig 4.7 (a) pH tolerance of PBM-1. Phage was incubated for 1 hour at different pH. The results presented here are the mean values with SD indicated by error bars from three independent experiments

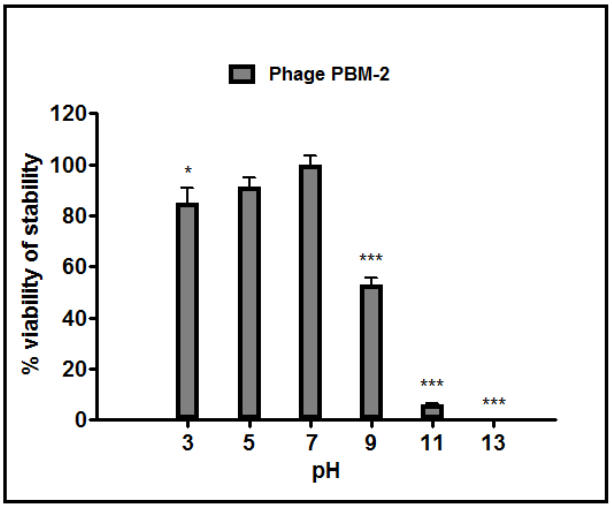


Fig 4.7 (b) pH tolerance of PBM-2. Phage was incubated for 1 hour at different pH. The results presented here are the mean values with SD indicated by error bars from three independent experiments

4.8 Kinetics of phage adsorption to host cells:

It was observed that both phages PBM-1 and PBM-2 efficiently adsorbed to APEC-O1 host cell suspension in short time interval. It was evaluated by calculating the phage titer at specific time intervals by double layer agar assay. PBM-1 showed over 90% adsorption in 15 minutes whereas PBM-2 showed over 95% adsorption in 9 minutes. Rate of phage adsorption was calculated by T/T_0 (T = phage titer at different time intervals, T_0 = phage titer at 0 time interval).

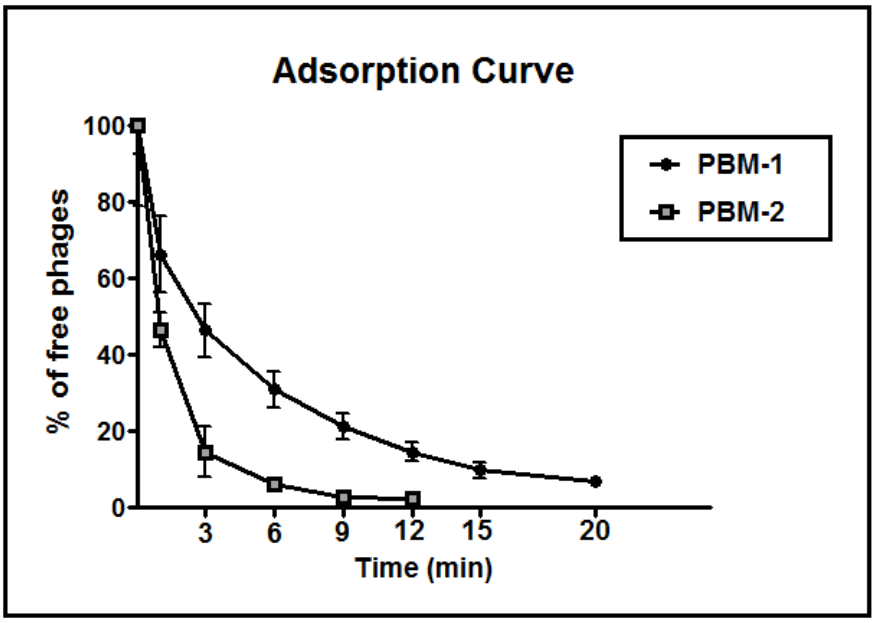


Fig 4.8 Rate of adsorption of PBM-1 and PBM-2 to APEC-O1. Phages PBM-1 and PBM-2 were added to APEC-O1 host suspension at MOI of 0.1 and the percentage of unadsorbed/free phages was calculated at different time intervals by $T/T_0 \times 100$. The results presented here are the mean values with SD indicated by error bars from three independent experiments.

4.9 One-step growth curve analysis:

Lytic development of phage PBM-1 and PBM-2 was evaluated by one-step growth curves. At 37^0C in LB broth, lytic development of both phages appeared to be complete in short time period. Burst size was calculated by taking the average of free phages on time points before plateau denoted as A. Taking the average of free phages on time points after plateau denoted as B and by subtracting the A from B, the value obtained was denoted as C. C represented the total burst /total number of new phages released. Burst size was calculated by dividing the C with infecting phages i-e, total number of phages at T_0- free phages at T_0. It was observed that PBM-1 has a

latent period of 20 min and a burst size of 102 per bacterium. PBM-2 has a latent period of 25 min and a burst size of 97 per bacterium.

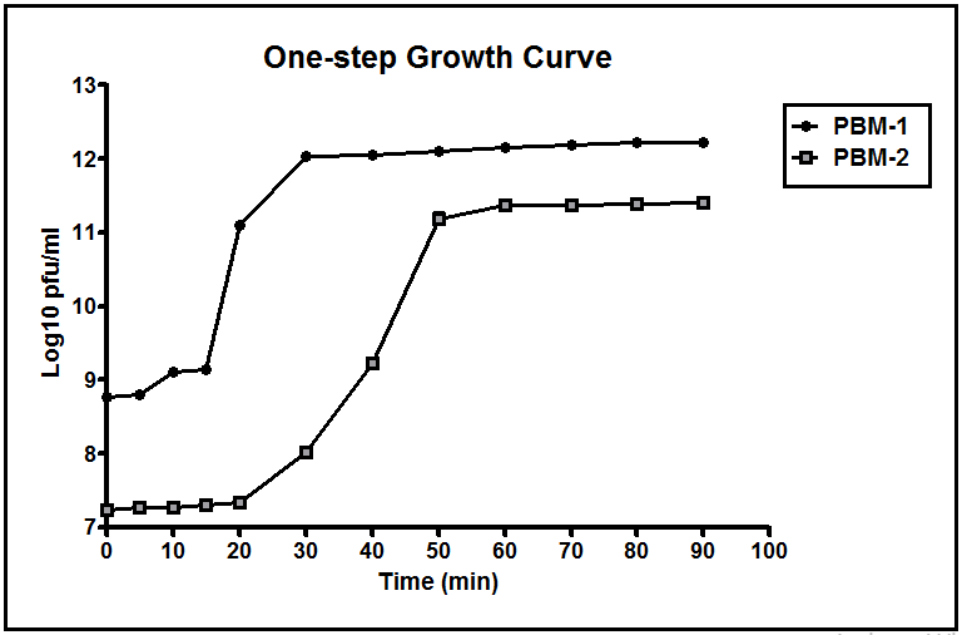


Fig 4.9 One-step growth curve of phage PBM-1 and PBM-2. The results presented here are the mean values with SD indicated by error bars from three independent experiments.

4.10 Phage DNA analysis on agarose gel:

In order to confirm the size of phage genome, we extracted the DNA and run on agarose gel. The bands observed showed that either the DNA is in supercoiled form or its size is greater than 10 kb. Usually, genome of Myoviridae family is double stranded DNA. Whole genome sequencing will further confirm the results.

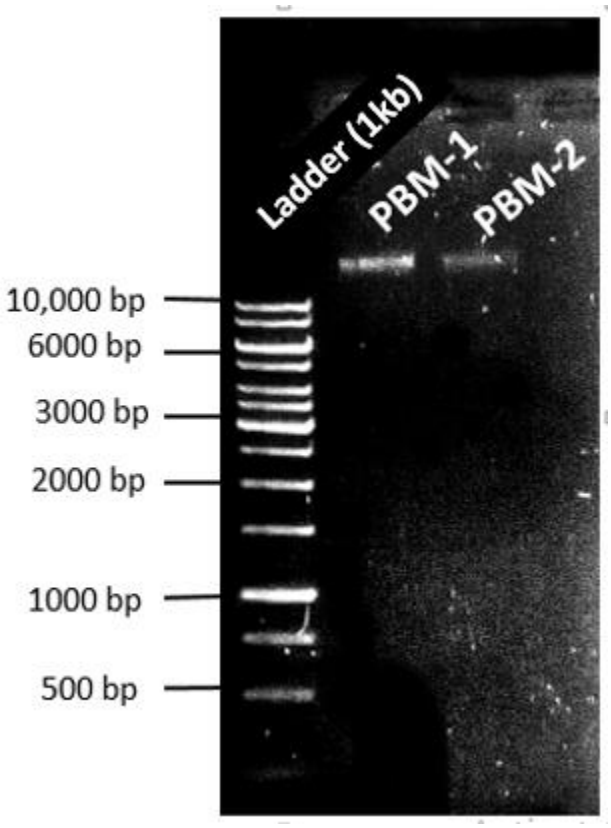


Fig 4.10 Bands of DNA of phages PBM-1 and PBM-2 visualized on 1% agarose gel. 1kb ladder is used as a marker

4.11 Effect of storage duration (one year) at different temperatures:

In order to check the efficacy of phages PBM-1 and PBM-2 after prolong period, PBM-1 and PBM-2 were stored for one year in LB broth at 4^0C, -20^0C and -80^0C. Lytic efficacy of both phages against APEC-O1 was evaluated by calculating the phage titer by performing double layer agar assay before and after storage. No significant reduction in titer of both phages before and after storage was observed ($p > 0.05$).

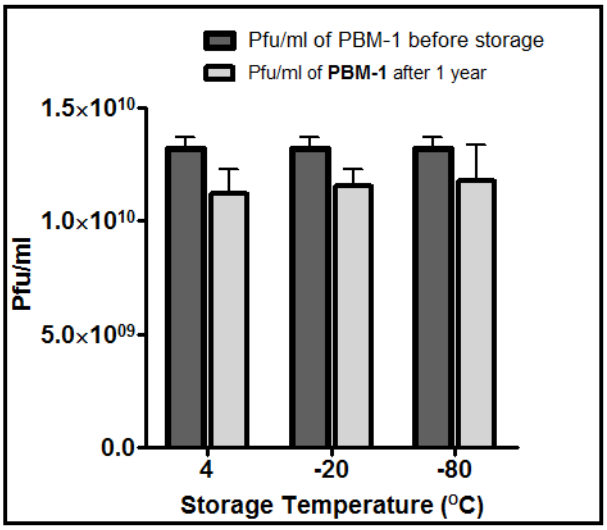


Fig 4.10 (a) Storage of PBM-1 at different temperatures. The results presented here are the mean values with SD indicated by error bars from three independent experiments

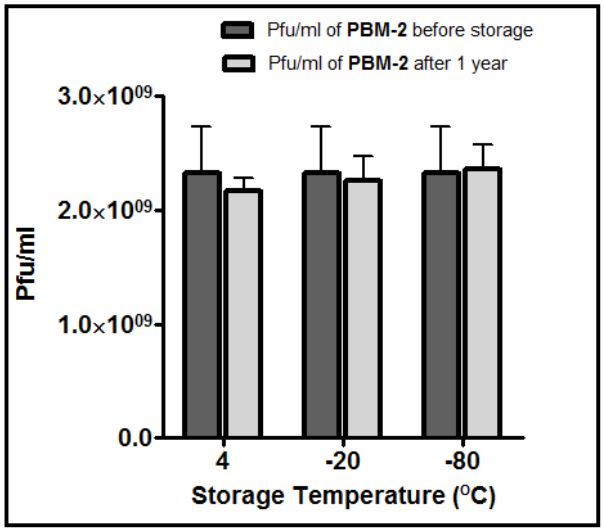


Fig 4.10 (b) Storage of PBM-2 at different temperatures. The results presented here are the mean values with SD indicated by error bars from three independent experiments

5. Discussion:

Emergence of antibiotic resistant strains have limited the use of antibiotics. Previously, antibiotics were added to poultry feeds to elevate the performance and prevent the disease challenges. In 2017, No Antibiotics Ever (NAE) system was introduced by Food and Drug Administration (FDA) of United States according to which no antibiotics would be added to poultry feed as growth promoters (Smith, 2019) that once imparted protection against pathogens. Phage therapy is one of the alternative option to eliminate bacterial pathogens. Numerous phages against APEC were isolated but phage resistance observed in bacterial strains is faster than antibiotic resistance. Therefore, it is important to discover different phages with novel genetic backgrounds to construct phage cocktail that prevents the resistance issue. In this study, two phages PBM-1 and PBM-2 against Avian Pathogenic *E. coli* (APEC) were isolated and biologically characterized. Both of the phages showed a narrow host range i-e, ability to lyse a specific host range make these phages suitable for phage therapy (Kutter, 2009). TEM analysis revealed that both phages belong to Myoviridae. According to literature cited, (Ackermann, 2009) most of the phages belonging to Myoviridae have large, double stranded DNA genomes and their size can range upto as large as >250kb (Hatfull, 2008). It was reported that large DNA phages usually form extremely small plaques on agar plates (Serwer et al., 2007). Genome extraction from both phages PBM-1 and PBM-2 and visualization of bands on 1% agarose gel confirmed the presence of DNA as their genome.

Planktonic cell lysis of bacterial cells was observed when APEC-O1 was co-incubated with both phages PBM-1 and PBM-2. This indicated phage potential to reduce the bacterial growth, as the time passed, bacteria co-evolved with phage and developed resistance against the phages which

resulted in increase in optical density after a certain time. However, the resistance problem can be overcome by synergistically using both these phages i-e make a phage cocktail that will have a better therapeutic potential or use antibiotics in combination with phages. As it is known that, when MDR bacteria acquire resistance against phages it loses its resistance against antibiotics ultimately these bacteria are susceptible to antibiotics again.

Environmental parameters such as temperature and pH plays an important role in efficacy of phage therapy (Silva et al., 2014). Both the phages PBM-1 and PBM-2 showed maximum stability from 4-37^{0}C and after that significant reduction in lytic ability of phages was observed as seen by fewer plaques. Temperature plays an important part in attachment of phage to its receptors. At higher temperatures, lysozyme enzyme is denatured and incapable of attachment with bacterial cell wall, resulting in decrease/loss of lytic ability of phages. Stability of both phages PBM-1 and PBM-2 at 37^{0}C showed that they can work efficiently at body temperature. Much like temperature, pH has an influence on phage multiplication, intracellular replication, infectivity and attachment (Jepson & March, 2004; Leverentz et al., 2001; Pirisi, 2000). pH can interfere with phage infectivity by disrupting the lysozyme enzyme and other capsid proteins thus, inhibiting the phage attachment to bacterial cell wall. Both phages PBM-1 and PBM-2 have the ability to withstand acidic pH conditions showing that they can be administered by oral route. However, at alkaline pH, lytic activity of both phages was reduced significantly as fewer plaques were observed showing the susceptibility of both of phages towards alkaline pH.

Both phages PBM-1 and PBM-2 have a high adsorption rate in 20 and 12 mins showing that short time is required by these phages for encountering and attacking the host bacterial cell. Phages having a higher adsorption rate would have a shorter lysis time (Shao & Wang, 2008). One step growth curve determines the latent period and burst size of bacteriophages that are affected by

media composition, host, incubation temperature and growth rate (Sun et al., 2012). Burst size and latent period have a progressive relationship, an optimal latent period give rise to efficient lytic ability of phage. Generation time of phage and burst size are positively linked; longer the generation time, higher will be the burst size and vice versa. A phage with too long lysis time would have a higher burst size but it will lose the opportunity of initiating many new infections; in the same way, a phage with a too short lysis time infect other cells in the environment earlier but with a reduced gain due to shorter burst size (Shao & Wang, 2008). Having an intermediate lysis time is required for higher efficacy of phage therapy. In this study, both phages PBM-1 and PBM-2 have a latent period of 20 and 25 mins whereas, burst size calculated was 102 and 97 per infected bacterial cell. Moderate latent period showed the efficacy of these phages to be used in phage therapy.

Both phages can be stored in refrigerator or at -20^0C and -80^0C for upto a year in LB medium without any loss in their lytic efficacy. No additional preservatives are required that immediately would help in reducing the cost of phage therapy.

6. Conclusion

The present study conclude that PBM-1 and PBM-2 phages can be considered as potential candidates for phage therapy. Specifically, following attributes are advantageous; clear plaques against APEC-O1 with no signs of lysogeny, ability of phages to reduce the bacterial growth upto 6 or 7 hours, ability to efficiently infect many though not all APEC strains, high stability towards varying temperature and pH range, high adsorption rate and a moderate burst size. It was concluded that further studies on genome characterization, protein characterization and dose optimization will lead to utilization of these phages in phage therapy.

7. Future Prospects

For further characterization, whole genome sequencing of both phages PBM-1 and PBM-2 may be done for identifying the therapeutic potential of phages by confirming the absence of resistant and virulent genes. For structural and functional characterization of capsid proteins, protein extraction and mass spectrometry may be done. For further evaluation of therapeutic potential of PBM-1 and PBM-2 against colibacillosis *in vivo* testing may be done.

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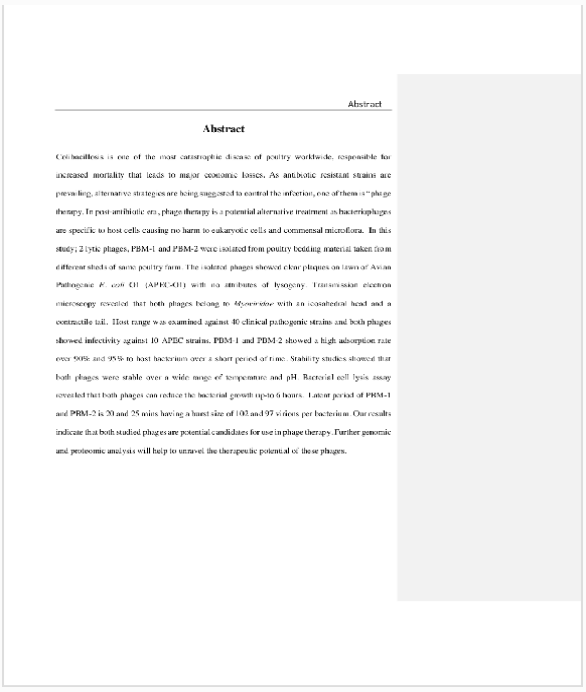
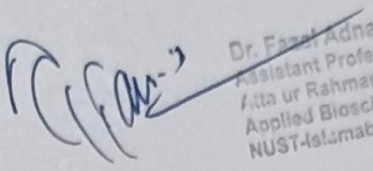


Abstract

Abstract

Colibacillosis is one of the most catastrophic disease of poultry worldwide, responsible for increased mortality that leads to major economic losses. As antibiotic resistant strains are prevailing, alternative strategies are being suggested to control the infection, one of them is "phage therapy. In post-antibiotic era, phage therapy is a potential alternative treatment as bacteriophages are specific to host cells causing no harm to eukaryotic cells and commensal microflora. In this study; 2 lytic phages, PBM-1 and PBM-2 were isolated from poultry bedding material taken from different sheds of same poultry farm. The isolated phages showed clear plaques on lawn of Avian Pathogenic *E. coli* O1 (APEC-O1) with the attributes of lysogeny. Transmission electron microscopy revealed that both phages belong to *Myoviridae* with an icosahedral head and a contractile tail. Host range was examined against 40 clinical pathogenic strains and both phages showed infectivity against 10 APEC strains. PBM-1 and PBM-2 showed a high adsorption rate over 90% and 95% to host bacterium over a short period of time. Stability studies showed that both phages were stable over a wide range of temperature and pH. Bacterial cell lysis assay revealed that both phages can reduce the bacterial growth up-to 6 hours. Latent period of PBM-1 and PBM-2 is 20 and 25 mins having a burst size of 103 and 97 virions per bacterium. Our results indicate that both studied phages are potential candidates for use in phage therapy. Further genomic and proteomic analysis will help to unravel the therapeutic potential of these phages.



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